# The corneal endothelium reflected

Studies on surgical damage to the corneal endothelium and on endothelial specular microscopy

Bart T.H. van Dooren

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# The corneal endothelium reflected

## Studies on surgical damage to the corneal endothelium and on endothelial specular microscopy

## Het cornea-endotheel ge- en bespiegeld

Onderzoek naar chirurgische schade aan, en spiegelmicroscopie van het cornea-endotheel

**Proefschrift**
ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties

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**door**
Bartholomeus Theodorus Herman van Dooren
geboren te Geleen

# Addendum concerning the thesis:

# The corneal endothelium reflected,
# by Bart T.H. van Dooren,

# December 21st 2006

Dr. Gerrit R.J. Melles has identified statements in the thesis concerning his own scientific work that he believes are incorrect. He was not offered to review the entire manuscript of the thesis before it was printed.

After discussion between the Rector Magnificus Prof.dr.S.W.J. Lamberts, the promotor Prof.dr.G.van Rij, Dr.G.R.J. Melles, and the promovendus it was decided to add the following:

- Dr. Melles' initial work on posterior lamellar keratoplasty was by no means influenced by, or based on the work by Ko et al. (reference 1139, page 201 of this thesis).

Errata concerning the thesis:

The corneal endothelium reflected,
by Bart T.H. van Dooren,

December 21st 2006

Three figures in this thesis were published previously, but unfortunately references were not properly made. Permission from the publishers of the original articles for the reproduction of these figures in the thesis was requested by the author, and has been granted for each of these figures.

- Figure 7.1, pages 150 and 329 of the thesis, is a reproduction of Figure 1, in the article: Melles GRJ, De Waard PWT, Pameijer, J.H., Beekhuis W.H. Trypan blue capsule staining to visualize the capsulorhexis in cataract surgery. J Cataract Refract Surg 1999;25:7-9. (Reference number 32 in this thesis).
- Figure 10.1, page 203 of the thesis, is a reproduction of Figure 1, in the article: Melles GRJ, Lander F , Rietveld FJR, Remeijer L, Beekhuis WH, Binder PS. A new surgical technique for deep stromal anterior lamellar keratoplasty. Br J Ophthalmol 1999;83:327-333. (Reference number 34 in this thesis).
- Figure 12.1, pages 235 and 330 of the thesis, is a reproduction of Figure 1, in the article: Van Dooren BTH, Mulder PGH, Nieuwendaal CP, Beekhuis WH, Melles GRJ. Endothelial cell density after posterior lamellar keratoplasty (Melles techniques); 3 years follow-up. Am J Ophthalmol 2004;138:211-217. This article is included in this thesis as chapter 12. (Reference number 1223 in this thesis).

## promotiecommisie

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*Aan mijn ouders*

*Voor Gertrude en Jonathan*

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part

I

# General introduction

# chapter

# 1

# Preface

The cornea, the transparent anterior structure of the eye, transmits and refracts light entering the eye, and forms a barrier to protect the interior of the eye against the outside world. It is composed of distinctive layers. In 1849, the corneal endothelium was first described as the distinctly identifiable 'delicate and perishable' innermost corneal layer. (Bowman W, 1849, as referred to in Waring et al.[1]) Leber demonstrated in 1873 the necessity of an intact endothelial layer for the deswelling and clarification of edematous corneas, using experimental studies on pressure-induced corneal edema.[2] In 1920, Vogt introduced a technique of visualising the human endothelium and its individual cells in vivo. He applied a biomicroscopic ('slit lamp') technique that used the direct reflection of light from the endothelium-to-aqueous interface to produce an image in the 'Spiegelbezirk' (specular area).[3]

Interest in endothelial function renewed in the 1940s and '50s, when laboratory research led to the concept that deswelling of the cornea involved a metabolic pump, which was mainly located in the endothelium.[4-6] An explanation for the opacification of the cornea in corneal swelling was proposed in a theory developed by Maurice, involving disruption of the regular organisation of the stromal collagen fibrils in corneal edema.[7] These concepts still are the pillars on which current corneal hydration physiology is based.

In 1968, Maurice described a contact-type 'specular microscope' for the viewing and photographing of the corneal endothelium in an intact globe,[8] and in 1970 Brown showed an example of an in-vivo endothelial 'macrophotograph' obtained with a non-contact macrophotography instrument.[9] Both techniques used the specular reflection principle described by Vogt,[3] although in neither paper references to Vogt's work were made. These developments coincided with important concepts that developed in ophthalmic surgery: the vulnerability of the endothelium during cataract extraction and intraocular lens implantation, endothelial cell loss after keratoplasty, and hence pre-operative evaluation of the endothelium in donor corneas. This led to the fast adoption of these prototypes of specular microscopes and further improved instruments in clinical practice, research, and eye banks, generating in its turn even more interest in the biology of the corneal endothelium.[10-27]

Since then, many aspects of the endothelium have been investigated. Several of these studies are reviewed in chapter 2 of part I of this thesis. In this chapter a tradition of research on the corneal endothelium is described, to which researchers in The Netherlands have made contributions as well.[28-31]

The present thesis may be considered to be a (collection of) new contribution(s) to the vast body of knowledge that already exists on the endothelium. It focuses on three main issues. The first issue is about the reliability

of endothelial morphometric assessments, obtained with a modern specular microscope and semi-automated endothelial evaluation. It was evaluated in methodological studies on calibration, validity, and reproducibility of measurements. Also, the systematic differences between specular microscopic in-vivo endothelial cell densities were compared with endothelial cell counts obtained from donor corneas, obtained with a different, in-vitro assessment technique. These studies are presented in part II, Chapters 3, 4, and 5, respectively. In chapter 6, optical phenomena associated with non-contact specular microscopy are elaborated upon, as they possibly accounted for systematic differences as observed in the study described in chapter 5.

In the part III of this thesis, the second issue, toxic effects on corneal endothelial cells caused by exposure to trypan blue, was evaluated. Trypan blue is a (supra-) vital stain that since long has been in use in cornea banks, for the evaluation of donor cornea endothelia. Interest in this dye increased significantly with the introduction of its application in cataract surgery for lens capsule staining.[32] Clinical and experimental studies on possible toxic effects of trypan blue are presented in Chapters 8 and 9, following a review of the literature relevant to this topic in Chapter 7.

The third and last issue is the evaluation of endothelial cell loss after deep anterior lamellar keratoplasty (DALK, Part IV, chapter 11) and posterior lamellar keratoplasty (PLK, Part IV, chapters 12 and 13). These studies were instigated by the introduction of new operative techniques,[33, 34] which further developed and popularized surgical concepts in lamellar corneal transplantation. A history of lamellar keratoplasty is reviewed in Part IV, Chapter 10.

In part V, Chapter 14, the findings of the studies presented in this thesis are summarized, and put into the perspective of other current developments and future prospects. By then the reader will already have understood, that in spite of the vast existing body of knowledge and the present new contributions, a lot of questions remain unanswered about the corneal endothelium.

## References

See page 287.

# chapter

# 2

# The corneal endothelium: a review of the literature

# I. Basic corneal and endothelial anatomy.

The cornea is the transparent, avascular, anterior- and outermost structure of the globe. Although the precorneal tear film is not a structural part of the cornea, cornea and tear film can be considered to be an integrated functional unit. The functions of this unit are to transmit and refract the light entering the eye and to form a protective barrier between the globe and the outside world against traumatic force, entrance of pathogens, and dehydration.[35-37] The human cornea is composed of three distinctive anatomical layers. These are, from anterior to posterior: the epithelium with its basement membrane; the stroma, which is subdivided into Bowman's layer, the lamellar stroma, and Descemet's membrane; and the endothelium. Part of Descemet's membrane is in fact the basement membrane of the endothelium, and therefore it is sometimes also considered to be part of the endothelium.[27 35-37] See figure 2.I.1.

The endothelium, the innermost corneal layer, consists of an approximately 5µm thick mosaic of cells forming a tesselated monolayer. This mosaic is configured of the apical, most posterior surfaces of the endothelial cells, typically with hexagonal forms.[1 27 35-40] See figure 2.I.2. Electron microscopic studies showed that central apical cell membranes usually are regular and flat, whereas more peripheral cells have cilia. In adults, towards the far periphery endothelial cells have been found to cover Descemet's membrane outcroppings called Hassall-Henle's warts.[41] The lateral cell membranes were shown to be extremely tortuous and interdigitating.[1 27 35-38 40] The basal, anterior sides of the endothelial cells are primarily hexagonal as well, albeit with more ruffled cell borders compared to the apical hexagons, due to larger interdigitating cell processes basally.[39 42 43] Endothelial cells contain a great number of mitochondria, as well as smooth and rough endoplasmatic reticula and a Golgi apparatus. This reflects a high metabolic activity. [1 27 35-37 40 44] Descemet's membrane, located just anterior to the endothelial cells, is 8-10 µm thick. It has an anterior embryonal zone that is mostly banded, and a thicker, unbanded, posterior layer that constitutes the basement membrane of the endothelial cell layer in adults.[1 27 35-37 40 44 45]

Descemet's membrane is composed of predominantly type IV collagen besides other types of collagen (III-VIII), and glycoproteins including laminin and fibronectin. The corneal stroma is composed of organized fibrils consisting mainly of type I collagen and to a lesser extent type V collagen; collagen types III, VI, VII, XII and XIII, have been found in the extrafibrillary stroma. The other important stromal proteins are the proteoglycans keratan sulfate (65 %) and chondroitin/dermatan sulfate (35 %), both of which can be constituted by several core proteins. Collagen fibrils together with microfibrils consisting of fibrillin are organised in flattened bundles, the corneal lamellae. The lamellar stroma is secreted and maintained by the stromal fibroblasts, a.k.a. keratocytes,

residing between the lamellae. Bowman's layer which lies adjacent to the epithelial basement membrane, is an acellular zone consisting of collagen fibrils and associated proteoglycans, densely woven in a felt-like matrix. [1 35 37 45-54]

The corneal epithelium is 5 - 7 cell layers (50 – 52 µm) thick in adults and has a stratified squamous organization, providing an absolutely smooth apical surface, by means of microplicae filled and covered with a glycocalyx. Its basement membrane is thin compared to Descemet's membrane, and also consists of predominantly type IV collagen. The outermost 3 or 4 cell layers consist of flattened squamous cells, resting on and resulting from 1 –3 intermediate layers of wing cells, and the innermost, single layer of mitotically active, columnar, basal cells. All epithelial cells have highly interdigitating and undulating membranes, with prominent desmosome junctions and gap junctions. Especially noticable is the band of tight junctions between the lateral membranes of the apical cells. The basal cells adhere to their basement membrane through extensive anchoring complexes. Epithelial anatomy and function is influenced by the subbasal nerve plexus.[37 45]

**Fig. 2.I.1** **The layers of the cornea.**

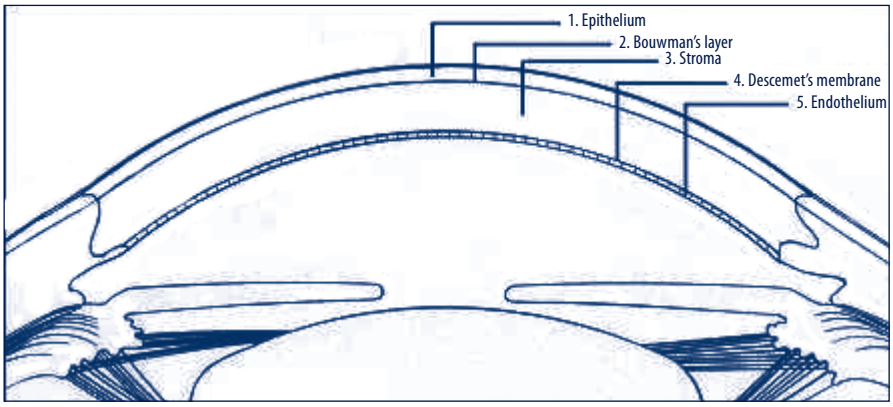
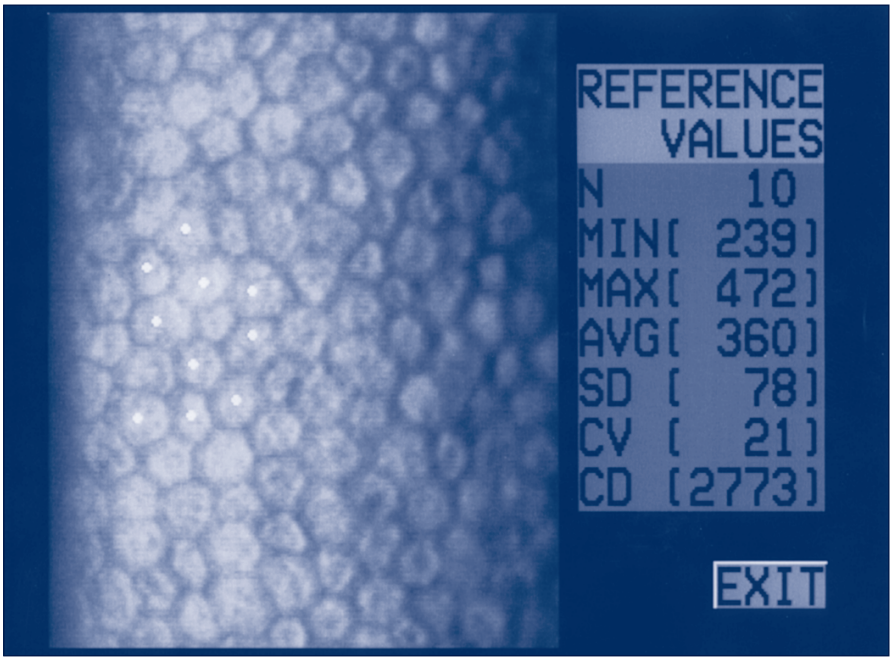


Fig. 2.I.2 Specular microscopic image of a (fairly) normal, homogeneous endothelium. Most cells are hexagonal, and there is little variation in cell size. Please note the white dots indicating counted cells with the "centres method" (see paragraph 2.VII.a).



## II. Embryology of the endothelium.

From gestational day 24, the optic pits pouch out from the cranial neurectoderm towards the surface ectoderm, and subsequently become optic cups through invagination and fusion. Upon adhesion with the optic cup, the surface ectoderm is induced to form the lens placode. The lens placode invaginates and separates on day 33 from the overlying surface ectoderm, which goes on to form the corneal epithelium and the epidermis of the eyelids. From the beginning of the sixth gestational week cells migrate in at least two consecutive waves from the periphery in the space between the basal lamina of the lens vesicle and the corneal epithelium. A double row of flattened cells is formed, which starting in the eighth week thins to a monolayer: the corneal endothelium.[1 40 44 55-57] Initially, these migrating cells and hence the endothelium were thought to have a mesodermal origin, in analogy to the vascular endothelium,[58] but later studies have proven them to be neural crest cells.[59-62] A second wave of neural crest cells immigrates soon after anterior to the endothelium to form the stromal mesenchymal cells, i.e. the keratocytes or fibroblasts. Migration posterior of the endothelium forms the iris stromal cells, as well as the covering of what will become the trabecular meshwork.[1 40 44 57].

The stromal fibroblasts produce the stromal extra-cellular components proteoglycans and collagen. Descemet's membrane results as the synthesis of a basement membrane by the endothelial cells or arguably by the lower stromal cells,[56] and becomes evident as a complete, identifiable layer at a gestational age of 16 weeks. Its anteriormost, in-utero formed part has a mostly banded appearance in electron microscopy, whereas the post-partum formed part of Descemet's membrane is non-banded.[1 35 40 44 57 63]
The importance of the presence of a normally developing lens for the induction of the development of a normal corneal endothelium has since long been recognized.[56 64] Cell adhesion molecules such as N-cadherine may play a role.[65 66] The vulnerability of this developmental sequence is demonstrated by the existence of a spectrum of congenital disorders called the mesenchymal dysgenesis disorders, including entities such as Axenfeld-Rieger syndrome and Peter's anomaly. Hallmark of these disorders is an abnormal development of the corneal endothelium, probably cause by abnormalities in the immigration of neural crest cells. This is also thought to result in incomplete or abnormal developmental separation between anterior chamber structures.[1 40 65 67]

## III. Concepts for corneal transparency, corneal hydration, and endothelial physiology.

In 1957 Maurice postulated that prerequisite for the cornea to be transparent was that the parallel collagen fibrils constituting the corneal stromal lamellae

had a hexagonal lattice arrangement. He demonstrated the necessity for such a regular arrangement of the fibrils through mathematical and physical calculations on the birefringent properties of the corneal lamellae and the light-scattering properties of the individual collagen fibrils in each lamella.[7] This theory basically still holds, although the development of new models and the use of newer experimental techniques have resulted in adjustments and refinements. The proteoglycans in the corneal stroma may serve as an interconnecting meshwork encircling the collagen fibrils to form Maurice's hexagonal array of fibrils. Uniform thickness of the fibrils and a uniform distance between the fibrils (usually smaller than ½ λ (wavelength) appear to be prerequisites for optimal light transmittency.[68-70] Maurice also postulated that swelling of the cornea would disrupt the regular arrangement of the fibrils, thus providing an explanation for the well known observation of opacification of the cornea in corneal edema.[7]

Intraocular pressures (IOP) of over 40 to 50 mm Hg have since long been known to cause corneal swelling.[71 72] In 1857 Donders showed that the cornea also has an intrinsic tendency to swell, to imbibe fluid, when immersed in hypotonic solutions.[73] Later experiments showed that a negative pressure inside the cornea existed, the so called Imbibition Pressure (IP). The imbibition pressure is an osmotic pressure developed in the charged mucopolysaccharide gel: the glycosaminoglycans of the interfibrillary substance in the corneal stroma.[1 4 74] The following relation was demonstrated between IOP, IP and swelling pressure (SP):

$$IP = IOP - SP.$$ [72 74]

The amount of swelling is different between stromal compartments: posterior stroma can osmotically attract and hold more water than the anterior compartment, in accordance with differences in stromal glycosaminoglycan content. Obviously it also depends on the (hypo-)tonicity of the experimentally applied solutions. [45 75-79] Evidence exists that in edematous corneas not only water intake rises, but also stromal glycosaminoglycans are lost.[80]

Normal average corneal water content is 78 %. The average central corneal thickness is 0.52 mm, with increasing values towards the periphery.[1 72 76] Although diurnal fluctuations in corneal thickness have been observed,[81] these fairly constant figures imply that mechanisms must exist to counteract the swelling pressure. These mechanisms include passive barriers and active transports, or 'pumps'. In the 19th century Leber thought, after he had shown in his experiment that the endothelium prevented corneal swelling, that the main counteracting mechanism was that the endothelium was an impenetrable barrier.[2] Studies of among others Davson, Maurice and Giardini, Harris and Nordquist, Mishima, and Maurice, provided evidence that the extrusion of water depended on metabolism dependent active pumping. Also, the corneal

epithelium appeared to have a considerably greater resistance to water flow than the endothelium. The fluid pump with the greatest activity had to be located in the endothelium, and not in the epithelium. There also is an epithelial pump however, which appears to provide about 15 % of total deturgescence of the stroma (in rabbits).[4-6 72 82 83] The prevailing theory of the endothelial functions that resulted is sometimes called the 'pump-leak' theory, with the endothelium being a leaky barrier against the swelling pressure, but providing a powerful deswelling counteraction through a metabolically active fluid pump.[1 84 85]

## IV. Ultrastructural and molecular functional anatomy of the endothelium and epithelium.

Studies on animal material have shown that the corneal epithelium is a relatively impenetrable barrier to ion flow and hence water flow. The cause for the impermeability was found in the presence of paracellular zonulae occludentes or tight junctions in the most superficial cell layer, combined with a high electrical resistance in the cell membranes. Nevertheless, fluid transport capacity and ion secretory capacity has been demonstrated in epithelial cells.[83 86-89] The endothelial cells also have an apical belt of tight junctions. In the endothelium these tight junctions are included in the so-called apical junction complex (AJC). Although they appear to grow during maturation, there is accumulating evidence that these AJCs remain discontinuous in adults. Furthermore, they appear to be of a different composition than the true zonulae occludentes found in the epithelium. These findings support the concept that the endothelial barrier is permeable, "leaky".[55 88 90-94] In an experimental setting the calcium ion concentration influenced the permeability of the corneal endothelium. Probably, the calcium-dependent transmembrane cadherine proteins in the adherens junctions play an important role in this equilibrium, as they are integral parts of the AJCs of endothelial cells.[94 95] The cytoskeletal protein F-actin, found to be connected to the AJC, was shown to be involved in maintaining the predominantly hexagonal cell shape of the endothelial cells. Hence it was implicated in the forming of polymegathism in unstable or injured endothelia. Dibutyryl-cAMP and forskolin induced condensation of F-actin at the cell borders and dispersion of the tight junction network in cultured bovine endothelial cells, resulting in decreased endothelial barrier function. The AJC therefore seems to play a central role in endothelial cell architecture and endothelial barrier physiology. The AJC appears furthermore to play a role in inter-cell connectivity. Contact inhibition preventing migration of endothelial cells is supposedly modulated by the AJC. Contact inhibition occurs under normal circumstances and provides a stable monolayer of endothelial cells. It also occurs during migratory and enlargement reactions of the endothelial cells after endothelial cell loss and wound healing.[94 96 97]

Regarding the exact nature and location of the endothelial pump there is still no unequivocal answer. Both a metabolically active $Na^+/K^+$ ATPase ion-exchanger, located in the basolateral membrane of the endothelial cell, and $HCO3^-$ (bicarbonate) efflux via the apical membrane have been indicated as the primary ion pump. Water transport is thought to concur through osmotic gradients provided by ion transport. Inhibiton of $Na^+/K^+$ ATPase with ouabain was found to almost abolish corneal deswelling. Bicarbonate efflux was found to play a role in the establishment of a negative electric potential across the endothelium towards the anterior chamber aqueous.[71 98-106] Later models integrated the role of both pumps, including a variety of mechanisms and ion-exchangers for bicarbonate extrusion. [1 4 40 45 79 93 107 108] Recently, integrated models for the mechanisms in water and ion transport in and out of the endothelial cells and through the corneal endothelium in and out of the stroma have become even more complex. New bicarbonate formation and transport mechanisms have been discovered, and also the presence in endothelial cells of the $Na^+$ - $K^+$ - 2 $Cl^-$ ion cotransporters and Aquaporin water channels (specifically: type AQP1) has been established.[109-117] The $Na^+/K^+$ ATPase pump appears to contribute about 80 % of the total endothelial pump function. (H.F. Edelhauser, personal communication, 2005).

## V. Endothelial mitotic capacity and endothelial wound healing.

Proliferative ability of the corneal endothelium appears to be species-specific. Rodent, bovine, and porcine endothelium has evident mitotic capacity that plays a major role in endothelial regeneration after (experimental) wounding. In contrast, in feline, primate, and human endothelia hardly any spontaneous mitotic activity is observed. Only relatively little cell proliferation - maximally 10 % in cats and monkeys, in contrast to up 40 % in rabbits - was observed after severe endothelial damage. These similarities between cat and monkey endothelium and human endothelium make these animals more suitable for experimental models for wound healing than the often used rodents.[28 118-127] On the molecular level, human endothelial cells have been shown to be arrested in the G1- phase of the cell cycle, as opposed to corneal keratocytes that have exited the cell cycle and are in the resting (G0) phase.[128-130] Contact inhibition of endothelial cells appears to induce an inhibitor protein, the cyclin $p27^{KIP1}$, that arrests the cell cycle in the G1 phase. Transforming Growth Factor (TGF)-β2 prevents endothelial cells of entering the DNA-synthesis S-phase that normally would follow the G1 phase. [129 130] However, human endothelial cells have not completely lost their proliferative capacity, as has been shown through human endothelial cell culturing. Especially endothelial cells from the corneal periphery ("endothelial stem cells") and cells from younger individuals and especially embryos have been shown to proliferate. These findings may provide prospects

for the future induction of endothelial regeneration by mitosis, or for a source of viable cells for endothelial transplantation.[129 131-133]

Notwithstanding observations of mitoses and the demonstration of mitotic contribution to wound healing, endothelial wound healing in humans, cats, and monkeys is currently thought to occur mainly through other mechanisms. These include endothelial cell enlargement and migration of individual cells, also called 'elongation and sliding', as well as spreading of collective groups of cells. Larger cells may later contract, but very large, multinucleated cells, created by amitotic cell division, may also be observed.[1 28 40 118-120 127 134-148] During endothelial wound healing, a posterior banded layer or posterior collagenous layer (PCL) is often secreted and added onto Descemet's membrane.[1 145 149] In experimental endothelial wounding, the expression of fibronectin changed in and around the cells as well as in the aqueous humour during the process of endothelial migration/spreading and PCL deposition. Fibronectin is an extracellular matrix protein that plays a role in the connection of endothelial cells to their basement membrane. These findings were therefore thought to reflect differences in the maintenance of attachment between endothelial cells and Descemet's membrane during the reparative process. Similar observations and conclusions were made in relation to the glycosaminoglycans keratan sulfate and chondroitin sulfate, and the wound healing protein thrombospondin.[150-154] The type (freeze vs. scrape) and size of the wound determined whether healing was found to occur mainly through collective cell spreading, or through both spreading and migration of individual cells. In small scrape wounds mainly healing through spreading occurred in which cell connectivity was maintained, without much of actin re-arrangement or extension of actin stress fibers. In contrast, after freeze-injury or more extensive scrape wounding, (myo-) fibroblastic transformation was observed in (part of) the endothelial cells. Both individual migration of endothelial cells with deposition of posterior collagenous layers, and en-block spreading of collective endothelial cells with maintenance of normal endothelial cell morphology were now observed. Usually, actin rearrangement and appearance of actin stress-fibers, as well as assembly/disassembly of microtubuli could be detected.[155-162]

These experimental results might explain why large wounds close slower than smaller wounds and why wounds close in different rates and patterns after heat-, freeze-, or scrape injury. Other factors that influence the endothelial wound healing rate are age and inflammation. Experimental wounds in older animals (rabbits) closed slower than in young animals. Wound healing in the presence of severe inflammation was retarded. It appears that the phenotype of endothelial cells in culture is modulated by polymorphonuclear cells.[134 163-166]

Regarding endothelial function, in healed wound areas the barrier function of the endothelium was found to recover earlier than the pump function. Usually after wound healing there was no difference in $Na^+/K^+$ ATPase pump site density as compared to non-injured endothelia. However, the presence of very large multinucleated endothelial cells, more often seen after larger wounds, seemed to be correlated to poorer pump function.[123 134 145 167-169]

Growth factors such as Mesodermal growth factor (MGF), Epidermal growth factor (EGF), Platelet derived growth factor (PDGF), basic and acidic Fibroblast growth factor (FGF), Transforming growth factor alpha (TGFα), and Vascular endothelial growth factor (VEGF) have been shown to increase endothelial wound healing rate, by promoting cell migration. Some of these growth factors, such as EGF, also promote mitosis in endothelial cells of species with more mitotic capacity.[146 147 170-193] Hepatocyte growth factor (HGF), and Keratinocyte growth factor (KGF) also stimulate cultured human endothelial cell proliferation, as did EGF.[194 195] EGF is probably the most powerful growth factor in endothelial wound healing. It promotes human and animal endothelial cell migration, through actions on protein-kinase-C-mediated alterations in the actin cytoskeleton. It also promotes endothelial cell proliferation, especially in susceptible species or cells. EGFs actions can be enhanced by agents including fetal human, respectively bovine serum, insulin, retinoic acid, and prostaglandin E2.[194 196-202]

Indomethacine, an inhibitor of prostaglandine E2 (PGE2) synthesis, has a direct stimulatory effect on endothelial wound healing through the promotion of the confluent spreading mechanism, which is the alternative to cell migration. However, it has an inhibitory effect on the individual migration of endothelial cells through the inhibition of PGE2, which in turn downregulates EGF.[146 201-205] Experiments have shown a detrimental effect of corticosteroids on endothelial wound healing, either in direct contact with the endothelium in in-vitro studies, or in vivo after topical or regional administration in animal experiments. This is interesting as topical or regional administration of corticosteroids is commonplace after keratoplasty. The mechanism of this inhibitory effect of corticosteroids on woundhealing is unknown.[206-208] Other drugs may also influence endothelial wound healing. Colchicine has been shown to delay endothelial wound healing, both by arresting cell division in species where this is occurs regularly, and by inhibiting cell migration. Both effects probably result from inhibition of cytoskeleton microtubuli assembly.[155 209] Cytochalasin is known to block actin filament polymerization, and has been shown to suppress endothelial cell migration after wounding.[155 210-212]

# VI. Endothelial biology.

## a. Natural history of endothelial cell loss, and its effect on endothelial morphometry.

At birth, normal human endothelial cell density (ECD) is around 6000 cells/mm$^2$, perhaps even higher. [213-217] As was discussed in paragraph V, the mitotic capacity of endothelial cells is restricted. The ECD is expected to drop rapidly in the first few years after birth, when the corneal surface still increases. As a result of the observation of apoptosis in endothelial cells, it also has to be concluded that these cells are subjected to ageing. Therefore a further decline in ECD during life is to be expected, even in the absence of major endothelial damage.[40 218]

Changes in both endothelial morphology and cell size occurring with increasing age had since long been documented and indicated age-related endothelial cell loss.[41 219] After the widespread introduction of the specular microscope, cross-sectional epidemiological studies showed that with increasing age the mean endothelial cell area increased, i.e. the ECD decreased.[21 213 220-227] Corneas remain transparent with high as well as with significantly decreased ECDs. Clear corneas with low ECD can for instance be seen years after corneal transplantation. This indicates that "normal" endothelial cell densities provide for a substantial physiological reserve. Furthermore, it is evident that ECD is not directly correlated to endothelial functional capacity.[1 40] However, ultimately there is a critical ECD of 300 – 500 cells/mm$^2$ below which endothelial barrier and/or pump functions become insufficient to maintain corneal transparency. Inversely a mean endothelial cell size of 2700 – 3200 $\mu^2$ is the critical level above which transparency cannot be maintained. Finding such a low ECD was found to predict corneal decompensation. [1 22] Remarkably, even when overall ECD was still substantially above the critical level, the presence of giant endothelial cells has been found to be related to decreased overall endothelial deswelling functions.[167] Anyway, until levels close to the critical threshold ECD are reached, it is rather the rate of endothelial cell loss than the ECD per sé that indicates a risk for endothelial decompensation. From prospective and histological studies it became clear that the estimated age-related, "physiological" endothelial cell loss in adults is 0.5 to 0.9 % annually. [40 214 228 229] Regression models have been constructed to evaluate patterns of ECD loss with increasing age. Initially, linear models were proposed.[16 220 221] Since in childhood ECD loss proceeds at a much higher pace than after adolescence,[21 214 229 230] a biphasic linear model,[213 216] a non-linear model,[27] and an exponential model[229] have been proposed. In other fields of biology often biphasic exponential decay patterns are encountered (W.J. Armitage, personal communication, 2004). Hence, recently such a biphasic exponential model for physiological ECD loss was proposed.[231]. The model is:

$$ECD_t = p * \exp(-at) + q * \exp(-bt)$$

In which $ECD_t$ is the ECD at time t; p and q are constants the sum of which is equal to the ECD at time zero (initial ECD); and a and b are exponential rate constants.

The residual standard deviation indicating the goodness of fit was 113.9 cells/mm$^2$. Half times for the two components of decay ('fast' and 'slow') are calculated as: 0.693/(the exponential rate constant). For physiological cell loss, the model found half times for the fast component of 3.1 - 3.5 years, and for the slow component of 224 - 277 years. See figure 2.VI.1. [231] these results were based on the data from Möller-Pedersen and Yee et al.[216 226] The half times illustrate the rapid ECD-loss in youth which may partly be caused by corneal growth and not only by the loss of endothelial cells. Under normal circumstances a large physiological reserve of endothelial cells exists. The very long half-time of ECD loss in the 'slow' phase also explains why for middle-aged and older individuals the ECD-loss can be quite adequately described using linear models. [227 232]

The advent of (semi)-automated endothelial analysis techniques made it easier to analyse the size and shape of individual endothelial cells. The initially interchangeably used morphological concepts of pleomorphism and polymegathism could now be quantified. [233-236] Pleomorphism means the amount of variability in shape: (increased) pleomorphism implies a decrease in predominantly hexagons, accompanied by an increase in pentagonal and heptagonal cells. See Figure 2.VI.2. Polymegathism represents variability in cell size; (increased) polymegathism implies the occurrence of substantial deviations from a normally fairly uniform and narrow cell size distribution. See figure 2.VI.2. A regular-sized, hexagonal pattern is considered to be the most stable and efficient two-dimensional array, both geometrically and thermodynamically. [39 235 237-241] See figure 2.I.2. Increased pleomorphism and polymegathism often indicates endothelial repair, possibly after cell loss. At the least it is a sign of endothelial mosaic instability or endothelial "stress".[123 144 219 236 241 242] At this point it should be pointed out that endothelial cell loss, although it may be present, is neither the only explanation, nor a necessary condition for polymegathism. An electron microscopic study demonstrated that in polymegathous corneas the lateral cell walls do not run orthogonal but oblique to the endothelial and Descemetal planes. This may provide an explanation for the occurrence of both smaller and larger than normal apical (and basal) cell areas, without notable changes in ECD. The individual endothelial cell volume remains the same in this case.[233 243 244] Both pleomorphism and polymegathism have been expressed in several morphometric measures. Some of these parameters that are currently more often used, are the coefficient of variation of cell size (CV, i.e. the standard deviation divided by the mean of the cell size) as a measure for polymegathism, and Hexagonality (i.e. the percentage of hexagonal cells on the total of observed

cells) as an inverse measure for pleomorphism.[233 236 241 242 245] A general finding was that both pleomorphism and polymegathism increased with increasing age.[213 220 226 229 232 246-248]. This might reflect a decrease in endothelial barrier function or (perhaps less likely) in pump function with age or in disease. However, significant functional deteriorations are probably foremost related to the prevalence of giant endothelial cells, especially when their number increases. [167 233 235 243 249-252] Some authors rate the importance of pleomorphism and polymegathism higher than ECD. Morphometric parameters for pleomorphism and polymegathism are more sensitive indicators of endothelial imbalance or endothelial distress and repair. They are probably also more closely related to endothelial function and endothelial functional reserve than ECD / cell size. [233 239 243 251 253-255]

Table 2.VI.1 shows data from four in-vivo studies concerning normal values of ECD, Coefficient of variation, and Hexagonality, in relation to age. Differences between ECD findings from these studies may reflect differences in methodology of measurements, exclusion criteria, and group size. Rigorous exclusion criteria and a relative small group size in one study[226] was opposed to inclusion of patients with diabetes and a significant number of included paired-eye ECD counts in a second study.[232] A third study did not mention the inclusion of patients with diabetes,[227], and a fourth reported on summated data from different groups with different but very little exclusion criteria, and used measurements with two different specular microscopes.[223] Furthermore the subjects investigated in different studies probably have significant differences in racial and perhaps socio-economic demographics. ECD in a Japanese population such as in the study by Inoue et al. is usually higher than in other populations.[232 256] The data of Yee et al. on CV and Hexagonality,[226] and from the prospective study by Bourne et al.[229] are much more in the same order of magnitude (CV: 0.26 – 0.29; Hexagonality 73 - 61 %) compared to those found by Inoue et al.( CV: 0.63 - 0.66; Hexagonality 39 – 36 %);[232] these differences may be explained by the same bias factors mentioned above for ECD.



b. **Regional distribution of ECD over the cornea and differences between contralateral eyes.**

The studies in the part a. of this paragraph all described central ECD findings, i.e. ECD measured from the corneal centre. Early studies found no significant difference in ECD between the central corneal areas and what they called peripheral areas. [136 221 226 257-259]. However, the investigated populations in these studies were either too small to detect significant differences,[136 221 257] or "peripheral" endothelial photographs were actually taken quite close (i.e. 2 – 4 mm) from the corneal centre,[226] or the statistical techniques used to evaluate differences were inadequate.[259 260] Several more thorough studies did demonstrate regional differences. Peripheral areas, and specifically the superior corneal area (in subjects under 50 years of age) yielded a higher ECD.[27 260] In light microscopy studies of

the far corneal periphery towards the limbus, significantly higher ECDs were found.[248 261 262]. A recent study comparing four different assessment methods confirmed that ECD increases with distance from the central cornea towards the periphery.[263] Increasing age appeared to cause more heterogeneity in these regional ECD differences, probably reflecting endothelial cell redistribution after endothelial cell loss.[264 265]. Independently of age however, ECD was higher in the far corneal periphery, specifically superiorly. This may reflect the presence of endothelial stem cells.[129 131 264-266]

Most studies agree that in healthy subjects with pairs of eyes with a similar medical history, differences in central ECDs between contralateral eyes are only small,[27 221 222 224 226 267] although such a difference may be statistically significant and therefore may need to be reckoned with.[222]

**Table 2.VI.1 ECD, CV and Hexagonality: age related findings (cross-sectional studies).**

| Parameters & studies | | | |
|---|---|---|---|
| **Age groups** | **20 – 29 years** | **50 – 59 yrs** | **80 – 89 yrs** |
| **ECD (in cells / mm²)** | | | |
| Hoffer & Kraff 1980 (n = 1523)[223] | N/A | 2355 | 2256 |
| Yee et al. 1985 (n = 32)[226] | 2942 | 2685 | 2316 |
| Abib & Barreto 2001 (n = 784)[227] | 2834 | 2344 | 2077 |
| Inoue et al. 2002 (n = 1819)[232] | N/A | 2605 | 2457 |
| **CV (coefficient of variation of cell size = s.d./mean of cell size)** | | | |
| Yee et al. 1985 [226] | 0.27 | 0.29 | 0.29 |
| Inoue et al. 2002 [232] | N/A | 0.63 | 0.66 |
| **Hexagonality ( % of hexagonals, on the total number of cells)** | | | |
| Yee et al. 1985[226] | 73 % | 64 % | 61 % |
| Inoue et al. 2002 [232] | N/A | 39 % | 36 % |

Fig. 2.VI.1. Bi-eponential regression model for physiological endothelial cell loss.
(From: Armitage et al, Invest Ophthalmol Vis Sci 2003;44:3326-31).

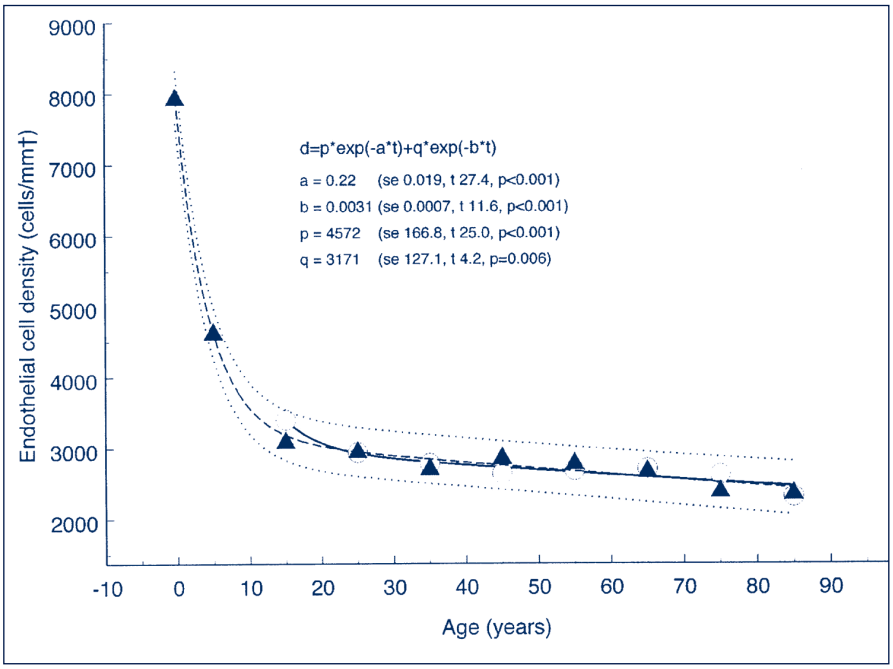
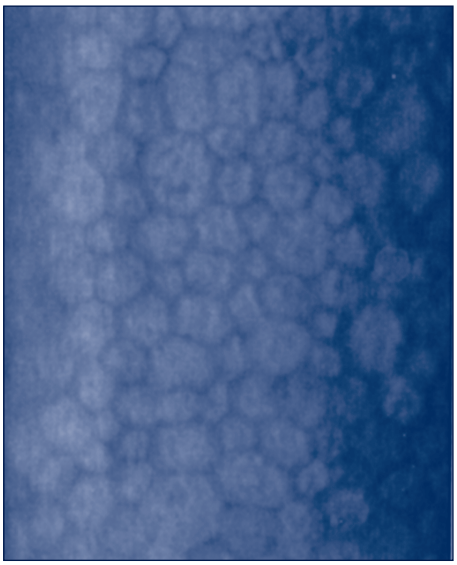



Fig. 2.VI.2. Specular microscopic image of an endothelium with substantial polymegathism and pleomorphism.



## VII. Endothelial morphometric assessment, in vitro and in vivo.

### a. Specular microscopy.

Vogt described the principles of viewing the living endothelial cells with a biomicroscopy (slit lamp) technique.[3 268] At the border of two structures with different refractive indices a small part of the incident light is directly, i.e. not diffusely, reflected. Thus in what Vogt called 'das Spiegelbezirk' (the specular area) of optical borders, 'Spiegelbilder' (mirror images) of the structures at such a border are produced. These can be observed when the observation angle equals the incident angle of the light. See figure 6.1. Vogt described and showed images of the corneal endothelium, bordering the corneal stroma and the aqueous in the anterior chamber, and of the lens epithelium, bordering the aqueous and the crystalline lens proteins. With regards to the endothelium, he discussed the influence of the dominant epithelial reflex on the image, and described the observation of Hassall-Henle warts, guttata, Descemet's folds and other phenomena.[3]. It is possible to also visualise with the biomicroscope some of these morphological findings in or close to the endothelium using a technique called 'indirect specular microscopy', in which light reflected from the anterior iris surface leads through indirect, diffuse retroillumination to an image in the indirect specular microscopic area, and which gives a more three-dimensional impression.[269] Cellular phenomena on the anterior surface of the crystalline lens or an IOL can also be observed using a direct specular microscopic technique. These observations however are extensively described elsewhere and are further left out of consideration here.[3 270 271]

In 1968 Maurice described the design of an instrument he called a 'specular microscope', for the viewing and photographing of the corneal endothelium in an intact globe which was based on specular reflection principle.[8]. This prototype of the contact-type specular microscope, equipped with a 'dipping cone' for applanation of the cornea, was quickly adopted in clinical practice to photograph the endothelium in vivo. Technical improvements regarding the focusing mechanism and light source were added along the way. It was also soon described as a method for the evaluation of donor cornea endothelium, although this caused restrictions on acceptable post mortem times of harvested donor corneas. Furthermore, concerns for microbiological contamination of donor corneas were raised. Specular microscopy was not generally adopted in American eye banks until 2001 (E. Pels, personal communication, 2006).[10 11 13-20 22-24]

In 1970 Brown showed an example of an in-vivo endothelial "macrophotograph" obtained with a non-contact technique that also used the specular reflection principle.[9] Subsequently, several studies using non-contact endothelial photography were published; either with special microscopes or with ordinary

slit lamps at high magnification. [12 21 213 222 224 225 228 272] There are fundamental differences in optical properties between contact and non-contact specular microscopy.[21 272-275] These will be discussed in detail and illustrated with some calculation examples in Part II, Chapter 6 of this thesis. To counteract the bright epithelial reflex that inevitably restricts the observable field width, (see figure 6.1) several approaches were reported. An adjustment to the dipping cone of a contact specular microscope, combined with the use of a soft contact lens was described. This modification both artificially 'thickened' the cornea-tear film complex and gradually (instead of abruptly) increased the refractive index from the tear film towards the microscope, thus allowing a much broader illuminating beam and hence a wider image.[26 27] A different approach to this problem, called wide field specular microscopy, involved the use of an illumination beam ('slit') oscillating at high frequency and scanning the image plane. Thus an apparently continuous wider image was created, which in fact was composed of a summation of very narrow images from consecutive optical sections. This principle is applicable to both contact and non-contact specular microscopes.[25 27] This same scanning slit principle was later applied in confocal microscopy, see part b of this paragraph VII. Wide field specular microscopy might also improve on sampling errors in polymegathous endothelia.[276]

In-vivo qualitative evaluation of the corneal endothelium became readily available with the introduction of the clinical specular microscope.[277] Moreover, in-vivo morphometric endothelial assessment could now be performed. The assessment of ECD was initially done by manual counting of the number of cells in an area of known dimensions on an endothelial image, after calibration of the magnification. This technique, called 'fixed-frame' assessment, should be performed using the strict counting rules (the so-called forbidden lines principle) described by Gundersen and Sperling and Gundersen. This principle aims at preventing the so-called edge effect; i.e. either counting cells on edges of the counting zone more than once or not counting them at all.[278-280] If these rules were applied and data of more than one count were averaged, acceptable to good (short-term) reproducibility in time was found, as well as good inter-observer reproducibility.[220-222 224 228 281] With the introduction of automated image-analysis, through automated planimetry, individual cell size measurement became available. This is the so-called variable frame analysis.[234 240 280 282-285] This allowed determination of mean cell area and ECD, and also assessment of polymegathism, through the calculation of the Coefficient of Variation of cell size or the calculation of cell size distributions. With other algorithms also cell shape factors such as hexagonality could be calculated.[234 240 285] Assessment options in (semi-)automated variable frame counting methods include the 'dot' or 'centre' method,[286-288] (derived from an earlier semi-automated method using nearest neighbour distances[289]) with which the centres of individual cells in a contiguous group are marked. See figure 2.I.2. This method is necessarily semi-

automated or 'interactive, i.e. manual marking of cells by an observer is mandatory. In the 'corners' or 'borders' method the borders or corners of cells are outlined. The borders method can be performed in a completely automated fashion with software that is based on the recognition of contrast differences between cells and cell borders.[286 287 290-294] Many of the currently commercially available specular microscopes use a CCD video camera to obtain images for analysis and are not dependent on hardcopy prints anymore. Some microscopes have in-built analysis software packages, whereas with other instruments commercial or custom-built software on a personal computer is used, and images are imported with a frame grabber. In most variable-frame endothelial analysis software however, fully automated cell border recognition is still far from flawless. This necessitates manual correction of software-defined cell borders before morphometric calculations are performed.[286 287 290-296] See figure 2.VII.1. For a representative sample in adults over 20 years old, at least 75 cells per image, box, or target area need to be counted. Under 20, the endothelium is usually more homogeneous. The more heterogeneous (i.e. polymegathistic and pleomorphic) the endothelium becomes, the less reliable a single sample from a single central cornea is for determining any of the morphometric parameters. And not only ECD but at least CV or another parameter for polymegathism should be presented in this case. Unrepresentative samples may also occur when the number of cells per sample is small, due to low density. In both these cases more samples from different (para-)central corneal areas should be obtained. [220 246 297-299] The validity of various manual, semi-, and automated assessment methods is discussed in more detail Part II, Chapter 3 of this thesis. The reproducibility of a modern semi-automated morphometric measurement method is reported in Part II, Chapter 4.

Early comparative studies did not report significant differences in ECD measurements between non-contact and contact specular microscopy, and emphasized the greater patient comfort and the lower risk for microbial contamination with the non-contact method. Therefore, non-contact specular microscopy seemed particularly suitable for immediate post-operative endothelial assessment.[275 281] Some of the more contemporary non-contact instruments, such as the Topcon SP-1000, the Konan Noncon Robo SP-8000, and the Topcon SP-2000P, have additional autofocus features, making them user-friendly.[286 287 291 292 294 300] Studies with recent specular microscopes with or without the use of semi-automated endothelial analysis did not find significant systematic differences in ECD measurements between contact- and non-contact specular microscopes, provided that the instruments all had a correct magnification calibration.[287 300 301] Landesz et al. however did find significant differences in ECD between several contact and non-contact types of calibrated specular microscopes with semi-automated analysis. Therefore, even when calibration and the application of correction factors for magnification have been performed adequately, it may still be prudent not to use instruments interchangeably.[291 302]

b. **Confocal microscopy.**

The development and principles of confocal microscopy were recently described in excellent review articles by Jalbert et al. and by Masters, and will only be summarized here.[268 303] The main optical principle is that the illumination (condenser) and the observation (objective) systems have common focal points, hence the name 'confocal'. Thus, light reflecting of structures surrounding the point of observation, which provides out of focus information and which obscures the image, is eliminated. A very high resolution results, allowing for magnification of up to 600 x. To improve the very limited field of view, the focal point is rapidly scanned across the sample, and a real-time on-screen image sequence is reconstructed. For scanning there are two techniques available. Tandem scanning, using a rotating Nipkow disc technique, provides through multiple pinholes multiple single spot illumination and whole area scanning. Slit scanning has also been used in specular microscopy and was described earlier in part a. of this paragraph.[268 303] Water-immersion, contact-type objectives are used with topical anaesthetics. Confocal microscopy provides in-vivo coronal optical sections through the eye. All the corneal layers from the epithelium to the endothelium, including the epithelial basement membrane, Bowman's layer, Descemet's membrane, and lamellar or micro-keratome wound interfaces, and even edema in these layers, can be imaged. Measurement of light reflectivity of different layers can provide valuable information.[303-305] Several clinical conditions, including keratitis, dystrophies and the iridocorneal-endothelial (ICE) syndrome, can be superbly imaged in-vivo.[304 306 307] See figures 2.IX.1-3. All corneal cells, including epithelial cells, corneal nerve plexus, antigen-presenting Langerhans' cells, keratocytes, and endothelial cells can be investigated. This allows morphologic and with image-analysis systems also quantitative evaluation, for instance keratocyte density measurement or ECD measurement. Wound healing processes can also be followed in vivo.[303-305 308-311] Stromal opacities or edema preventing imaging of the endothelium in specular microscopy need not be a problem in confocal microscopy. [303 312] It is however not yet clear what the reliability of confocal ECD measurement is. Studies that evaluated reliability and reproducibility of ECD measurements with confocal microscopy,[313] or compared confocal ECDs with ECDs obtained with contact[296] and non-contact specular microscopy[312] did not take into account factors related to the calibration of magnification. Moreover, some of these studies suffered from inadequate statistical methods. [312 313]

c. **Light microscopy of donor corneal endothelium.**

In the seminal reports of the 1970s, specular microscopy was sometimes used for donor cornea endothelial evaluation.[10 15 19 314 315] Only since 2001 this technique is mandatory in Eye Banks in the United States for the evaluation of donor corneas before they are stored. In the US, almost all corneas are preserved with cold storage techniques. Widespread introduction of specular microscopy for

donor evaluation was delayed, because of the necessity for imaging of a clear cornea, which restricted the post-mortem time (i.e. time between donor cornea procurement and endothelial evaluation; E. Pels, personal communication, 2006). Also, there was fear of microbiological contamination. Both endothelial cell density and morphology, and the presence of guttata can be evaluated with specular microscopy, comparable to in vivo.[316-321] In Europe, simultaneously with the introduction of the organ culture preservation method for donor cornea storage (instead of cold storage, see also paragraph XVI section e.), light microscopy techniques for endothelial evaluation were developed. These are applied several times during the storage process. In some eye banks both specular microscopy and light microscopic techniques that include trypan blue staining are used before the donor corneas are immersed in organ culture medium. This combination of evaluation techniques is often found in German eye banks.[322] However, specular microscopic techniques are of no use anymore after corneas have been preserved in organ culture medium because of corneal swelling. At that time light microscopic evaluation has to be used (E. Pels, personal communication, 2006).

Light-microscopic examination of donor corneas is done usually at the beginning of the preservation period, and always after preservation prior to transplantation. This takes place before transfer of the donor cornea from the storage medium into the transport medium. The criteria that are used to assess whether an endothelium is suitable for transplantation are: the extent of damage to endothelial cells, the endothelial cell density, and endothelial cell and mosaic morphology.[323] Currently, in many eye banks a direct test for endothelial function is not used. Instead, the evaluation of endothelial morphology is used as an indirect assessment. As has been discussed before, morphology reflects stress or damage to the endothelium, and hence to some extent the functional reserve capacity.[324] Damage can for instance be assessed by the recognition of reformation figures, which lead to pleomorphism and polymegathism.[249 325]

To evaluate the extent of endothelial damage, since long vital stains have been used. Actually, 'supra-vital stain' would be a more appropriate term, as not living but dead and severely damaged cells are stained. In 1966, Stocker et al. and other authors experimented with para-nitro-blue-tetrazolium. However, this is not a vital but irreversible stain which makes the stained corneas useless for transplantation. Therefore Stocker later investigated and favoured trypan blue for reversible evaluation of endothelial damage in donor corneas. [326-329] Trypan blue (TB) staining of donor corneas was later adopted by Sperling, Pels, and others.[325 330-333] Staining with 0.1 – 0.3 % TB for 30 – 60 seconds has now become routine practice for endothelial damage assessment in many cornea banks. The properties, toxicity, and different applications of trypan blue will be described more extensively in Part III, Chapter 8 of this thesis.

Possibly more sensitive techniques to assess endothelial cell damage and viability have been proposed as alternative assessment methods, but most of these are mainly suited for research purposes. These include:

- Fluorescein di-acetate vital staining;[322 329]
- Combined acridine orange and ethidimium bromide staining, to identify both viable and nonviable cells. This staining however may be toxic to the endothelium, especially after exposure to light;[334]
- Janus green photometry, which gives a direct (i.e. without stained cell counting) quantitative assessment of endothelial cell damage;[335-337]
- Photometric MMT-assays (see also chapter 7 and chapter 9);[338]
- In-situ TUNEL, short for "TdT dUTP mediated nick end labelling", a technique that is specifically used to demonstrate apoptosis.[339]

Except the fluorescein and acridine orange/ethidimium bromide staining these are all irreversible techniques, which can therefore only be used for research purposes and not for donor cornea evaluation.

In contrast, confocal laser scanning microscopy assay after calcein AM and ethidium homodimer-1 dual vital staining, redox fluorophotometry (in combination with specular microscopy), and $^{31}P$ magnetic resonance spectroscopy, all allow for non-invasive, non- destructive assessments of donor corneal endothelial viability c.q. metabolism.[338 340 341] These techniques may provide different and perhaps more accurate information, especially on endothelial function, but require extensive investments in time and instruments – in contrast to the extremely cheap and fast trypan blue staining (E. Pels, personal communication, 2006).

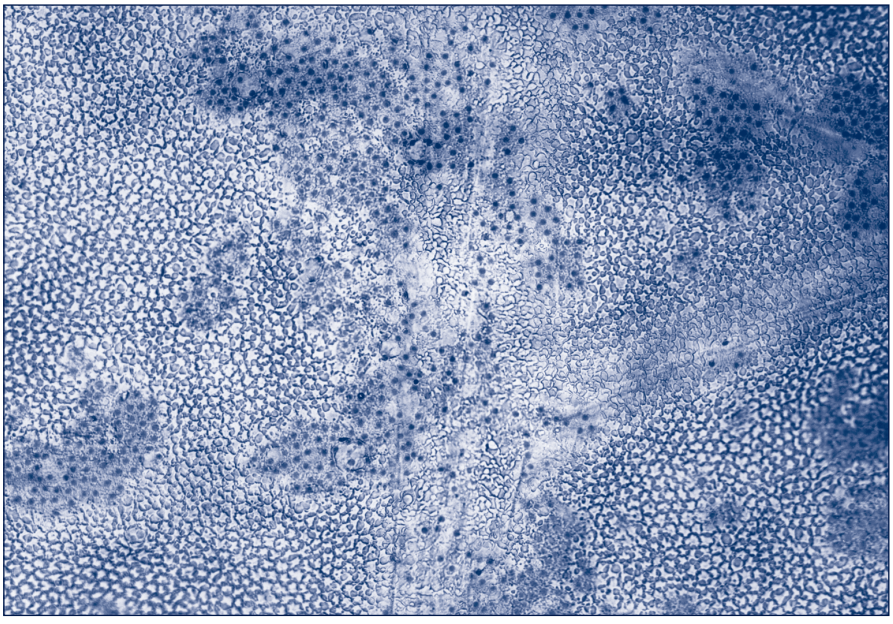


Another dye, lissamine green, was proposed early on by Hassard and Kirk for supra-vital staining of the donor cornea endothelium.[342] This vital stain did not gain popularity, but another observation in the same study, that application of hypotonic solutions can visualise endothelial cell borders by provoked swelling of the intercellular spaces, proved to be very valuable and was further developed by Sperling.[343 344] In order to be able to estimate ECD and assess endothelial mosaic and cell morphology in light microscopic donor cornea evaluation, it is necessary to visualise the endothelial cell borders. For research purposes, silver staining had been an often used technique.[345] In combination with TB staining, the dye Alizarin red S gained much popularity and is currently still in use for accurate light microscopic ECD-assessment and evaluation, in endothelial research.[346-354] Both silver and alizarin red S staining needed tissue fixation and were irreversible, and therefore not suitable for donor cornea evaluation. For this purpose now the provoked intercellular swelling principle, in which application of a hypotonic solution (e.g. 1.8 % sucrose, or 0.45 % NaCl) is applied.[332 343 344] Exposure to the hypotonic solution is often performed directly in

combination with TB staining. This sequence, when performed under sterile conditions, allows a complete, reversible light microscopic evaluation of the endothelium of transplantable donor corneas. See figure 2.VII.2. Both solutions are easily removed by thorough rinsing in buffered saline. [323 332 333 355] The ECD is estimated with a calibrated graticule in the ocular of the light microscope, using an empirically established estimation nomogram.[323] More recently, semi-automated image analysis methods have been developed to obtain more accurate endothelial cell density assessment and more quantitative morphometric evaluation,[356-359] as had earlier been proposed for specular microscopic donor cornea assessment.[360] However, these systems appear to be very sensitive to calibration errors, image quality, and surprisingly also to the composition of the preservation medium. [361 362]

Fig. 2.VII.1 **Example of a specular microscopic image, analysed with the "borders" variable frame endothelial assessment method.** See colour figure on page 327.

Fig. 2.VII.2 **Donor cornea endothelium after trypan blue supra-vital staining and sucrose provoked intercellular swelling. Note that the cellborders are clearly visible due to swelling, that damaged cells have trypan blue stained nuclei, and that intercellular swelling and cell mosaic morphology is poor over Descemet's folds.**



## VIII. Endothelial function tests.

### a. Pachymetry.

Changes in corneal thickness reflect alterations in both the endothelial barrier and the pump functions. Baseline corneal thickness is an individual characteristic for each person.[72 363 364] Corneal thickness measurements a.k.a. 'pachymetry' or 'pachometry' (from the ancient Greek παχύς, thick, or το πάχος, thickness) are clinically usually performed with either optical or ultrasound measuring instruments.[303 363 364] The traditional optical principles used for pachymetry included: 1. focussing on anterior and posterior corneal surface (successively or simultaneously), 2. the use of specular reflections from the anterior and posterior corneal surfaces, and 3. the use of scattered light reflections from the anterior and posterior boundaries of an optical section through the cornea.[72 84 85 303 363-367] Slit lamp pachymeters, using principle 3., and specular microscopes with pachymetry capacity, using principle 2., have been described and are available commercially.[294 300 363-365 367] Confocal microscopes can also be used to perform pachymetry, using principle 1.[303] Other optical pachymetry techniques include optical coherence interferometry and optical coherence tomography, both based on differences in refractive indices, causing changes in speed of light and back reflections at corneal boundaries,[363 364] The technique of the Orbscan corneal topograph (Bausch and Lomb), uses instead of the conventional Placido disc images, the scanning slit principle described earlier combined with advanced image analysis techniques. In this way it can measure both the anterior and posterior corneal curvatures, and also the thickness of the cornea.[363 364 368] Recently the Pentacam has been introduced, a commercially available topograph and pachymeter based on a rotating camera using the old Scheimpflug principle. With this instrument cross-sectional images through the cornea (and crystalline lens) can be obtained, and hence also reliable corneal thickness measurements.[369]

The other major principle currently applied for pachymetry is based on ultrasound. Both 'regular' ultrasound probes (about 20 MHz) and the higher frequency (50 MHz) Ultrasound Biomicroscope (UBM, Humphrey Instruments) are contact instruments. The thickness measurement is based on the time lag between an emitted ultrasound pulse and the reflected echoes from the acoustic interface at the corneal posterior curvature, related to the velocity of sound in the cornea.[363 364]

Several studies compared many different instruments based on both ultrasound and optical pachymetry. Considerable differences in thickness measurement and reproducibility were found and moreover, these differences appeared to vary between the different studies. It is therefore advisable not to use instruments with different measurement principles interchangeably in prospective studies.[288 300 303 368-370]

Normal values of pachymetry from a larger study with one of the older optical

techniques showed a central thickness of 0.523 mm and a peripheral thickness of 0.660 mm. There was a small (but not significant) difference in men having higher values than women, and there were also differences in different age groups, with significant thinning in the corneal periphery in the higher age groups.[366]

b. Hypoxic stress test.

The function of the endothelial pump can be assessed in vivo by measuring changes in corneal thickness following hypoxic stress. Corneal hypoxia is produced by exposing the anterior surface of the cornea to a low-oxygen/high-nitrogen atmosphere, or by placing a thick contact lens onto the eye. The accumulation of lactic acid produced by anaerobic metabolism in the epithelium causes stromal swelling, either through osmosis or by directly inhibiting the endothelial pump. After reinstating normal oxygen tensions to the anterior surface, deswelling occurs over the next two to four hours at a rate consistent with endothelial pumping. As evaporation from the anterior surface contributes to corneal deturgescence, ideally the relative humidity should be controlled, or the investigated eyes should be kept closed during the experiment. Very accurate and reproducible pachymetry is needed to assess corneal deswelling properly. A possible relation between endothelial morphology or ECD and hypoxic stress may be influenced by other factors, including contact lens wearing history.[40 255 371-379] As an alternative method for inducing corneal edema, cold stress may also be used. Especially in eyes with subnormal corneal sensitivity cold stress invokes corneal swelling.[380]

c. Fluorescein fluorophotometry.

The barrier function of the endothelium can be assessed in vivo by measuring the permeability of the endothelial barrier to fluorescein. Fluorescein can be administered systemically via the oral or intravenous route, and enters the anterior chamber via the uvea (corpus ciliare). Alternatively, it is topically instilled into the conjunctival sac, and is taken up through the cornea into the aqueous, with or without the use of iontophoresis. Subsequently the fluorescein concentration in both the cornea and the anterior chamber is measured fluorophotometrically at repeated time intervals. Using farmacokinetic formulas, from these concentrations, their differences, and their change over time, the permeability of the endothelial layer to fluorescein in the anterior chamber can be determined. The method is time-intensive and calls for a fluorophotometry instrument.[1 40 84 85 364 377 379 381-384]

d. Investigational perfusion of corneae.

From the 1970s onwards, experiments have been performed in which (donor or animal) corneae had been mounted in a perfusion chamber, followed by exposure of the endothelial side to a continuous flow of solutions with varied compositions. Morphologic assessment of the endothelium via light, electron, and specular

microscopy, as well as functional evaluation of the endothelial function by means of pachymetry can be performed before, during, and after the experiment. In this way, in many studies toxic effects of medications and other substances were evaluated. Another application was the determination of the optimal constitution of intraocular perfusion solutions and the concentration of its constituents. The use of this technique to test the viability of endothelial cells on donor corneas, an application with direct clinical implications, has also been suggested.[385-396]

e. **Endothelial pump site quantification.**

As was discussed in paragraph IV, the $Na^+/K^+$ ATPase is a very important ion pumping (and hence water transporting) mechanism in the corneal endothelium. It is thought to constitute up to 80 % of total endothelial pumping capacity (H. Edelhauser, personal communication, 2005). These $Na^+/K^+$ ATPase pump sites can be quantified in vitro with Tritium-($^3$H-)labeled ouabain. Ouabain binds selectively (and blocks) $Na^+/K^+$ ATPase, in the ratio: one molecule ouabain to one $Na^+/K^+$ ATPase pump site. Scintillation spectroscopy quantifies $^3$H-uptake, and hence the number of molecules of ouabain bound, by a disc of endothelial cells on Descemet's membrane with known dimensions and known ECD. With these known parameters, the number of $Na^+/K^+$ ATPase pump sites per cell can be calculated.[100 101]

## IX. The primary endotheliopathies.

Primary endothelial disorders, also known as neurocristopathies, result from abnormal migration, proliferation, or differentiation of the neural crest cells, the embryological progenitors of the endothelial cells. Hence, most primary endotheliopathies are congenital. An acquired primary endothelial abnormality is endothelial metaplasia. This may accompany any of the congenital primary endothelial disorders, and implies that endothelial cells assume the phenotype of another, similarly differentiated tissue. Epithelioid metaplasia implies the loss of contact inhibition, increased keratin expression, and proliferation of surface microvilli (e.g. in posterior polymorphous dystrophy and in the iridocorneo-endothelial syndrome). Fibroblastic metaplasia implies that the cuboidal cells become fusiform, may loose apical junctional contact and possibly become multi-layered; this is a non-specific reaction to stress and can be seen in many endothelial disorders, and after many noxious stimuli including (experimental) trauma. Most endotheliopathies usually are accompanied by the deposition of a posterior collagenous layer (PCL), formed of long-spaced collagen, posterior (i.e. more towards the anterior chamber) to Descemet's membrane [40 397 398]

Abnormal migration of neural crest cells results in the mesenchymal dysgenesis syndrome, including Peter's anomaly and the Axenfeld-Rieger syndrome which

were briefly addressed in paragraph III (Embryology of the endothelium) earlier in this chapter.[65]
Abnormal differentiation of neural crest cells causes the endothelial dystrophies: Congenital Hereditary Endothelial Dystrophy (CHED), Posterior Polymorphous Dystrophy (PPD), and Fuchs' Endothelial Dystrophy (FED). All endothelial dystrophies are bilateral disorders, although not always with a completely symmetric presentation, particularly not in PPD.

CHED, a rare disorder with both autosomal recessive and dominant inheritance, presents at birth with a mild to severe complete corneal edema, due to endothelial failure. The endothelium demonstrates fibroblastic metaplasia, of Descemet's membrane only the anterior banded part exists, and a disorganised PCL is present. The anterior segment is usually normal, in contrast to the mesenchymal dysgenesis syndromes. In severe cases penetrating keratoplasty is necessary.[1 27 40 217 397 398]

In contrast, PPD is usually a more slowly progressive disorder. It is suggested that its onset is in the perinatal period, since in some affected areas (so-called 'pits') only a very thin portion of the posterior non-banded part of Descemet's membrane exists. PPD is usually inherited in an autosomal dominant pattern, but there is a variable expression. Clusters of blister-like endothelial vesicular lesions are seen, in groups, traumatic-break-like bands, geographic forms, or even over large areas of the cornea. In these lesions endothelial cells are either large and pleomorphic, or small with indistinct boundaries. Some endothelial cells show marked epithelioid metaplasia. See figure 2.IX.1. Usually a thick PCL is deposited. Penetrating keratoplasty is often not necessary. [1 27 40 217 397-401]

Fuchs' endothelial dystrophy (FED) is the most common endothelial dystrophy, with autosomal dominant and sporadic forms. It is more common in women, and usually becomes apparent around the age of 40, with the biomicroscopic appearance of cornea guttata. Guttae are excrescences of collagen protruding from Descemet's membrane into the anterior chamber. Pigment phagocytosed by endothelial cells can also be observed. Over the collagen deposited in guttae lie abnormal, enlarged endothelial cells. Current concepts of the pathophysiology agree that abnormal, diseased endothelial cells produce the collagenous material that is accumulated in the guttae. See figure 2.IX.2. Relating to the pathophysiologic mechanism causing the dysfunctional endothelial cells however there are many theories, none of which is proven. The fibrinolytic system, hormonal influences, increased apoptosis, and mitochondrial abnormalities have all been proposed as mechanisms. The result is in any case that the affected endothelial cells progressively thin and are finally lost, and that the ECD progressively drops. The remaining cells also thin and spread out. Apical junctional complexes may be lost, preceding fibroblastic metaplasia. A PCL is de-

posited. Barrier function is thought to be lost gradually, whereas pump function may initially be increased, delaying the onset of stromal edema. However, eventually the pump-function deteriorates, demonstrated by the decline in functional $Na^+/K^+$ ATPase. When more guttae are formed and more endothelial cells are lost, perhaps accelerated by intra-ocular surgery, stromal edema and even bullous keratopathy may occur. Corneal dehydration by hypertonic saline drops or ointment or by blow-drying usually provides mostly temporary improvement of edema and vision. Eventually, keratoplasty is often necessary. [1 27 40 101 217 397 398 402-406]

The Irido-Corneal Endothelial (ICE) syndrome cannot easily be classified as a dystrophy, because of several characteristics. For one, abnormal *proliferation* of neural crest cells is thought to cause some of the typical changes. Epithelioid metaplasia of these cells is a common and striking feature, which gives this syndrome sometimes a phenotypic resemblance with PPD. Diminished numbers of endothelial cells have also been encountered in ICE syndrome, probably caused by necrosis and/or apoptosis, which indicates that degeneration plays a role as well. As pathophysiologic mechanism, besides neural crest cell abnormalities or embryonic ectopia, inflammation (iridocyclitis and endotheliitis) has been mentioned and observed. Viral infection (HSV, EBV) has been suggested as a cause, and indeed viral DNA has been detected in some but not all examined patients. A "two-hit" hypothesis has been proposed, with abnormal pluripotent neural crest cell nests or ectopic embryonal epithelial cells accounting for the first hit. Inflammation or infection later in life delivers the second hit, causing proliferation and metaplasia, and hence the late manifestation and/or progression. ICE-syndrome is usually unilateral, with no or only minimal morphological changes in the other eye. It occurs mostly sporadic (isolated cases of familiar involvement exist), and typically affects women (female to male ratio 5 : 1) with an onset usually in the third to fifth decade. Occasionally bilateral involvement is encountered, especially in men or in familiar cases. On bio- and specular microscopy the endothelium has a 'beaten-metal' appearance. The pathognomonic 'ICE-cell' is recognized with specular microscopy. It is very irregularly shaped, has oval dark and light intracellular bodies, and has a 'negative image', i.e. a dark cell surface and light cell borders. (Normal endothelial cells have light cell surfaces and dark cell borders on specular microscopy). See figure 2.IX.3. On an ultrastuctural level, the "ICE-endothelium" shows characteristics of epithelial metaplasia. In partial corneal involvement there is a sharp demarcation line between affected areas and areas with normally appearing cells that have either higher or lower densities than in the unaffected eye. Descemet's membrane under a thick PCL is normal, implicating a post-natal onset. Secondary features, including corneal edema, peripheral anterior synecchiae, glaucoma, and changes in iris architecture and pigmentation, result from degeneration of endothelial cells on the cornea and paradoxical proliferation of endothelial cells onto the iris or trabecular meshwork. Collagen (extended basement membrane) under

the ectopic endothelium produces peripheral anterior synecchiae, ectopic pupil, and traction tears of the iris. Along with the predominance of some of these (secondary) features, the ICE syndrome used to be subdivided into essential iris atrophy, Chandler's syndrome, and Cogan-Reese syndrome (also known as iris nevus syndrome). This labeling of subtype does not seem to be very useful however, and eyes can progress from one phenotype to another. Glaucoma needs to be controlled, and sometimes keratoplasty is indicated. [1 27 40 217 398 407-409]

It has been implied that the aetiology of PPD, CHED, mesenchymal dysgenesis, and ICE syndrome may to some extent be related, in that common genetic defects may play a role. PPD and CHED have been linked to a defect on chromosome 20. Even in Fuchs' (FED) a similar genetic defect may be implied; a defect in the COL8A2 gene coding for collagen VIII has been identified in families with FED and in a family with PPD. Alternatively, common observed features may also be attributed to a final common pathway in pathophysiologic mechanisms, such as non-specific secondary dysplasia, instead of to a common primary genetic cause. In this respect also phenotypic similarities between ICE syndrome and Axenfeld-Rieger anomaly can be pointed out. PPD has further been found in association with Alport syndrome, a basement membrane disorder characterized by heriditary nephritis and sensorineural hearing loss.[1 27 40 217 364 397 398 401 409]

Fig. 2.IX.1 **Confocal microscopic image of a localized endothelial abnormality, typical for Posterior Polymorphous Dystrophy. (Courtesy of C. Weenen, MD).**

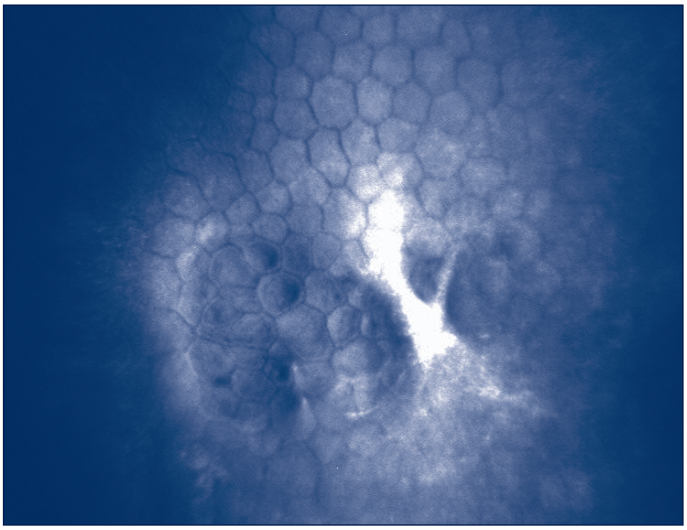


Fig. 2.IX.2 A confocal microscopic image of Fuchs' endothelial dystrophy. De dark and the highlighted white areas are abnormal endothelial cells over guttata. (Courtesy of C. Weenen, MD.)

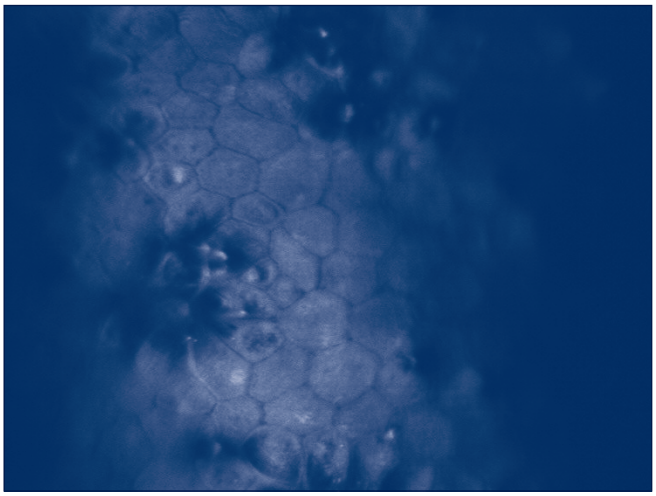
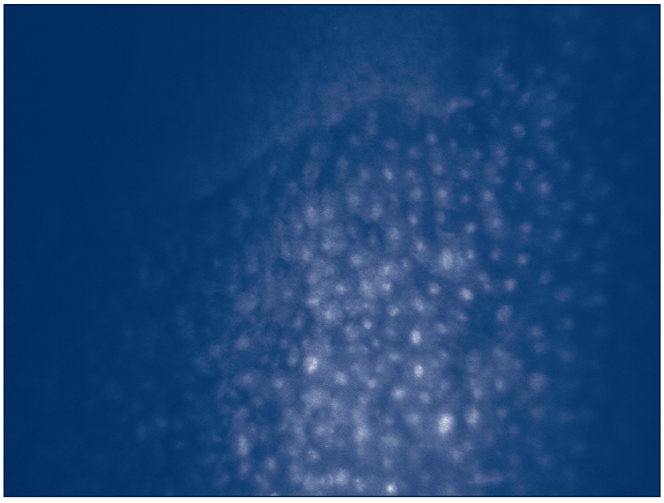


Fig. 2.IX.3 Confocal microscopic image of an endothelium affected by the irido-corneal-endothelial (ICE) syndrome. Note the "negative image" of the abnormal "ice"-cells with dark cytoplasm and light nuclei. Also note the demarcation line between normal and abnormal endothelium. (Courtesy of C. Weenen, MD.)



## X. Secondary endogenous endotheliopathy: ocular and systemic disease.

In uveitis, leukocytes in the anterior chamber sometimes migrate between and beneath endothelial cells causing keratic precipitates. In mild cases this is usually reversible with no notable endothelial cell loss. However, in more severe and/or chronic uveitic episodes, as well as in cases of herpes simplex uveitis with or without endothelitis, the ECD centrally or in the area of the keratic precipitates may stay decreased with increased pleomorphism and polymegathism. Endothelial function may be importantly diminished during uveitic episodes.[1 23 40 410-415]

In acute angle-closure glaucoma, significant endothelial damage often occurs, related to the duration of significantly raised IOP. Hypoxia due to stagnant aqueous, as well as concomitant inflammation may be pathophysiologic factors.[27 416 417] Corneal swelling in angle-closure glaucoma seems to occur when the endothelium has indeed been damaged.[416] Endothelial cell loss may occur in pigmentary dispersion glaucoma and especially in pseudo-exfoliative glaucoma. Pseudoexfoliation syndrome without glaucoma led to a lesser decrease in ECD.[27 40 418-420] The endothelial cell loss observed after glaucomato-cyclitic crises, also known as Posner-Schlossmann syndrome, may (in part) be caused by the inflammation.[27 40] Lower ECDs found in congenital/infantile glaucoma may have resulted from the IOP, from breaks in Descemet's membrane (Haab's striae), or be secondary to corneal enlargement.[1 27] In primary open angle glaucoma, affected eyes have been reported to have fewer endothelial cells and greater annual losses, but this remains controversial.[27 40 364 421-423] In one study the ECDs were inversely proportional to the means of IOPs.[424] Although not entirely excluded, a clinical detrimental effect of topical medication has not been demonstrated.[27 40 364 421]

Diabetes mellitus, both the insuline-dependent and the non-insuline dependent types, has been shown to affect the endothelium. Polymegathism and pleomorphism were observed, indicating a higher susceptibility to stress (eg. surgery). ECD may also be decreased in long-standing diabetics, but this is not an unequivocal finding. Endothelial pump and/or barrier functions may be affected. Descemet's membrane is usually normal.[40 253 364 377 425-428] Argon laser photocoagulation in diabetics has been found to induce endothelial cell loss that is correlated to the amount of energy delivered to the eye by the photocoagulation.[429]

Cystic fibrosis has also been implicated in endothelial pump and barrier dysfunction, but remarkably, a higher rather than lower ECD has been observed. This remained unexplained.[426]

## XI. Ocular trauma, and effects of radiation, topical, and regional medication.

Birth trauma to the cornea by forceps delivery is the cause of Descemet's membrane ruptures which in turn may lead to localized endothelial cell loss. Penetrating corneal trauma causes focal, and in larger trauma also generalized, endohelial cell damage and loss. The amount of endothelial cell loss correlates with the dimensions of the wound. Blunt traumata, including airbag trauma and the so called annular keratopathy, cause focal endothelial cell damage. Irreversibly damaged endothelial cells are shed, and the bare Descemet's membrane is repopulated by the wound healing mechanisms described earlier.[1 23 40 364 400 417 430-436] Epithelial downgrowth sometimes occurs after a penetrating injury or intraocular surgery. The epithelial cells stemming from the conjunctiva may overgrow the endothelial cells, especially in case of a pre-existent endothelial disease, and also overgrow chamber angle, iris and ciliary body.[23 27 40 437]

UV-B radiation may induce endothelial damage. No effect by electromagnetic irradiation of other wavelengths has been reported.[1 40]

As discussed in the previous paragraph, endothelial damage by current topical ocular medications was not unequivocally demonstrated in clinical trials. In animal experiments some indications for endothelial damage by the drug timoptic was found. Topical epinephrine and phenylephrine were found to have some detrimental effect, but toxicity may also have been related to the preservatives in the preparation. The carbonic anhydrase inhibitors may have an action on endothelial physiology, i.e. by blocking (part of) the endothelial pump. It is however not certain whether (prolonged) administration causes irreversible effects.[23 364 377 421 438-441] Much more substantiated is the endothelial damage that can be caused by preservatives in ophthalmologic topical drugs. Benzalkonium chloride, chlorobutanol, methylparaben, propylparaben, thimerosal, and sodium bisulphite are examples of these preservatives. All are associated with allergies, dose-dependent epitheliotoxicity, and dose-dependent endotheliotoxity. Endotheliotoxicity occurs in a dose much lower than needed to damage epithelial cells. Subconjuntival injections containing these preservatives may also cause toxic effects.[40 387 442-445] There is a report that chlorhexidine, when used as a preoperative antiseptic for preparation of the surgical field in (non-ocular) head-and neck surgery, may cause not only epithelial defects and keratitis, but also endothelial damage even leading to bullous keratopathy.[446]

## XII. Effects of contact lens wear.

The effect of contact lens wear on the corneal endothelium became an issue of concern when, within minutes of placement of a contact lens, the formation of blebs within endothelial cells and widening of the intercellular spaces were observed.[447] These reversible phenomena represent intra- and inter-endothelial cell edema, caused by stromal acidosis as a result of epithelial hypoxia. This mechanism was discussed earlier in this chapter with the hypoxic stress test, in paragraph VIII.b..[364 448 449] Induction of hypoxia differs among the various contact lens materials. Poly-methyl-methacrylate (PMMA), rigid gas-permeable (RGP), soft hydrogel, silicone, and silicone hydrogel lenses each have different oxygen transmissibilities. The oxygen permeability of a contact lens is usually expressed as Dk/L; D is the diffusion coefficient of the material; k is the solubility coefficient of the material, and L is the average thickness of the lens. Furthermore, the pattern of wear is important, as is the individual susceptibility. [448-450] All corneal layers may be affected by hypoxia, [450] but in this paragraph we will only focus on the endothelium. Prolonged contact lens wear causes chronic hypoxia and hypercapnia. Almost all contact lens types, including RGPs, induce pleomorphism and polymegathism within a few months of the start of wear. [364 450-460] Highly oxygen-permeable ('high Dk') silicone hydrogel and silicone elastomere soft contact lenses, as well as piggyback lenses (an RGP lens on top of a high-water content soft lens) may be exceptions.[450 461-463] The changes become more pronounced and are more slowly reversible with increasing duration of wear over time and the number of hours of wear per day.[364 450 451 457-459] As discussed earlier, polymegathism is not necessarily related to endothelial cell loss.[244] Most studies have been unable to confirm lower ECD in contact lens wearers,[451 456 458 459 464] although this has been observed after PMMA contact lens wear,[433 465] and in subsets of soft contact lens wearers,[450] as well as in contact lens wear after penetrating keratoplasty.[466] Some authors believe increased pleomorphism and polymegathism to precede endothelial cell loss. [457 364 450] Neither endothelial barrier nor pump functions may suffer directly from contact lens wear, and they may even be increased. However, the barrier function may be decreased in case of deep stromal opacities – which may be related to preservatives in contact lens cleaning fluids. At times of stress, eg. in a hypoxic stress test as described in paragraph VIII.c. of this chapter, the functional reserve of the endothelium pump function has been shown to be reduced.[364 375 377 450 458 459 467]

Corneal reshaping with nighttime contactlenses, also known as orthokeratology and as corneal refractive therapy, is discussed in paragraph XIV.

# XIII. Effects of cataract surgery.

## a. Developments in techniques of cataract surgery, intra-ocular lens design and implantation, in relation to endothelial cell loss.

After the introduction of the specular microscope in the 1970s it soon became apparent that both the performance of cataract surgery and the implantation of intraocular lenses (IOLs) could cause significant endothelial cell loss.[14 17 18] Substantial endothelial cell loss may result in the long term in the complication bullous keratopathy (either aphakic, ABK, or pseudophakic, PPBK).[251 468] Factors that are of influence on the severity of endothelial cell loss were now identified. Pre-operative factors should influence decision making, whether and how to operate. Some of these, such as pre-existent endothelial dystrophy, pre-existent endothelial pleomorphism and polymegathism, or a low ECD, are identifiable with a specular microscope. Other factors include previous surgery, trauma, glaucoma, pseudo-exfoliation, and uveitis. Intra-operative factors include direct endothelial trauma; placement, position, and type of an intraocular lens (IOL); toxicity of irrigating fluids; and the surgical technique of cataract extraction. The development of less damaging surgical techniques and IOLs, and the application of safe or even protective intra-operatively used solutions, substances, and pharmaca, has gained much interest. Prevention or early treatment of post-operative factors are further possibilities for the minimization of endothelial cell loss. These include elevated intraocular pressure, persistent inflammation (with or without anterior synecchiae), vitreo-endothelial touch, IOL dislocation, and intermittent IOL touch.[40 251 468-475]

In early studies, the effects on the endothelium of intracapsular cataract extraction (ICCE) and contemporary (anterior chamber and posterior chamber) phacoemulsification were evaluated. IOL placement, which had not been commonplace before, was also subjected to investigation. IOL types used at that time included anterior chamber (angle-supported and iris-attached) IOLs, iris-attached pupillary plane IOLs, and early types of posterior chamber IOLs .[14 17 18 20 23 27 476-481] Measured a few months postoperatively, ICCE caused ECD loss of around 12 %; whereas anterior chamber phacoemulsification caused an ECD loss of 14-34 %; this latter technique was soon after these reports abandoned in favour of posterior chamber phacoemulsification.[23 468 477 478 480] The placement of an IOL was found to independently cause significant ECD loss,[17 23 477 482-487] causing scrutiny of the type and position of IOL. Especially pupillary fixated lenses, which for a while were widely used in combination with ICCE, appeared to be the cause of substantial of endothelial cell loss. This observation initiated changes in preference towards the placement of posterior chamber IOLs and surgical techniques that – in contrast to ICCE - left the capsular support for these IOLs intact. The result was that the extracapsular cataract extraction (ECCE) technique became more popular. By itself however, ECCE did not differ

much from ICCE regarding endothelial cell loss.[237 468-470 472 474 478 480 486-492] Another consequence of the finding of increased ECD-loss associated with IOL implantation stimulated attempts to protect the endothelium during implantation. For this purpose first air, and later viscoelastics (see part b of this paragraph) were used.[468 493] A different approach was the search for IOL materials or coatings that showed less adherence to endothelial cells.[494-496]
The next step in the development of cataract extraction techniques that preserved the lens capsule, was improved posterior chamber phacoemulsification. At 6 to 12 months after modern phacoemulsification with implantation of an IOL in the posterior chamber - either foldable or rigid, and preferably within the capsular bag - central ECD-losses of between 7.5 and 16 % are commonly reported. Only in one study a remarkable 24 % loss was found.[497-501] The amount of cell loss appears to be correlated with incision dimensions. It may be somewhat larger with clear-corneal incisions than with same-sized or even larger scleral tunnel incisions.[498 501 502] Other factors that increased ECD loss included shorter axial length, older age, small pupil diameter, greater infusion volume, and the amount of phacoemulsification energy that was used. Phaco-energy in turn was related to the presence of a harder nucleus.[499 503 504] The technique used during the procedure may influence the ECD loss. Several techniques were developed with the aim to diminish the amount of ultrasound energy needed during phacoemulsification, such as divide-and-conquer nucleofraxis, 'phaco-chop', 'stop-and-chop', 'chip-and-flip', and 'reversed tip and snip'. Most of these techniques were to some extent effective in lowering the surgically induced endothelial cell loss, or to result in less postoperative deterioration in endothelial pump and barrier functions.[505-509] In contrast, prospective studies comparing the older ECCE with manual nuclear expression technique to phacoemulsification, did not find differences in ECD-loss (10-18 %) after 1-12 months. Only small and temporary differences in endothelial function were observed (see also part c of this paragraph) . In the case of a very hard nucleus, endothelial cell loss was in one study even found to be more severe after phacoemulsification than after traditional ECCE.[510-513]

New developments in cataract surgery focus on smaller incisions, to allow the creation of a more closed system regarding intra-ocular fluid currents ("better fluidics"). This closed system diminishes intraoperative pressure changes as well as the intra-operative loss of viscoelastics, which both enable better maintenance of anterior chamber depth. Also, surgical control of nuclear fragments and cortical remnants is enhanced. Additional advantages include faster wound healing and less astigmatism. [514-516] A second important goal in improving phacoemulsification techniques is the reduction of intra-ocular energy transfer, especially heat production. Improved fluidics, which is also aimed for by the use of smaller incisions, provides higher vacuum and necessitates less ultrasound energy. New software modulations for ultrasound energy delivery increase the

length of pausing intervals - the so –called "off" time - between microbursts of phacoemulsification energy delivery. This provides for lower intraocular energy transfer, and lower temperatures at the incision. This last feature may enable phacoemulsification without a cooling irrigation sleeve, through a very small incision (0.9 – 1.4 mm). [515-523] One of these techniques has already been shown to diminish endothelial cell loss.[524] A third factor that is subject to technical improvement is the occurrence of many tiny air bubbles ("cavitation"). This phenomenon may also be detrimental to the endothelium. Oscillatory handpieces have been developed, in order to diminish cavitation and also aiming at decreased intra-operative heat production.[525-527] Many of these developments converge in bimanual phacoemulsification a.k.a. microincision cataract surgery (MICS) techniques that currently get a lot of attention. These involve two small instead of one larger clear corneal incisions. Each of these incisions gives access to the phacoemulsification handpiece, and the other incision is used for simultaneous continuous irrigation with a second instrument. This second irrigating instrument enables enhanced lens (fragment) manipulation including nuclear chopping, which further decreases phaco-energy delivery.[521 522 528-530] Besides the inevitable learning curve for a new surgical technique, currently the most important limitation of this technique is the size of available foldable IOLs. Unfortunately, enlargement of a small incision or even adding a third, larger, incision is still necessary at the moment.[515 530] The few studies reporting on endothelial cell loss after bimanual phacoemulsification showed at 3 months postoperatively a wide range of endothelial cell losses, from 4.6 % to 15.6 %. Again, cell loss was related to nucleus hardness grade.[521 529 531] Longer experience with and improvements in the technique, as well as improved IOLs, may be necessary to translate enhanced intra-operative manipulation and other (theoretical?) advantages into reproducible reductions in endothelial cell loss.

Besides efforts directed at improving phacoemulsification, research is also conducted on alternative techniques for lens fragmentation, among which laser-, water jet-, and sonic-oscillation-assisted lens removal.[516 520]

b. **Endothelial protection during (cataract) surgery: viscoelastic materials.**
Both the use of viscoelastic substances and the insertion of an air bubble in the anterior chamber were attempts to protect the endothelium during IOL insertion. [23 478 532-534] However, since air bubbles themselves had been found to cause endothelial damage after (prolonged) contact,[11 535 536] the use of viscoelastics soon became the preferred technique. They proved to be inert and to provide good protection for the endothelium against endothelial trauma. Not only IOL insertion but other surgical trauma, including air bubble (cavitation) trauma from phacoemulsification appeared to be diminished. Additional advantages included the creation and maintenance of space in the anterior chamber facilitating surgery. Viscoelastics were therefore increasingly used, during ICCE,

and in ECCE, both in non-automated nuclear evacuation techniques and in phacemulsification .[450 478 526 532 533 536-547] Concern arose about intra-ocular pressure increase after the use of viscoelastics, especially Na-hyaluronate. Experimentally, increased aqueous outflow resistance was observed. Meticulous irrigation of the anterior chamber and aspiration of the viscoelastic material after surgery was advocated to prevent or diminish this phenomenon [533 534 538 542 548-550] Current viscoelastics are usually subdivided into two main groups: dispersive, coating substances that are foremost endothelial protectors, and cohesive substances that predominantly maintain space. However, many viscoelastics have both capacities to some extent.[551 552] The materials used include Na-hyaluronate, highly purified (hydroxypropyl-) methylcellulose (HPMC), hypromellose, chondroitin sulfate, polyacrylamide, and combinations (eg. Na-hyaluronate and Na-chondroitin sulfate), with Na-hyaluronate and polyacrylamide providing more cohesive effects, and the other substances as well as the Na-hyaluronate/Na-chondroitin sulfate combination more dispersive effects. Most clinical studies demonstrated less endothelial cell loss with the more dispersive viscoelastics. [532-534 536 542 545-547 549-557] After the introduction of their use in cataract surgery, soon viscoelastics were also used in other types of anterior segment eye surgery such as filtering procedures for glaucoma, and penetrating keratoplasty.[532]

c. **Endothelial function and ongoing endothelial cell loss after cataract surgery.**
After ECCE both the endothelial pump and barrier functions have been measured. Hypoxic stress testing revealed decreased pump function up to 24 weeks after surgery. However, corneal thickness, which was increased during the first postoperative month, returned to normal thereafter. Endothelial permeability as measured by fluorescein fluorophotometry was significantly increased at 3 months but not at 12 months postoperatively.[510 558 559] After phacoemulsification, fluorescein permeability may or may not be increased. Corneal pachymetry was significantly increased one day postoperatively, but returned to normal at 3 months.[500 510 560] Epithelial but not endothelial $Na^+/K^+$ ATPase abnormalities were found after cataract surgery.[561]

Some older long-term studies showed stabilization of the endothelial cell density 3 moths postoperatively, regardless of the type of surgery or IOL.[562 563] In most studies from this period however indications were found for continued endothelial cell loss years after cataract surgery with several different techniques, including ICCE, ECCE, and phacoemulsification, with or without IOL implantation (of different types). The initial ECD-loss ranged between 12 % and 25 %.[493 564-567] In one long follow-up study, the annual loss at 10 years postoperatively was 2.5 %, regardless whether or not an IOL was implanted, and also regardless of which type of IOL was used[492] In contrast, other studies reported that ongoing ECD-loss was related to the type and position of IOL.[480 568] Mechanisms that were suggested for continuing, accelerated ECD loss after cataract

surgery include ongoing endothelial remodelling, chronic low-grade inflammation, exposure to vitreous components, breakdown of blood-aqueous barrier and other changes in aqueous flow. None of these supposed mechanisms have been proven unequivocally.[136 480 486 487 492 564]

Only one prospective study on ECD-loss after modern phacoemulsification and (posterior chamber) IOL implantation with a follow-up of longer than 2 years could be retrieved. In this study an initial loss of 8 % (corrected for physiological loss) 1 year post-operatively increased to a cumulative loss of 11.5 % (corrected) after 3 years, whereafter the ECD-loss stabilized at the physiological level.[569] In line with these findings another study reported at 2 years postoperatively an annual endothelial cell loss of 0.9 %, only slightly higher than the physiological loss.[570] A retrospective study confirmed that several years after phacoemulsification no increased endothelial cell loss was observed, even when an anterior chamber IOL had been inserted.[571]. A tentative conclusion may be that the increased central endothelial cell loss that is observed in the first postoperative years after modern phacoemulsification mainly reflects endothelial mosaic remodelling. This serves to increase ECD in areas with the largest initial loss, such as for instance at the corneal end of the tunnel incision. After the first years, central endothelial cell loss seems to return to physiological levels.

The study by Armitage et al., discussed earlier in paragraph VI.a. of this chapter, looked into biphasic exponential regression models for ECD loss after cataract surgery. It reported a shortened half time (26 years) of ECD-loss in the slow phase after cataract surgery that correlated with higher than physiological ongoing cell loss. However, the data used were from a study in which older techniques of cataract surgery had been applied. The model needs to be updated for ECD-loss after modern phacoemulsification.[231 492]



## XIV. Effects of refractive surgery.

There are two main principles to alter the refractive status of an eye with a (surgical) intervention. One principle is to change the refractive power of a cornea by modifying its shape. For this purpose several techniques are currently used or were used in the past. These include: corneal incisions; excimer laser ablation of corneal tissue; lamellar corneal surgery; the insertion into the cornea of (pretreated) donor corneal tissue or "inert" materials such as hydrogel, silicone, or PMMA; and altering the corneal shape mechanically with contact lenses or thermally with cautery. The other principle is the implantation of intra-ocular lenses of a specific refractive power into the eye, either in adjunction to, or instead and in the position of the crystalline lens. These techniques are respectively called phakic IOL implantation, and refractive lens exchange.[423 572 573]

(Epi-)Keratophakia, corneal alloplastic inlays, manual keratomileusis, and holmium laser, conductive, and thermokeratoplasty,[30 572 574 575] are left out of consideration in the following overview of refractive surgical procedures because they are not widely used anymore.

Radial keratotomy (RK) is an incisional procedure in which 8 to 16 peripheral radial corneal incisions of 90 to 100 % corneal thickness are made, with the aim to flatten the central cornea in myopic patients. In the 1980s and early 1990s this procedure was very popular, especially in the USA. Since then it has been largely abandoned in favour of excimer laser ablation surgery. Three to 12 months after the RK procedure, central endothelial cell loss has been reported to range from 0.4 to 10 %, with case reports of 25-50 % ECD loss in case of perforations. Prolonged endothelial cell loss after RK is questionable. Risk factors for ECD loss in RK include: intra-operative microperforations, increased number of incisions, and a smaller diameter optical zone. Possibly, inflammation also plays a role. Endothelial permeability is not significantly altered after RK.[572 574 576-584]

The 193-nm (= ultraviolet wavelength range) argon fluoride (EXCIted di-MER gas) excimer laser is a powerful instrument for the ablation of corneal tissue. With the excimer laser predictable and stable corneal shape alterations and hence predictable and stable refractive results can be obtained. Because of this predictability and stability, its relative easiness to use, and the favourable safety profile, refractive surgery with this instrument replaced RK soon after its introduction in the clinical practice in the early 1990s.[30 572] Experimental research in rabbits and monkeys has shown that the deeper excimer laser stromal ablation was performed, the stronger the detrimental effects on endothelial cell density, morphology, and barrier function became. Deposition of extra material onto Descemet's membrane was also observed.[423 585-593] The first excimer laser refractive surgery technique that was introduced was photorefractive keratectomy (PRK), in which laser stromal ablation was performed after manual, mechanical abrasion of the epithelium.[30 572] Following PRK, a wide variation of changes in ECD has been observed, including slightly increasing, unchanging, and slightly decreasing central ECDs. Slightly improving, unchanging, or slightly deteriorating endothelial morphology have also all been reported.[594-602] Peripheral endothelial cell densities may be decreased more constantly.[572] Many explanations and influencing factors were proposed in discussions about the different observations regarding endothelial cell density and morphology after PRK. The procedure may have caused no or only minor central endothelial damage, and observed losses may be attributed to remodelling or even to merely physiological cell loss. The discontinuation of contact lens wearing may even have caused improvement of the endothelium. The age of the patient may have influenced whether or not endothelial cell loss occurred. Magnification changes in specular microscopic images by changes in central corneal steepness and thickness after PRK may have influenced ECD measurements (see also chapter 6 of this thesis). [572 596-598 601 602]

Laser in situ keratomileusis (LASIK) was developed to overcome some shortfalls of PRK, in particular the discomfort and pain that subjects experience before the epithelium has healed, and the slower visual recovery. In LASIK excimer laser ablation is performed on a stromal bed, after the creation of a hinged anterior lamellar flap using an automated microkeratome. [603] As the laser ablation is usually performed closer to the endothelium than in PRK, there was some concern for more damage to the endothelium.[423] One study indeed found that morphometrical changes occurred within 15 minutes after LASIK, but these lasted only 24 hours.[604] Up to 1 year after LASIK a wide variation of observations in the endothelium has been reported, very similar to the observations after PRK. Endothelial cell density and morphology were reported to remain unaltered, to have deteriorated only minimally, or to have improved slightly. Again, just as with PRK, many factors were mentioned as explanation for this variation in findings..[604-610] Laser assisted subepithelial keratomileusis (LASEK) is a procedure that seeks to combine some advantages of PRK and LASIK. In LASEK a superficial epithelial flap is made, usually with help of ethanol. Hydro- and viscodissection techniques have also been described. Studies on endothelial changes after LASEK could not be retrieved. The deleterious effect of alcohol on the epithelium has been extensively studied, but no reports were found of its effect on the endothelium in this procedure.[611]

Intrastromal corneal ring segments (ICRS) are PMMA ring segments of 150˚ of arc, with diameters of 6.8 (inner) to 8.1 (outer) mm, and available in several thicknesses (0.25-0.45 mm). They are inserted pairwise into the corneal stroma, for refractive purposes usually in a superior to inferior direction, in order to flatten the corneal centre and steepen the corneal mid-periphery for correction of myopia up to 3 to 4 diopters. Follow-up studies up to 2 years post-operatively have shown no or only minimal (physiological) central ECD-loss, with slightly more loss at the 6 o'clock and 10 o'clock positions.[423 612 613] A confocal microscopy study reported undisturbed endothelium under the ICRS.[614] The predecessor of the ring segments, the intrastromal corneal ring (360˚ of arc), also did not lead to significant decrease in central ECD.[615 616]

Orthokeratology (OK) is actually not a true surgical technique, but aims at altering the corneal shape and refractive power using contact lenses. This technique was first introduced in the 1960s but disappeared from practice after the mid-1980s because of lack of effectiveness and predictive results. However, from the mid 1990s onwards OK has regained interest. The widespread introduction of corneal topography, as well as the availability of new higher Dk contact lens materials, and the concept of 'reverse geometry' design, all contributed to this revival. The reverse geometry design entails flatter contact lens centres and steeper mid-peripheral back surface shapes, which provides for good centration and a central posteriorward pressure. OK has now been renamed "corneal

refractive therapy" (CRT) or "corneal reshaping with contact lenses". The current principle implies to reversibly meld the cornea overnight with nocturnal RGP contact lens wear, so that lower degrees of myopia are corrected. This should obviate the wear of refractive correction during daytime. Night-time wear needs to be ongoing to prevent regression.[617 618] Nighttime CRT with reverse geometry lenses has been shown to cause central epithelial and corneal thinning, which is currently thought to be the mechanism of action; and perhaps also mid-peripheral stromal / epithelial thickening, of which the mechanism and effects are less understood. Epithelial thinning may occur by cell volume loss, cell loss, cell redistribution, or a combination. [618-623] Obviously, of great concern with nighttime CRT is that the epithelial changes seem to lead to an increasing incidence of epithelial complications such as punctate epithelial keratopathy and hence to an increased incidence of microbial infection, especially with Pseudomonas aeruginosa.[624-626] Studies on extended wear soft contact lenses had already shown a manifold increased risk for bacterial corneal ulcers. The incidence of ulcers was 0.2 % with extended wear soft lenses, compared to 0.04 % daily wear soft lenses, and 0.01 % with daily wear RGP lenses.[627-630] In nighttime CRT, the insults to the epithelium, the effects of lower oxygen pressure, and the risk for microbial keratitis may be similar to, or exceed extended wear soft contact lenses.[624 625 631] Studies showed that after CRT, Pseudomonas binding to the corneal epithelium was significantly increased. Even though RGP materials, as used in CRT lenses appeared to bind Pseudomonas much less than high-Dk hydrogel materials, as used in extended-wear soft contact lenses.[632 633] The age of the patient, and the patient's compliance with instructions on wear and care may be an important factor in the rate of keratitis in nighttime CRT. This stresses the importance of good instructions and regular check-ups. Children seem to be more affected by CRT-related keratitis, which is especially troubling as this group may have the best indications for nighttime CRT. They are not yet suitable for LASIK because of unstable refraction. In contrast, CRT can be adapted as the refraction changes with increasing age. It may be less expensive overall. Furthermore, there are hypotheses that CRT might slow down progressive myopia. [620 634 635] In contrast, the ECD did not change significantly in a clinical study on the older OK treatments. ECD nor endothelial morphometry has deteriorated in a recent study with 1 year follow-up, nor in recent case reports.[636-638]

Refractive lens exchange, also known as "clear lens extraction" may be a valuable refractive surgery technique, especially for presbyope, no longer accommodating individuals. The technique and hence the consequences for the endothelium are similar to regular cataract phacoemulsification surgery, except that usually less phaco-energy is needed as crystalline lenses are less hard in non-cataractous eyes. This obviously causes less of a threat to the endothelium.[30] In a retrospective study on phacoemulsification that included cases for refractive indications in high myopes, a 1-year ECD-loss of 2.1 % was reported, increas-

ing to 6.8 % after 4 years.[639] In a study on clear lensectomy for hyperopia (7 to 14 Diopters) using manual ECCE and phacoemulsification techniques, mean endothelial cell loss at 12 months was 7.38 %. This is similar to after cataract extraction with phacoemulsification.[640]

Phakic IOLs are usually implanted in the anterior chamber. One type of IOLs, the Implantable contact lens (ICL®), is positioned in the posterior chamber in front of the crystalline lens, in the ciliary sulcus. The endothelial cell loss after implantation of ICLs appeared to continue for three years (up to 8.9 –12.9 %), after which stabilisation of remodelling of the endothelium was observed.[641 642] In the Netherlands, the most often used type of phakic IOL is the anterior chamber iris fixated Artisan® Iris Claw lens. When implanted for myopia, the endothelial cell loss with this lens was reported to be 4.8-5.5 % at 6 months, 7.2-8.9 % at 12 months, 9.1 % at 24 months, and 9.8-10.9 % at 36 months, implying a loss of only 0.7 –1.8 % in the last two years. This indicates a stabilisation of cell loss, at or just slightly higher than the physiological level after the first year.[643-645] The more recent United States Food and Drug Administration Ophthec clinical trial reported much lower, non-significant endothelial cell losses at 6, 12, and 24 months postoperatively, of 0.09 %, 0.87 %, and 0.78 % from baseline. When corrected for physiological cell loss these losses were even less.[646] In a study on toric iris claw lenses, implanted in myopes with high astigmatism, central ECD increased 2.9 % from baseline at 1 year, which was tentatively explained by endothelial remodelling after discontinuation of contact lens wear.[647] Artisan® phakic IOLs are also implanted for hypermetropia/hyperopia. In one study, the 1-year ECD loss in hyperopic patients was found to be 9.4 %,[648] whereas in another study there was a gain of 1.0 – 3.8 % in central ECD in the first year followed by a decrease from baseline of 8.5 % after two years and of 11.7 % after three years.[649] In this latter study a significant negative correlation between ECD-loss and anterior chamber depth was observed, which had not been the case in an earlier study on myopic implants.[643 649]

In some studies on older types of iris-claw IOLs, including the Worst-Fechner iris claw type, the reported ECD loss was significantly higher. In some cases even bullous keratopathy requiring penetrating keratoplasty was observed. As factors causing ECD-loss were mentioned: pre-existing corneal guttata, low scleral rigidity in highly myopic eyes, eye rubbing, and IOL design factors like high "vault" and prominent optic edge, causing proximity of (parts of) the IOL to the endothelium.[30 572 650] However, in other studies endothelial cell loss was comparable to the later Artisan® models and also appeared to approach physiologic levels at 3 and four years after implantation.[651 652]

With a different type of phakic IOL, the anterior chamber angle-supported Baïkoff phakic IOL, an ECD-loss of only 1.5 % after 5.2 years follow-up was

reported.[653] However, with older types of angle-supported phakic IOLs such as the Domilens®, substantial larger endothelial cell loss was reported, especially in the paracentral areas and along the border of the optic. This was attributed to lens vaulting and eye rubbing, both possibly leading to intermittent endothelial touch. Implantation of this type of IOL has been dicontinued.[654 655] Similar observations had been made in a primate model using angle-supported Kelman Multiflex® phakic IOLs.[656 657]

## XV. Effects of glaucoma surgery.

As mentioned earlier in paragraphs X and XI of this chapter, glaucoma probably has a detrimental effect by itself on the corneal endothelium. Toxic effects of topical glaucoma medications have not been demonstrated unequivocally. Trabeculectomy used to be the mainstay in glaucoma filtration surgery. In this procedure, open connections between the posterior chamber, the anterior chamber, and the extra-ocular subconjunctival/subtenon filtration bleb area are created. Uncomplicated trabeculectomy has been found to result only in minimal central endothelial cell loss at three months postoperatively (3.7 %). In case of overdrainage, leading to a flat anterior chamber with iridocorneal touch, central endothelial cell loss increases to 7.1 % -11.6 % at 3 to 6 months postoperatively. When lenticulo-corneal touch occurred, a loss of 50 % or more has been reported.[416 421 658-660] After combined cataract extraction (phacoemulsification), implantation of a posterior chamber IOL, and trabeculectomy a 1-year ECD loss of 10.4 % was reported, which is similar to ordinary cataract extraction with implantation of an IOL (see paragraph XIII. of this chapter).[661]

As a result of observed complications of overdrainage and hypotony after filtration surgery, in the last decades there has been great deal of interest in non-penetrating filtration surgery, eg. deep sclerectomy and/or viscocanalostomy. Studies on endothelial cell loss after non-penetrating filtration glaucoma surgery could not be retrieved. As the anterior chamber is not entered in this procedure, and as iridocorneal or lenticulo-corneal touch is less likely to happen, endothelial damage may be expected to be less than after trabeculectomy.

Inadequate IOP reduction by or even complete failure of a trabeculectomy also received a great amount of attention. Adjunctive use of the antimitotica mitomycin C (MMC) intraoperatively, and 5-fluorouracil (5-FU) postoperatively aimed at impairing fibroblast and vascular endothelial proliferation. Increased patency of the filters with lower resulting IOPs have been observed, but also a higher incidence of complications such as hypotony, thinner avascular blebs with a higher risk of leakage and subsequent endophthalmitis. Toxic effects on

corneal, scleral, and conjunctival epithelial cells sometimes resulted in necrosis of tissue.[31] Furthermore, even in eyes in which the anterior chamber had not yet been entered, measurable aqueous concentrations of MMC were found after intra-operative application. Therefore, toxic effects of the adjunctive use of these antimetabolites on the corneal endothelium is of great concern. Animal and in-vitro studies showed a dose- and exposure-time-dependent endothelial toxicity of MMC and 5-FU.[662-664] Clinical studies showed a central ECD-loss of 7 – 14.5 % at 3 –12 months after intraoperative MMC or postoperative 5-FU application in trabeculectomy. Factors found to influence postoperative endothelial cell loss were: the occurrence of postoperative hypotony with shallow anterior chamber and endothelial touch; opening of the anterior chamber before the intra-operative application of MMC; the concentration of MMC and 5-FU that was used; the exposure time to MMC; and the application site of the MMC, i.e. episcleral/subtenon vs. under the scleral flap.[660 665 666] Combined cataract surgery and trabeculectomy with application of MMC after wound closure resulted at 3 months post-operatively in a non-significant slight increase in endothelial cell loss compared to after placebo application (7.8 % vs. 3.6 %).[667]

An alternative surgical approach to obtain a more rigorous IOP control is the use of a glaucoma drainage implant a.k.a. aqueous drainage device/shunt/seton. In this technique, a tube in the anterior chamber drains aqueous humour to a subtenon filtering device. Examples of aqueous drainage devices are Baerveldt, Molteno, and Ahmed shunts. Endothelial damage can obviously be caused by overdrainage and flat anterior chamber, and further by intermittent tube-endothelial contact. Clinical studies reporting on amount of ECD loss with glaucoma drainage implants could not be retrieved. The length of the tube has been thought to be a factor in the occurrence of endothelial damage. The material of the tube may also play a role. [668-671]

Surgical and laser peripheral iridectomy and laser trabeculoplasty may be expected to cause focal endothelial cell loss in some instances. In one study a significant decrease of ECD and corneal decompensation has been observed. Especially argon laser iridotomy has been implicated as a cause for bullous keratopathy. Not only direct laser damage to the endothelium but also shear-stress from aqueous current contributes to this adverse effect.[40 416 421 433 672-675]



## XVI. The endothelium after penetrating keratoplasty.

### a. Introduction

In this paragraph, the most important factors for graft survival in penetrating keratoplasty (PK) are discussed. The mechanisms for endothelial cell loss, before, during, and after PK will be addressed. A more general background on the

development, indications, and techniques, of both penetrating and lamellar keratoplasty is presented in Chapter 10. In that chapter also the literature on endothelial cell loss after lamellar keratoplasty (LKP) is discussed.

b. **Transplant failure: statistics and causes.**

Primary penetrating corneal transplants are often considered to be highly succesful.[676] However, in the literature a wide range of 10-year overall graft survival rates is reported for primary grafts, varying from 50 – 82 %..[677-683] Probably, corneal transplant survival rates differ for different patient (=recipient) populations. But the rates definitely differ for different transplant indications, i.e. diagnoses. Transplants for keratoconus have reported 10-year survival rates of over 90 %, followed by transplants for Fuchs' endothelial dystrophy with a survival of 77 – 90 %. Pseudophakic and aphakic bullous keratopathy transplants had a reported 33 – 85 % 10-year survival. These figures are further influenced by presence, type, location, and the removal/exchange/implantation of (secondary) IOLs. Transplants for Herpes Simplex Virus had a reported 56 - 87 % survival.[677-683] For some other indications the graft survival rate is even substantially lower: especially for re-grafts long-term graft survival rates may be smaller than 50 %.[682] 'Therapeutic' keratoplasties are performed when corneal perforation is present or imminent, or in case of refractory corneal disease. Transplants for active infections appear to survive relatively well, but therapeutic PKs for indications such as ocular surface problems, penetrating trauma, and immunological melts and immunological eye diseases usually have long term survival rates of less than 50 %.[681 683 684] Eyes may be considered to be "high risk" for graft failure due to allograft rejection, because of the existence of extensive deep corneal neovascularisation in two or more quadrants, or because of ongoing, active, inflammation, or previous immunological graft rejections. These grafts also have mid- to long-term survival rates of (considerably) lower than 50 %.[680 682 683 685]

In table 2.XVI.1, the main causes for graft failure are presented. The figures are composed from data from an editorial in the Br J Ophthalmol in 2000 that reviewed studies in the UK and Australia, and further from a French and two American studies. The two most important causes, allograft rejection and late endothelial decompensation, together constituted 53 % of graft failures. Endothelial allograft rejection causes graft failure by means of loss of endothelial cells. Late endothelial decompensation refers to graft opacification by ongoing, non-rejection loss of endothelial cells. Additionally, there is another endothelial cause for graft failure. Primary graft failure occurs when donor corneas stay opaque and do not clear in the post-transplantation period. Most often this is caused by insufficient donor endothelial densities or endothelial function, which may result from inadequate preoperative donor cornea evaluation and/or flaws in donor cornea preservation. Thus the total percentage of graft failures due to endothelial reasons may even be higher than 53 %. Therefore, the con-

clusion is justified that endothelial failure, by whatever mechanism, is the single most important pathophysiological factor in PK graft failure. [319 678 680-682 686]

Earlier in this section, the importance of the preoperative diagnosis and/or indication for graft survival was discussed. Other risk factors were statistically identified,[678 685 687] and in one study even correlated to one of three main causes of graft failure: allograft rejection, endothelial cell loss, or ocular surface disease.[685] Risk factors included Herpes Simplex Virus and (deep) stromal vascularisation, which were risk factors in grafts failed because of allograft rejection. Diabetes mellitus, peripheral anterior synecchiae, recipient race, and small trephination size, were risk factors for graft failure due to endothelial cell loss. Glaucoma and topical glaucoma medication were also found to be risk factors for graft failure, in all failed grafts.[678 685 687]

Notably, donor age is probably not a strong predictor of graft survival in these studies. An extensive meta-analysis of many different studies was not able to identify donor age as a strong predictor for graft survival either.[688 689] This may be caused by opposing effects of donor age on graft survival. A higher donor age has been correlated to lower donor endothelial cell density which may predispose for faster graft failure (see also later this paragraph). However, younger donor age has been correlated to a higher risk for allograft rejection, which will also cause poorer survival.[687-690]

In contrast, increasing recipient age was found to be related to decreased graft survival. This was thought to be related to several factors. Elderly recipients were transplanted more often for bullous keratopathy, for which graft survival is poorer than for many other indications. Also, they experienced more complications and co-morbidities after transplant.[687 690 691]

Other factors that have been mentioned to increase the risk for graft failure include: longer operation time, less surgical experience of the operating physician, aphakia/pseudophakia, and larger diameter graft size. Studies on these factors however did not produce unequivocal data. [678 680 687 691 692]

### c. Corneal allograft rejection and its effects on the endothelium.

Corneal allograft rejection has been reported to occur in as little as 2.3 % ranging up to as many as 68 % of all PK cases.[693] Experimental research has shown that immunological rejection mechanisms can occur against cells in all layers of the donor cornea: the epithelium, stroma, and endothelium. The distinctive rejection lines described by Khodadoust and Silverstein were observed in both the epithelium and the endothelium. A clinical pattern of sub-epithelial infiltrates may occur independent from, or concurrent with both epithelial or endothelial rejection signs. Both epithelial and sub-epithelial rejection usually

represent transient immunological phenomena, which usually react well to immunosuppressive treatment and leave no visually disturbing scars. However, endothelial rejection, which may occur as the typical endothelial rejection line (Khodadoust line) but may also have a more diffuse appearance with vascular engorgement, ciliary flush, keratic precipitates, and anterior chamber cell and flare, is potentially much more harmful. In contrast to rejected epithelial cells, rejected and lost endothelial cells are for the largest part not replenished by mitosis, as was discussed in paragraph V of this chapter. Significant endothelial cell loss leads to a breakdown of endothelial barrier and pump functions, and hence to edematous opacification of the graft. Graft opacification may become irreversible when too many endothelial cells are lost.[693-699] Several studies observed significant endothelial cell loss after corneal allograft rejection. The most important factor in the occurrence of more than average ECD loss was a delay in diagnosis and management of the rejection episode. This delay may be explained by the often insidious and atypical onset of clinical symptoms and signs. However, up to 50 % of rejections may be iatrogenically induced, for instance by suture removal. Although the immunological mechanisms in allograft rejection are potentially suppressible with medication, still up to 50 % of rejection episodes are irreversible. Therefore, allograft rejection remains the single most important cause of endothelial cell loss and hence of transplant failure, as was discussed earlier in this paragraph.[678 679 687 698 700-706]

Allograft rejection involves an immunological sequence or " arc". In the efferent arm of the arc, the target cells, in casu the (endothelial) cells of the donor cornea, are specifically killed by the effector mechanisms. Effector cells and antibodies are only activated after the cells of the donor cornea have been recognized as alloantigenic, in the afferent arm of the arc. The recipient Langerhans cells either capture at their resident site circulating corneal alloantigens from the aqueous, or migrate into the donor cornea to phagocytose these alloantigen (the so-called indirect pathway). Antigens are then processed and presented. Consecutive antigen recognition takes place by a recipient T cell. This depends on the presentation of the antigen by the Langerhans cell in context with the Major Histocompatibility Complex (MHC) a.k.a. Human Leukocyte Antigen (HLA) Class II molecules on both cell surfaces. The effector mechanisms in corneal allograft rejection involve mainly cellular killing of target cells. The role of antibodies and B-cells in corneal rejection is probably small. An important part of the cellular effector mechanisms is constituted by the delayed-type hypersensitivity reaction, in which recipient CD4-positive T helper-cells have a central position. CD8-positive T cells and other cytotoxic T cells have also been found to be involved. Furthermore, macrophages are important effector cells found in rejected corneas. [707-716]

Compared to solid tissue organ transplants, corneal transplant survival without immunosuppressive therapy is relatively good. This is thought to result from the

immune privilege of the (grafted) cornea and anterior chamber, which is based on several factors: 1. Absence of blood and lymph vessels in the graft and its bed - in non-high-risk corneal transplants - impairs the influx of leukocytes into the target tissue, the cornea. 2. Presence of a blood-aqueous barrier, formed by the tight junctions between non-pigmented epithelial cells and iris vessels that are non-fenestrated. This does not allow entry of macromolecules or leukocytes into the aqueous under normal conditions. 3. Absence of MCH class II molecules-expressing antigen presenting cells such as Langerhans cells from the (donor) corneal centre. 4. Expression by donor corneal endothelial cells of Fas ligand, which protects them from an attack by Fas(CD95)-positive T effector cells. 5. Presence, both in the aqueous and on corneal cells, of immunosuppressive molecules (among which interleukins, and membrane proteins that impair complement dependent immune effector mechanisms). 6. Active immunomodulation, called Anterior Chamber Associated Immune Deviation (ACAID), by the induction of tolerance to specific antigens, by the graft itself.[712 715 717-719].

In the meantime, the mainstay of current clinical rejection management involves suppression of the efferent arm by intensive topical or regional corticosteroid treatment.[693 699 720 721] Topical or subconjunctival/peribulbar steroids have been shown to reach higher intra-ocular concentrations compared to systemic corticosteroids.[722] In a randomized clinical trial, additional systemic corticosteroid treatment did not result in better rejection reversal, nor in less late recurrences of graft rejection, nor in better graft survival, when compared to topical steroids alone.[723] However in some patients, especially those who are at higher risk for transplant rejection and whose general condition permits it, systemic steroids may still be valid treatment options. These can be administered either orally or via a single pulse intravenously. Systemic immunosuppressants and topical or regional cyclosporine or tacrolimus (FK-506) application have also been studied.[446 693 699 720 724-727].

Alternative approaches to the problem of corneal allograft rejection deal with the afferent phase of the immune arc. Pre-operative Ultraviolet-B irradiation of donor endothelium has been shown to provide lower rejection rates in animal experiments, however at the cost of substantial numbers of primary graft failures. Hypothesized mechanisms for rejection prevention include suppression of Langerhans' cell function, or activation of donor specific suppressor cells.[728] A more common approach to prevent rejection is by ABO-blood group and HLA matching, in analogy of solid organ transplants. HLA-matching can be applied to class I HLA (the classical A, B, and C) antigens, and class II (DR, DQ, and DP) antigens. For high-risk cases, i.e. previous graft failures due to rejections or two or more quadrants of deep stromal vascularisation, HLA-A, -B and -DR matching has become standard practice in our country. Unfortunately in the literature the definition of high risk is not standardized, nor is there consistency in the

weighing of the importance of the different HLA antigens.[729]. Matters have even become more complicated. In addition to the major class I and the class II antigens, there are non-classical class I antigens E, F, and G; however these may contribute to the immune privileged status of the cornea instead of to allograft rejection. [730 731] HLA-A and -B can, and possibly should be typed on the split level (molecular typing) instead of the broad level (serological typing), with special attention to empirically known 'permissible' and 'taboo' mismatches, in order to (rapidly) identify matches with a higher chance for prolonged graft survival.[732-735] Similarly, with more precise molecular HLA class II DR typing, DR mistypings and hence unintentional mismatches may diminish.[736] And then there is the group of minor, HLA-H, antigens, which may be important targets of rejection in failed PKs.[737] These recent refinements may provide an explanation why studies on graft survival after HLA-matching, both in high-risk and in normal-risk keratoplasties, have contradictory results.[680 734 736 738-742] Alternative explanations for different results involve different more or less intense steroid regimens post PK,[740] the above discussed immune privilege factors that may have become increasingly active,[680] or a reduced potential for allograft rejection in older individuals because of a diminished capacity to expand truly novel T-cell clones due to thymic involution in older adults.[741]

For the experimental study of rejection after PK, as well as for endothelial cell loss as described in the next section, PK in cats and monkeys provides an excellent model, with very comparable mechanisms and reactions. Some of the data in this section and in the next of this paragraph stem from such animal models.[743-745]

d. **Endothelial cell loss after PK, not caused by allograft rejection.**

Since long it has been observed that endothelial cell loss from corneal transplants takes place at an accelerated rate, even in the absence of additional surgical interventions or of episodes of clinically overt allograft rejection. Endothelial cell loss appeared to occur until many years after transplantation, up to a reported maximum of 33 years. Whereas initial endothelial cell loss was found to be substantial and rapid, later cell loss appeared to be slow. As discussed in paragraph VI, as long as the ECD remained above a critical level (300 – 500 cells/mm$^2$), the grafts usually stayed clear, even many years after keratoplasty.[1 12 22 27 746-750] The so-called 'clinical reserve' of the endothelium however is significantly diminished, so that after additional insult (e.g. cataract surgery, allograft rejection) the endothelium may rapidly decompensate, causing graft failure that necessitates re-transplantation. [1 40 364] The barrier and pump functions of the endothelium are also still altered long after PK, with a diminished measurable pump rate, which is compensated by an increased barrier function (reduced permeability). Epithelial metabolism of the corneal graft is also altered as compared to the normal situation.[40 364 377 751 752]

Gradual decompensation and failure of corneal grafts without apparent cause,

unresponsive to corticosteroids, and without a history of a rejection episode correlated to the time of graft failure, was called 'late endothelial failure' (LEF) by Bourne and co-workers. LEF was reported to be responsible for more than 90 % of graft failures beyond 5 years post-keratoplasty in their series. Grafts with LEF had a lower ECD pre-operatively, and also a significantly higher cell loss in the first two postoperative months; thereafter the rate of endothelial cell loss was not higher than in the surviving grafts. Light- and Electron Microscopical studies of LEF-grafts revealed irregular-shaped endothelial cells of varying size with many abnormal features (for instance elongated processes, approximating nuclei in adjacent cells, the presence of multinucleated cells, increased microvilli density), lying on abnormal, thickened Descemet's membrane (PCL, see paragraph IX). Degenerating endothelial cells and bare areas of Descemet's membrane were also often observed. All these phenomena can be interpreted as signs of an unstable and highly stressed endothelial cell population. No evidence of acute or chronic rejection was found at the time of graft failure, as had also been described in an older study by Polack.[310 364 678 753-756]

The observations on accelerated loss of endothelial cells after PK gathered in the above mentioned cross-sectional studies have been confirmed by longitudinal studies on (central) endothelial cell loss. The most important of these longitudinal studies has been conducted by the group of Bourne and co-workers. They reported consistently and comprehensively on a cohort of 393 patients, who by now have been followed for at least 15 years, and for 20 years in many cases. At both time points, the loss relative to the donor ECD was 72 % and 77 %, respectively. It should be noted that in their series the number of patients ("n") examined at each of the consecutive follow-up time points decreased. The cause of this decrease was death or unavailability of the patients, but more importantly also the occurrence of graft failure in the previous interval. The mean endothelial cell density measured at each time point is therefore undoubtedly an overestimation.[24 231 310 678 757-760]

Studies from other groups provide data that do not compare easily with Bourne et al.'s data. Differences in results between the various studies should be interpreted with caution, because of significant differences in group sizes; in operative techniques; in whether or not donor corneas had been preserved, and if so, which preservation technique had been used (see also section e of this paragraph); and in methods and instruments for the prospective assessment of ECD. Furthermore, some studies did not provide comprehensive data on endothelial cell loss for their entire study population, but rather gave results for subdivided populations. For example, subdivisions into groups were made for if and how donor corneas had been preserved, for indications for PK/pre-operative diagnosis, as well as for technique of operation (only PK, or additional surgery such as cataract extraction, IOL implantation and/or exchange, and so on).[24 231 310 567 678 701 748 749 757-782]

Remarkable endothelial cell losses were found, especially in the first postoperative year, in donor corneas that had been cryopreserved. In later postoperative years some of these corneas still had sufficiently high ECDs.[761 771 774 783]
In several studies on ECD-loss after PK, attempts have been made to fit regression models to the decrease in ECD. Linear and exponential decay models have been described, both providing a reasonably good fit.[567 678 764 767 773 778] However, since the initial ECD-loss appeared to occur at a substantially higher rate than late loss, and in analogy of other biological decay processes, Armitage recently introduced a bi-phasic exponential decay model (see also paragraphs VI and XII of this chapter, and figure 2.XVI.1):

$$ECD_t = p * \exp(-at) + q * \exp(-bt)$$

In which $ECD_t$ is the ECD at time t; p and q are constants the sum of which is equal to the ECD at time zero (initial ECD); and a and b are exponential rate constants.[231]

He applied this model to the data provided by Bourne in 2001.[310] The residual standard deviation demonstrating the goodness of fit was 109.6 cells/mm$^2$. The co-efficient p representing the initial proportion of ECD applied to the fast phase was 1457.6, with a rate constant of 0.0802; q applied to the slow phase and was 1396.6 cells/mm$^2$ with a rate constant of 0.0027. Half times were calculated as: 0.693/(rate constant) and amounted to 8.6 and 257 months for the fast and slow phases, respectively. The relatively long fast phase indicates that not only surgical trauma may be severe, but also that other factors such as postoperative complications, immunologic reactions, and redistribution must play a role. Armitage found similar half times and also similar annual cell losses (2.4 and 2.5 % respectively) for the slow phase after PK and cataract extraction (ICCE and ECCE); he interpreted this as a common mechanism for late endothelial cell loss, possibly stemming from an innate, non-specific immunologic response (as opposed to allograft rejection), for instance initiated by breakdown of the blood-ocular barrier.[231]

The most recent report on Bourne's cohort of PK patients by Patel et al. showed data from the 15-year's follow-up.[760] These were different from those in Bourne's own report in 2001 on his cohort and on which Armitage's study was based.[231 310] At 15 years post PK Patel found an annual loss of 0.2 %, which is at (or below) the physiological level.[760] This contrasts with Armitage's finding of annual cell losses of 2.4 % in the late phase.[231] It should be noted however that Patel's data were from only 67 grafts out of an original figure of 388 grafts. All graft failures and regrafted eyes had already been excluded, and these 67 represented 30 % of the available clear grafts. Patel's results were therefore biased towards grafts with low late endothelial cell losses.[760]
As mentioned before, Armitage hypothesized that late endothelial cell loss

may be caused by an aspecific, non-rejection-like inflammation, or perhaps a chronic break-down of the blood aqueous barrier, but not an allograft rejection mechanism.[231] More evidence that this was not a T-cell mediated immune response was provided by a recent study that found that chronic ECD-loss was not diminished by HLA class I and II matching, with current high-standard HLA-typing. [782] Other possible immunological mechanisms however were not excluded by the authors, and these may involve antibody mediated reactions, or minor HLA antigen incongruence.[782] As discussed earlier, the pathologic findings in LEF did not point to allograft rejection either. Contradictory data on chronic endothelial cell loss result from studies on autokeratoplasty. In autokeratoplasty, chronic endothelial cell loss can not result from allograft rejection. Three studies, of which two were experimental studies in cats, found late endothelial cell loss similar to after (allogenic) PK. One other study in humans, found endothelial cell loss of nearly physiological levels after autologous rotational PK. The authors of the latter study concluded that after allogenic PK, immunological factors are responsible for chronic endothelial cell loss.[743 784-786]

Factors possibly influencing intra-operative and short-term post-operative ECD loss include, besides allograft rejection, the indication (original diagnosis) for PK, as discussed before. The pre-operative lens status (phakia/aphakia/pseudophakia), has been mentioned as an important factor and the simultaneous placement or exchange of different types of IOLs in different positions has also been implicated. However, several other studies found no clear effect of aphakia or pseudophakia, or placement or exchange of IOLs.[757 787-790] Lens status however probably has some correlation with the indication for PK (i.e. aphakic or pseudophakic bullous keratopathy) and may hence not be an independent risk factor. The triple procedure, i.e. simultaneous PK, "open sky" ECCE, and IOL placement, resulted at a mean follow-up of 10.5 months postoperatively in postoperative ECDs that were comparable to after PK alone.[791] Other factors that do affect postoperative endothelial cell loss are the occurrence of early postoperative (technical) complications,[701] and the amount of surgical trauma. Surgical trauma may be related to several aspects. The trephination technique that is used has been found to influence surgical damage to the endothelium.[792-800] Also technique of and proficiency in suturing influenced graft survival rates and endothelial cell loss.[680 796] The use of intraoperative viscoelastics probably preserves endothelial cells to some extent, although results are not unequivocal. Possible advantageous effects of viscoelastics do not seem to be caused by a protection against immune rejection.[784 801 802] The indication/original diagnosis for PK determines the preoperative state of the recipient endothelium, i.e. the preoperative recipient ECD. Redistribution of donor endothelial cells on to recipient corneas with poor endothelium, a phenomenon that has been demonstrated experimentally,[803 804] may contribute to a more rapid drop in central (donor) ECD in patients with poor recipient ECD. Conversely, endothelial redistribution phe-

nomena from the recipient onto the graft have been observed in patients with PK for keratoconus, for a scrofulous corneal scar, and in patients with primary graft failure or after total graft rejection who experienced spontaneous graft clearing. It was also shown experimentally in damaged grafts transplanted into a host animal with normal endothelium.[805-808] Hence, redistribution phenomena may explain some of the differences in ECD-loss that were observed in prospective studies on ECD-loss after PK in patients with keratoconus (good recipient endothelium), Fuchs' endothelial dystrophy (centrally more than peripherally deteriorated recipient endothelium), and pseudophakic or aphakic bullous keratopathy (overall bad recipient endothelium).[776 777 780] In bullous keratopathy cases, relatively less post-operative ECD loss was found in larger-diameter PKs, in which a larger absolute number of donor endothelial cells had been transplanted and in which the area of the recipient cornea that needed to be repopulated with donor endothelial cells was smaller.[781] The observation that postoperative endothelial cell loss increases in older recipients may similarly be based on the fact that the (peripheral) recipient ECD usually is lower in older recipients. Furthermore, older recipients are less likely keratoconus patients, but more likely patients with Fuchs' endothelial dystrophy or bullous keratopathy.[776 809 810]

e. **The influence of the donor cornea: donor ECD, influencing factors including age and post-mortem time, and storage techniques.**

As discussed earlier in this paragraph, PK graft survival related to late ECD loss depends mostly on initial (donor) ECD and perioperative ECD-loss. The rate of ECD loss in the late, slow phase, is less important, as this is relatively constant.[231 310 811] See figure 2.XVI.1. Applying a minimum donor ECD in eye banks, as for instance recommended by the European Eye Bank Association,[812] is therefore justified.[231 310] Most, but not all studies investigating the influence of donor age on endothelial cell density after transplantation, found that ECD after PK was higher with younger donors.[767 776 779 809 811] Younger donors generally had a slightly higher ECD than older donors. The variation in ECD in corneas of older donors was much larger than those in younger donors. Therefore corneas of younger donors were more often suitable for transplantation, but still a significant proportion of older donors had suitable ECDs.[762 813 814] However, a correlation between donor age and the amount of (chronic) endothelial cell loss after PK was inconsistently found.[678 762 765 776 781 810 815] Donor age per sé therefore does not have to be a restrictive factor for accepting donor tissue; (costs of) a higher discard rate when permitting older donors should be weighed against shortage of donor tissue.[320 321 323 324 762 765 813 815-817] Similarly, even donor corneas from pseudophakic eyes might be considered after thorough pre-operative examination.[818] The cause of death of the donors, especially trauma, and also cancer and sepsis, has been shown to strongly affect endothelial cell loss and/or metabolism during organ culture. Renal failure did so to a lesser extent, and cardiovascular causes affected the endothelial cells the least. [815 819 820] However,

donor corneas surviving the organ culture period after any of these causes of death may do equally well after transplantation. In contrast, other authors found that corneas from donors that had died from injury had less risk of graft failure compared to other causes of death.[821]

It is common knowledge that "fresh donor" eyes cannot be kept and used indefinitely, as there is time-dependent endothelial cell death. This was the reason that several different methods of donor cornea preservation have been developed.[767] The so-called post-mortem time, i.e. the time interval between excision of the corneoscleral button from the donor globe and the storage of the tissue seems to have only a small influence on the suitability of the cornea for PK and on endothelial cell loss after keratoplasty.[779 781 813] Besides donor endothelial cell density, an important factor to consider is the viability of the donor endothelial cells, which is reflected in their morphology. In this regard not only the ECD but also the morphology of the donor endothelium is relevant when assessing suitability of a donor cornea for transplantation.[319 323 324] In paragraph VIII c morphologic and morphometric assessment of donor corneal endothelium is described. Comparable preoperative ECDs in "fresh donors" and after different methods for donor corneal preservation may, due to differences in endothelial cell viability, result in different postoperative ECD losses.[747 761]

For instance in cryopreservation endothelial cells are not lost during preservation but instead in the freezing, and especially the thawing processes. When combined with organ culture preservation, additional endothelial cell loss occurs during organ culture after thawing, possibly due to wound healing processes. The amount of cell loss is such, even with current freezing and thawing techniques and the application of extracellular or intracellular cryoprotectants, that it prevents cryopreservation from being in large-scale use in eye banks. Nevertheless, there are reports of patients with long-term clear grafts after cryopreservation.[747 761 771 774 822-830]

All other techniques of corneal storage: cold storage (4 °C), in moist chambers or in media such as MK (McCarey-Kaufman) and Optisol, and the organ culture-derived techniques (at 31 –37 °C), show time-dependent loss of endothelial cells during the preservation period.[332 364 761 813] MK and Optisol are both examples of preservation media used to prolong endothelial viability compared to moist chamber storage under cold (4 °C) conditions. MK medium is a modification of tissue culture medium 199 (TC 199) and includes Hepes buffer and sodium bicarbonate with added dextran and antibiotics (currently gentamicin); it is still in use in eye banks all over the world. After much research it became apparent that chondroitin sulfate in preservation media provided even better endothelial protection, and thus prolonged preservation time. Optisol, developed from predecessors K-Sol and DexSol, has both Minimal Essential

Medium and TC 199 as base medium and contains chondroitin sulfate, dextran, bicarbonate, pyruvate, antioxidants and adenosine triphosphate precursors. All cold-storage type preservation media contain antibiotics, most often gentamicin alone or in combination with vancomycin. Optisol allows preservation for up to 14 days, and is currently the most often used storage medium for donor corneas in the US.[769 831-847] Cold storage is not routinely used in The Netherlands.

The principle of 'Organ culture' donor cornea preservation is based on maintaining endothelial cell metabolism and allowing repair of damaged cells, by incubation at body temperature or slightly below (31-37 ºC), in media providing nutrients and growth factors. The technique was introduced in the United States by Doughman and co-workers in the 1970s, then introduced in Europe and further developed by Sperling and co-workers, and introduced in The Netherlands and further developed and popularized by Pels, Völker-Dieben, and co-workers.[323 332 762 848 849] Different approaches to organ culture have also been investigated.[850] The exact composition of the incubation medium may vary but usually includes Minimal Essential Medium (MEM) or a variant (with Earle's salts it is called Eagle's MEM (EMEM)), supplemented with Hepes buffer, with extra L-glutamine, fetal calf serum, and sodium bicarbonate in appropriate concentrations. Chondroitin sulfate may or may not be administered. Antibiotics and antifungal medications are added. T500 (high molecular weight) dextran was either added to the culture medium (Sperling), and later, when it was seen to accumulate in the endothelial cells after long exposure, only to the transport medium (Pels), to ensure thinning of the donor cornea prior to transplantation.[323 330 332 333 848 849 851-857] An incubation temperature of 31 –34 ºC was found to result in less endothelial cell loss during incubation than incubation at 37 ºC.[323 330 332 333 813 849] This method allows for longer storage times than with the cold storage techniques. Storage longer than 28 days is possible but often results in substantial endothelial cell loss. However, occasionally storage up to even 48 days still provided transplantable corneas.[323 324 813 849 858]

As cells are lost in a time-dependent way during the preservation period organ cultured corneas may lose more endothelial cells during the preservation period. However, the viability of the remaining endothelial cells on organ cultured corneas may be higher.[848 859] Endothelial cell metabolism is not arrested by low temperatures and the culture medium provides nutrients and growth factors. Therefore, the culture period allows for irreversibly injured cells to be shed, reversibly injured cells to heal, and corneal wounds to be repaired. This is demonstrated by endothelial repair figures that were observed during endothelial assessment. Whether or not it is through apoptosis that donor corneas lose some of their endothelial cells during organ culture is controversial; differences in techniques for establishing the presence of apoptosis may be responsible for incongruous data on this matter.[135 325 330 848 860 861] Anyhow, endothelia of donor

corneas that are suitable for transplantation after a (prolonged) organ culture preservation period may be considered to have passed a 'stress test' for viability of the endothelial cells.[323 324 333 862 863] Additional advantages of organ culture include identification during the storage period of remaining bacterial and fungal contamination. Hence transplantation of contaminated donor corneas may be prevented.[813 864] Longer storage times enable a more elective scheduling of corneal transplantation.[762 848 862 863] In Europe many eye banks currently use the organ culture technique,[812] and the method is also used in Australia and New Zealand.[333]

Clinical studies usually failed to find significant differences in post-transplant endothelial cell losses or graft failure rates between organ-cultured corneas and cold-stored corneas.[762 770 775 862 863 865 866]

**Table 2.XVI.1 Causes of graft failure**

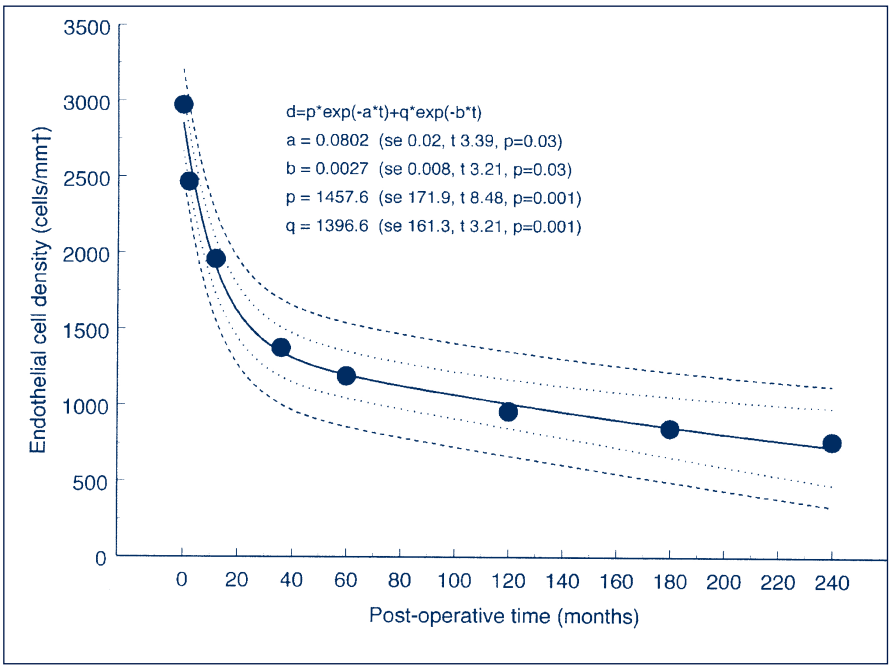
| Cause of failure | ACGR (1997) ref 680 | CTFS (1996) ref 680 | Ing et al. (1998) ref 678 | Muraine et al. (2003) ref 681 | Thompson et al. (2003) ref 682 | Combined data |
|---|---|---|---|---|---|---|
| (Irreversible) graft rejection | 32 % | 34 % | 28 % | 26 % | 27 % | **31%** |
| Endothelial decompensation | 20 % | 17 % | 24 % | 35 % | 29 % | **22 %** |
| Infection including HSV * | 14 % | 12 % | 13 % | 9 % * | 8 % * | **≤ 12 %** * |
| Ocular surface disease * | N/A | N/A | N/A | 14 % * | 18 % * | **≤ 17 %** * |
| Primary technical/donor failure | 6 % | 13 % | 16 % | 2 % | N/A | **± 7 %** *** |
| Glaucoma ** | 8 % | 0 % | 3 % ** | 0 % | 4 % | **± 6 %** ** |
| Disease recurrence | 0 % | 7 % | N/A | 2 % | N/A | **± 1 %** *** |
| Others/unknown */** | 20 % | 17 % | 16 % ** | 12 % * | 15 % * | **≤ 18 %**/**/*** |
| **Number of graft failures in series** | **985** | **201** | **68** | **43** | **385** | **1682** |

*) Numbers for "Infection" and "Ocular surface disease" were deducted in some studies and either included in the category "Others/unknown" or not available in others. These 3 categories therefore may, and probably do overlap in the combined results which are tentative ("≤" signs).

**) In one study this number was taken from another category ("Pupillary block"); not known was how often glaucoma as cause of graft failure occurred in the group "Others/unknown" in this study. Combined results in these categories are therefore tentative and given with "±" or "≤" sign.

***) In some studies, these data were not available or included in the group "Others/unknown". Combined results in these categories are therefore tentative and given with the "±" or "≤" signs.

Fig. 2.XVI.1 Bi-exponential endothelial decay after PK.
(from: Armitage et al., Invest Ophthalmol Vis Sci 2003;43:3326-31.)




## XVII. Effects of intra-operatively used intra-ocular substances, solutions, tamponades, and drugs.

Possible effects of topical drugs on the corneal endothelium were discussed in paragraph XI of this chapter. Intra-operatively used viscoelastics were discussed in paragraph XIII. Anti-proliferative adjunctive drugs in glaucoma filtering surgery were discussed in paragraph XV. Other substances, solutions, and drugs are discussed in the present paragraph. These include substances such as sterilization agents and preservatives that are normally not purposefully applied in the anterior chamber. However, in inadvertent intracameral introdcution they can have devastating effects on the endothelium. Furthermore irrigating solutions; intraocular/intracameral medications including antibiotics, anaesthetics, miotics and mydriatics; and surgical adjunctives and retinal tamponade materials in vitreoretinal surgery will be reviewed briefly.

In 1990 the toxic endothelial cell destruction (TECD) syndrome after routine ECCE was described. Its main feature was corneal decompensation, illustrated by Descemets folds and increased corneal thickness. Pathophysiologically, an endothelial cell loss of 72 % was observed, resulting in a breakdown of endo-

thelial barrier function. The cause was (unintentional) intracameral injection of a toxic detergent residue, which had been stuck with residual viscoelastic material inside reusable irrigating cannulas. Some enzymatic sterilization detergents have been shown to contain the exotoxin subtilisin and α-amylase, which in animal and in-vitro experiments had endotheliotoxic capacity [31 445 867-871] A variant of TECD is the toxic anterior segment syndrome (TASS) in which even more extensive damage to the anterior segment (iris, trabecular meshwork, lens) is caused. This is often accompanied by substantial anterior segment inflammation early i.e.within 24 hours after surgery, and it needs to be differentiated from bacterial endophthalmitis.[872] The variety of possible causes of TECD and TASS is large. For instance, gas sterilization has also been demonstrated to be the cause of TECD and TASS, by producing copper and zinc residues from brass oxidation and degradation. The copper and zinc could be experimentally shown to damage the corneal endothelium.[872-874] Probably, no single sterilisation agent that adheres to residual material on or in instruments, which thus may enter the anterior chamber, can be considered to be safe for the endothelium and the anterior segment. Up to now the safest approach for the endothelium is not to use reusable hollow cannulas, and to clean other instruments meticulously before sterilisation..[445 871 872 874 875] Even ophthalmic ointment inadvertently entering the anterior chamber has been shown to cause TASS. [872 876]

TECD-like keratopathy has further been observed after inadvertent intraocular irrigation with chlorhexidine, cetrimide, and cialit.[877] Tissue glues used to repair corneal perforations, such as cyanoacrylate, are not intended to be used intraocularly but will obviously sometimes enter the anterior chamber. These cyanoacrylates do not seem to destroy endothelial cells directly, but do provoke a severe inflammatory response in the anterior chamber.[124]

To maintain endothelial barrier and pump functions the composition of intraoperatively used intraocular irrigating solutions should be similar to aqueous humour. Their buffer, ionic balance, and pH must be correct (around 300 mOsm and around 7.4, respectively.) Furthermore, their constituents must include contain calcium (barrier function) and a buffer, should include an energy source, and may include an anti-oxidant (e.g. glutathione).[40 385 386 388 392 423 443 854 878]. Examples of well tolerated irrigating solutions are enriched balanced salt solutions as BSS Plus® (glucose glutathione bicarbonate solution), Opeguard MA, and glutathione bicarbonate Ringer's solution. Lactated Ringer's solution has been shown to induce endothelial damage, whereas Dextrose bicarbonate Lactated Ringer's has shown some good results.[388 878-880] The necessity of bicarbonate in intraocular irrigating solutions has been questioned. It does not seem to be a necessary prerequisite for adequate endothelial pump function after surgery. The endothelial pump has been hypothesized to 'hibernate', temporarily shut down, in the absence of bicarbonate.[391 393 394]

Intracamerally used anesthestics such as lidocaine, mydriatics including cyclopentolate phenylephrine and epinephrine, and miotics including acetylcholine, pilocarpine, and carbachol, all have demonstrated dose-dependent endotheliotoxic effects. However, in the currently used doses and concentrations of intracameral drugs such as adrenaline 1:10000, preservative-free cylopentolate 0.1 %, phenylephrine 1.5 %, and lidocaine hydrochloride 1 %, however, no (additional) clinical endothelial cell loss has been observed [396 423 443 881-885] For many common antibiotics including gentamicin, amikacin, cephalotin, cefazolin, ceftazidime, ofloxacin, amfotericine-B, and tobramycin, dose-and /concentration-related endothelial toxicity has been observed. Dosage needs to be low as dose-related retinotoxicity is even a greater concern than endotheliotoxicty.[31 40 395 408 445 886-895] Povidone-iodine in the infusion solution, as an alternative to intracameral antibiotics, needs to be diluted, because of concentration-related endothelial toxicity.[896] Preservatives used in topical medication, should not be used intraocularly, neither should topical or regional drugs containing preservatives be applied when an open connection to the anterior chamber exists. [40 387 442-445]

Pars-plana vitrectomy may have direct detrimental effects on the endothelium, which may be enhanced in the case of aphakia. A contributing factor to endothelial damage in pars-plana vitrectomy may be increased hydrostatic pressure of the infusion fluid during parts of the operation.[897 898] Gases and fluids used as surgical adjunctives or tamponades in vitreoretinal surgery, including air (see also paragraph XIII.), $SF_6$, $C_3F_8$, perfluorocarbon, and silicone oil, have all been correlated to endothelial cell dysfunction and death when these substances are in direct proximity to the endothelium. Damage appears to occur mainly through impaired endothelial metabolism due to decreased aqueous imbibition, rather than through direct toxicity, and increases when exposure times are longer.[11 40 535 899-908]



## References

See page 287.

part

II

# Specular microscopic endothelial assessment and its reliability

# chapter

# 3

# Validity of endothelial cell analysis methods and recommendations for calibration in Topcon SP-2000P specular microscopy

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## Abstract

### Purpose

To report on the calibration of the Topcon SP-2000P specular microscope and the Endothelial Cell Analysis Module of the IMAGEnet2000 software, and to establish the validity of the different endothelial cell density (ECD) assessment methods available in these instruments.

### Methods

Using an external microgrid, we calibrated the magnification of the SP-2000P and the IMAGEnet software. In both eyes of 36 volunteers, we validated four ECD assessment methods by comparing these methods to the gold standard Manual ECD, manual counting of cells on a video print. These methods were: the Estimated ECD, estimation of ECD with a reference grid on the camera screen; the SP-2000P ECD, pointing out whole contiguous cells on the camera screen; the Uncorrected IMAGEnet ECD, using automatically drawn cell borders, and the Corrected IMAGEnet ECD, with manual correction of incorrectly drawn cell borders in the automated analysis. Validity of each method was evaluated by calculating both the mean difference with the Manual ECD and Bland and Altman's Limits-of-Agreement.

### Results

Preset factory values of magnification were incorrect, resulting in errors in ECD of up to 9 %. All assessments except one of the Estimated ECDs differed significantly from Manual ECDs, with most differences being similar, (< 6.5%), except for Uncorrected IMAGEnet ECD: 30.2 %. Corrected IMAGEnet ECD showed the narrowest limits of agreement (-4.9 to +19.3 %).

### Conclusions

We advise to check the calibration of magnification in any specular microscope or endothelial analysis software as it may be erroneous. Corrected IMAGEnet ECD is the most valid of the investigated methods in the Topcon SP2000P/IMAGEnet 2000 combination.

## Introduction

The endothelial cell density (ECD) of a cornea may be important in the planning of intra-ocular surgery, notably in assessing the risk of corneal decompensation. Postoperatively, the ECD can assist in evaluating a surgical technique.

The Topcon SP-2000P is an easy-to-use, auto-focus specular microscope that combined with the Endothelial Cell Analysis Module in the IMAGEnet 2000 software package (both Topcon Corp., Tokyo, Japan) provides several methods for obtaining ECD measurements *in vivo.* As the SP-2000P is a non-contact instrument, endothelial imaging with it carries no risk of epithelial damage or microbiological contamination and is easily tolerated by subjects. Therefore this instrument seems particularly suitable for obtaining ECD measurements before and after intra-ocular surgery.

However, whether the combination of SP-2000P with any of the available assessment methods yielded reliable ECD measurements was yet to be confirmed. In the present study, we checked the magnification calibration of the SP2000P camera and the IMAGEnet software. We investigated the validity of the available ECD assessment methods, in particular of the (semi-) automated methods compared to basic manual counting. The general principles of our methods may also apply to other specular microscopes and endothelial analysis methods.

## Methods

### Calibration

Figure 3.1a presents a screen print obtained with our SP-2000P camera; the double-headed arrow indicates the mask slit width. Each *individual* camera has its own specific mask slit, an aperture that is approximately, but not exactly, 0.2 mm. (In the camera we used we found it to be 0.1914 mm). This mask slit width is a central referential parameter in the calibration of the magnification of a SP-2000P. For proper calibration of the magnification, one should first measure and store the mask slit width of a particular camera, and then use this value to adjust the magnification value in the appropriate menu of the IMAGEnet2000 software according to the formula:
New magnification value =

$$\left( \frac{\text{trueMaskSlitInsideDimension}}{0{,}2} \right) \text{x } 0{,}00115$$

.

(0.00115 is the standard, pre-programmed magnification value in the IMAGEnet2000 software; it uses 0.2 as a standard for the mask slit width.)
The true mask slit width was obtained with an external, micro-grid calibration device manufactured by Topcon for calibration of their specular microscopes. This device was snugly fixed onto the optical emitting/receiving head of the specular microscope. To obtain clear images of the micro-grid on the camera screen without using the SP-2000P's internal flash, an external continuous light source was applied behind the translucent micro-grid. Images of the microgrid were captured and displayed on the camera screen. Suitable images were similar to the diagram shown in figure 1B. With the camera's software, the mask slit and the standard distance between the reference microgrid lines A and B (see fig. 3.1b) were measured and related, resulting in a new slit width. In order to eliminate both observer bias and distortion caused by the position of the microgrid, this procedure was repeated in ten different images each obtained after a slight change in position of light source and grid, and three observers took turns in making the measurements. The mean value of ten measurements was stored in the camera's memory as the true mask slit width.

### Validity Study

Seventy-two eyes of 36 volunteers, aged between 23 and 80 years and with no known ocular or systemic disease were included in the study. From each eye 3 consecutive endothelial images of the central cornea were obtained with the SP-2000P. These were printed in hardcopy (Fig. 3.1.a) using a video printer, and then evaluated on the SP-2000P using the so-called Simplified Cell Analysis (see below). Finally the images were captured and saved on the hard disk of a personal computer with IMAGEnet2000 frame-grabbing and Endothelial Cell Analysis Module software. The combination of SP-2000P and IMAGEnet2000 endothelial cell analysis software provided us with four different ECD assessment methods, as did its predecessor, the SP1000 with contemporary versions of the IMAGEnet.[292] Each of these analysis methods was investigated.

First, from one randomly chosen printed image the ECD was estimated by three different observers, using estimation grids displayed on the SP-2000P screen next to the image of the endothelium. These grids consisted of examples of cell densities of 1000, 1500, 2000, 2500 and 3000 cells per $mm^2$ (Fig. 3.1.a). We estimated ECD in steps of 250 cells/$mm^2$ in order to enhance the accuracy of this Estimated ECD; we did not consider smaller steps in accuracy to be feasible. Each image contained at least 100-150 endothelial cells. Per eye an Estimated ECD was obtained by each observer. Observer 1 was an experienced corneal surgeon, whereas observers 2 and 3 were Ophthalmology residents.

Second, all three captured images of each cornea were analysed by one of us (W.v.S.). using the Simplified Cell Analysis method in the SP-2000P itself, which

basically is a "centre" or "dot" technique.[286 287] In the SP-2000P, this assessment method requires the identification of the centres of ten or more contiguous cells in an endothelial image by pointing them out on the camera screen (clicking them with the camera mouse). Only whole cells that did not sit on any edge of an image were included. As instructed by the Topcon SP-2000P user manual, we identified the centres of on average 15 (10 –20) cells per image. The software in the SP-2000P measures the distances between *individual* cell centres, then computes the mean endothelial cell area, and hence the endothelial cell density. For each eye, the results of these three ECDs were averaged and called the SP-2000P ECD.

Third, all images saved on the hard-disk of the personal computer were subjected to a fully automated analysis by the IMAGEnet2000 Endothelial Cell Analysis Module. This method is based on a planimetric variable frame technique as described by Waring et al.[282] The software determines the borders of at least 100 endothelial cells per image by the detection of contrast differences between cells and intercellular borders in a black-and-white image. *Individual* endothelial cell area is measured by the software, which then calculates the mean cell area and other morphometric parameters including the ECD. A standard setting of contrast and gain in the analysis software was used after preliminary results (not shown) had demonstrated that altering the settings of contrast and gain did not result in a better performance in the recognition of cell borders by the software. The mean of three analyses from each eye was called Uncorrected IMAGEnet ECD.

Finally, one of the authors (W.v.S.) corrected software-defined cell borders in all analysed images in IMAGEnet. This correction entailed erasing any incorrectly drawn or incomplete cell boundaries and redrawing them as they were deemed true. Then the software reperformed the planimetric endothelial cell analysis, and the mean of three analyses of each eye resulted in the Corrected IMAGEnet ECD.

As in the SP-2000P ECD, only whole cells with continuous cell borders were accepted by the IMAGEnet analysis software in the Uncorrected and Corrected IMAGEnet ECDs. Edge-effects, dealing with incomplete cells on edges of images to be analysed, therefore did not have to be taken into account in any of these methods; in Estimated ECD edge-effects obviously were irrelevant.

All of these four differently measured ECDs of each eye were compared to what we adopted as our gold standard ECD: the Manual ECD, a basic fixed-frame counting method.[241 282] We chose this method as gold standard because it was a completely non-automated assessment method, and we were particularly interested in the validity of (semi-)automated methods in comparison to a

non-automated method. The Manual ECD was obtained as follows: a counting box of 2.0 by 5.0 centimetres was placed over a hardcopy videoprint of the photographed endothelium. All complete cells within this box were counted, as well as any incomplete cells on the left and upper borders and on the left hand corners. A count was thus obtained according to the counting rules described by Gundersen, and by Sperling and Gundersen, to reduce counting bias caused by edge effects.[278 279] Each box contained on average about 75 cells (range: 50 - 100). The number of cells we counted in the box was multiplied by a magnification factor to obtain to obtain an ECD (per square millimeter). The ECDs of three images of one eye were averaged to obtain the Manual ECD for that eye. For the calculation of the magnification factor in this method, we micrometrically measured the mask slit width on several hardcopy videoprints (Fig 3.1a, double-headed arrow). The ratio between the printed mask slit width and the true mask slit width, described earlier in the Calibration paragraph of this Methods Section, was calculated to be the linear magnification. This linear magnification was squared to obtain the two-dimensional magnification. The two-dimensional magnification factor for our box was found to be 31.56 x the number of counted cells.

### Data analysis in the validity study:

Results of both eyes of our 36 subjects were pooled, which was justified because we studied differences between assessment methods rather than between eyes. Both eyes of each subject contribute independently to a possible difference between assessment methods. All the various ECDs were scatterplotted against the Manual ECD. Using Pitman's test, we determined whether paired variances were significantly different between ECDs obtained by the several assessment methods and the Manual ECD.[909] A significant difference in variances would indicate a difference in variability due to "noise", i.e. a difference in coincidental, non-*systematic* variability, as opposed to a *systematic* difference between the assessment methods.

The comparability of the two assessment methods was evaluated by calculating the 95 % limits-of-agreement according to Bland and Altman.[910] These Limits-of-agreement were calculated as mean difference ± 1.96 x standard deviation of the difference. The *absolute* differences between the ECDs obtained by each assessment method and the manual ECDs were plotted against the averages of both ECD measurements. In these so-called Bland Altman plots, three horizontal lines are drawn, the middle one representing the mean difference, and the upper and lower lines representing the respective Limits-of-agreement. Ideally, the mean difference between assessment methods is near zero and the Limits-of-agreement remain constant throughout the whole range of measurement values. However, we anticipated the possibility that the scatter of the differences might increase with an increasing absolute value of the ECD, i.e. that

the differences might be proportional to the average level. Therefore, we also performed a logarithmic transformation on the observed ECDs, and repeated the calculation of the Limits-of-agreement and of the mean differences, in order to assess any possible *relative* differences between the measurement methods.

## Results

### Calibration

In the camera and software combination we examined, we found that preset factory values of camera calibration, i.e. the mask slit width, was incorrect, and furthermore that the magnification value had been entered in an incorrect field in the IMAGEnet software. As a result, the IMAGEnet software computed an assumed ECD that was incorrect.

We calculated a correction factor to compensate for the incorrect assumed mask slit width in images that had already been stored. The mask slit width is the linear reference measure for the magnification. The true observed mean endothelial cell area is related to the assumed mean endothelial cell area as the ratio of the squared true mask slit width to the squared assumed mask slit width. The true ECD is the inverse of the true mean endothelial cell area. This resulted in the following formula to obtain the true ECD:

$$\left( \frac{\text{assumedMaskSlitInsideDimension}}{\text{trueMaskSlitInsideDimension}} \right)^2$$

If the magnification value had been put in an incorrect field in the IMAGEnet software this usually resulted in an overestimation of the observed area, for the ECD would then be calculated using the standard 0.2 mm for mask slit width instead of the – in our experience invariably - smaller true mask slit width. This leads to an underestimation of the ECD. In this case, the correction factor to multiply the assumed ECD with to obtain the true ECD in already stored images could be calculated by:

$$\left( \frac{0{,}2}{\text{trueMaskSlitInsideDimension}} \right)^2$$

When considered separately, each calibration error resulted in a different error in ECD, up to 9 %. Because in our example the errors occurred simultaneously, we multiplied both correction factors to obtain the true ECD, which resulted in a smaller error in ECD of 6,9 %. The effects of the two simultaneously occurring

errors opposed each other. Two other important endothelial morphometric parameters provided by the IMAGEnet2000 endothelial cell analysis module obviously did not suffer from these calibration errors. The Coefficient of Variation of cell size (CV = standard deviation of cell size divided by mean cell size) was unaffected because it is a dimensionless parameter in which errors in cell size are divided away, and Hexagonality did not change because it does not depend on cell size but on the ratio of cells with 6 corners to the total number of cells identified.

### Validity Study

Figures 3.2a-c, 3.3, and 3.4a-b represent scatter plots of the different ECD assessment methods, plotted against Manual ECD for each eye. The Estimated ECDs (figs. 3.2a-c, a separate plot for each of the observers) and the SP-2000P ECDs (fig. 3.3) were spread symmetrically but substantially on both sides of the identity line. The Uncorrected IMAGEnet ECDs were almost invariably higher than the manual ECDs, and were widely spread (fig. 3.4a). The corrected IMAGEnet ECDs showed less variation than all the other assessment methods; however, they were higher than the manual ECDs (fig. 3.4b).

With Pitman's test, no significantly different variances were found among the measurement methods, except in one of three Estimated ECDs (p=0.015). Limits-of-agreement were calculated. Figures 3.5a-c show for each observer the Bland Altman plot of the difference between the Estimated ECD and the Manual ECD. Differences between the SP-2000P ECD, Uncorrected, and Corrected IMAGEnet ECD vs. the Manual ECD are shown in figures 3.6 and 3.7a-b. The mean differences and standard deviations, and their statistical significance, with the limits-of-agreement, as well as the mean *relative* differences after logarithmic transformation and their statistical significance, have been summarized in Table 3.1. The only statistically non-significant mean *relative* difference was the Estimated ECD of Observer 1 (1.5 %, p = 0.316). The other statistically significant mean *relative* differences were found to be between – 6.3 % and 6.5 %, with the exception of the difference of Uncorrected IMAGEnet ECD with Manual ECD, which was 30.2 %.

## Discussion

The basic principle of (re-)checking the calibration of any measuring instrument was demonstrated by our findings. Ohno et al. and Modis et al. already showed this in specular microscopy.[287 300] In our study, unchecked preset factory values were found to result in substantial errors, up to 9 %.
We focused on the SP-2000P and the IMAGEnet 2000 endothelial cell analysis software, since this combination is being used for preoperative planning and

post-operative follow-up in several ophthalmic departments in our country. The details of the methods we used are therefore specific to the SP-2000P and the IMAGEnet 2000 analysis software. However, we believe that the underlying principles, such as the formulas we used for the calculation of the true ECDs or the methods applied in the investigation of the validity of assessment methods, may be applicable to other combinations of specular microscopes and endothelial analysis software.

In the study on the validity of endothelial assessment methods we adopted as gold standard assessment method the Manual ECD, a fixed-frame method.[241 282] Although variable-frame methods may give more accurate ECD estimates and more information on other morphometric characterisctics,[241] we chose the Manual ECD because this is a very basic non-automated assessment method to which we wanted to compare the available (semi-) automated assessment methods. The counting rules in fixed-frame methods in order to obtain unbiased counts when dealing with edge effects, were extensively described by Gundersen[278] and by Sperling and Gundersen[279]. Additionally, random aberrations possibly introduced by the counting rules were diminished by averaging counts from three images.

We investigated as factors contributing to validity: 1. Magnitude of any *systematic* difference with the gold standard, i.e. a *systematic* error in the measurement method, and 2. The amount of spread in the differences from the gold standard, reflecting the size of *individual* measurement errors.

The Estimated ECD, when performed by an experienced observer, provides a fast method of ECD assessment, albeit with wide limits-of-agreement (see table 3.1). This implies that this assessment method may be considered as less valid when very accurate ECDs are needed, for instance in the evaluation of endothelial cell loss caused by a surgical technique. It may however be a valid method when a fast, semi-quantitative endothelial evaluation suffices, such as in the preoperative assessment of the risk of a decompensation of a cornea.

The SP-2000P ECD showed similar *systematic* differences with Manual ECD compared to Corrected IMAGEnet ECD and most Estimated ECDs. The SP-2000P ECD also suffered from wide limits-of-agreement (see table 3.1), indicating a low level of accuracy in *individual* measurements. Therefore, in this study the validity of SP2000P assessment is less than that of Corrected IMAGEnet ECD, and does not offer any real advantage over the Estimated ECD, because it gives a false impression of accuracy. It should be noted that with this method we counted only 10 to 20 cells per image, as was indicated in the User Manual. This is considerably lower than the number of cells that was counted with each of the other assessment methods: 75 cells or more. Doughty et al. have shown

that for accurate estimates, samples of at least 75 cells are needed.[299] With larger samples one might obtain narrower limits-of-agreement with the SP-2000P ECD assessment method; however we still found considerable inaccuracies with some larger samples (unpublished results).

Fully automated recognition of endothelial cell borders by the IMAGEnet endothelial cell analysis software was still far from flawless, as was demonstrated by the results of the Uncorrected IMAGEnet ECD. Both the limits-of-agreement and the *systematic* difference with the Manual ECD were of such a magnitude that this method should be considered the least valid and thus the least useful of all, despite of its theoretical advantage in objectivity and its short analysis time. So the ultimate goal of having an endothelial cell analysis method that is both completely objective and valid was not reached with the IMAGEnet 2000 Endothelial Cell Analysis Module.

It is remarkable that both Uncorrected and Corrected IMAGEnet ECDs are systematically higher than Manual ECDs. The reason for this remains unclear. The fact that variable-frame counting methods are compared to a fixed-frame counting method may have introduced a *systematic* difference. However, by adopting the counting rules discussed before, edge-effects are most likely avoided. Perhaps the difference is due to some other, unknown factor in the IMAGEnet-software data processing or calculation. In contrast to the Uncorrected IMAGEnet ECD, the Corrected IMAGEnet ECD is more laborious and required subjective intervention. However, the Limits-of-agreement (table 3.1), which are comparable to those found by other authors[292] indicated that the Corrected ECD is the most accurate of all assessment methods, with the least *individual* measurement errors. The *systematic* difference of the Corrected ECD with the Manual ECD is on the same order of magnitude as for the other assessment methods in this study except the Uncorrected ECD. Most other studies on endothelial assessment methods report a slight *systematic* difference in ECD when compared to either manual counts or other analysis methods[286 287 291-293 302]

In conclusion, when comparing Corrected IMAGEnet ECDs to the Manual ECDs or ECDs obtained with other assessment methods, a correction factor has to be taken into account. However, the Corrected IMAGEnet ECD has a high validity when very accurate ECD measurements are needed, as for instance in follow-up studies. For purposes where less accurate ECD measurements may be satisfactory, such as in preoperative assessment of the risk of corneal edema, estimation of ECD by a trained observer may suffice.

## References

See page 287.

### Acknowledgements

The authors wish to thank E.Pels, Ph.D., L.A.M. de Hollander, and J. Kingma, respectively head, staff, and former staff of the Cornea Bank of the Netherlands Ophthtalmic Research Institute, Amsterdam, the Netherlands, for their methodological advice on, and assistance with manual counting of the endothelial images. Also thanks to P. Gelissen and E. van Buitenen, Topcon Europe B.V., Capelle aan de IJssel, The Netherlands, for their kind assistance in the calibration of the specular microscope and the endothelial cell analysis software.

**Table 3.1 Parameters of the endothelial assessment methods.**

| | Mean difference with manual ECD in cells / mm$^2$ | Standard deviation | Significance (p-value) | Limits of Agreement (absolute) | Mean relative Difference ECD (%) | Limits of Agreement (relative) |
|---|---|---|---|---|---|---|
| **Estimated ECD** | | | | | | |
| Observer 1 | 32.7 | 296.1 | 0.352 | -547.7 , 613.1 | 1.5 %* | - 20.6 % , 29.8 % |
| Observer 2 | 126.4 | 324.6 | < 0.0001 | -309.5 , 562.3 | 5.6 % | - 12.2 % , 26.9 % |
| Observer 3 | - 147.9 | 222.4 | < 0.0001 | -783.3 , 487.5 | - 6.3 % | - 29.3 % , 41.4 % |
| **SP-2000P** | 126.1 | 324.6 | 0.002 | -510.1 , 762.3 | 5.6 % | - 18.8 % , 37.2 % |
| **IMAGEnet** | | | | | | |
| Uncorrected | 725.5 | 349.0 | < 0.0001 | 41.5 ,1409.5 | 30.2 % | 0.2 % , 69.1 % |
| Corrected | 156.4 | 143.8 | < 0.0001 | -125.4 , 438.3 | 6.5 % | - 4.9 % , 19.3 % |

*: The only non-significant mean *relative* difference ECD (p= 0.316); all other mean *relative* difference ECDs had p = 0.001 or less.

Figure 3.1a Example of a video print of the endothelial image obtained by the SP2000P. The double-headed arrow indicates the mask slit width that is essential in the calibration. Just right of the endothelial image are the reference grids for the estimation of ECD (in cells/mm$^2$).

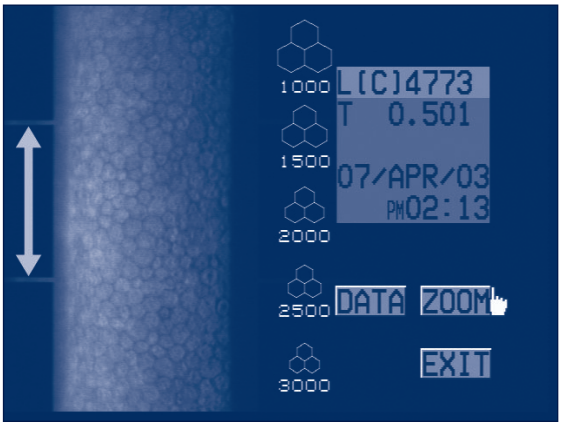



Figure 3.1b Picture of external microgrid used with calibration device as it appears on the SP-2000P screen. The double headed arrow indicates the mask slit width. The distance between lines A and B is a standard distance used to calculate the mask slit width.

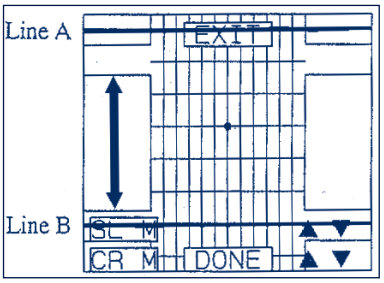
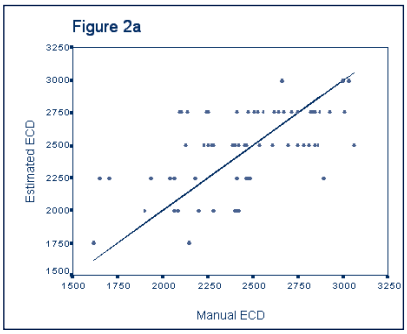
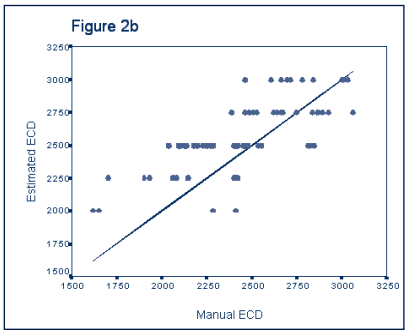


Figure 3.2 Scatter plots of the Estimated ECDs, each by one of three observers (fig. 2a-2c, respectively), against the Manual ECD.



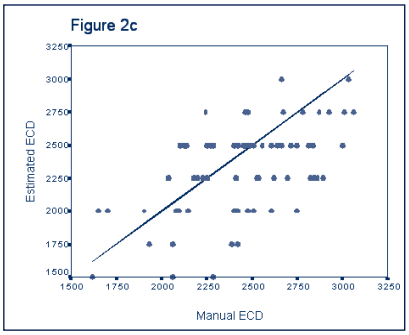




Figure 3.3 Scatter plot of the SP-2000P ECD ("Simplified Cell Analysis") against the Manual ECD.

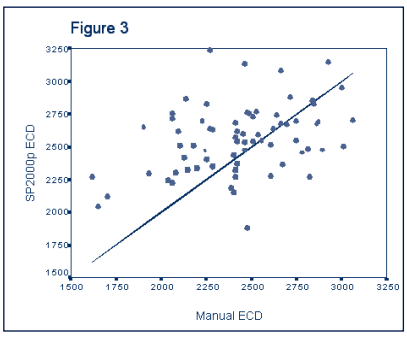
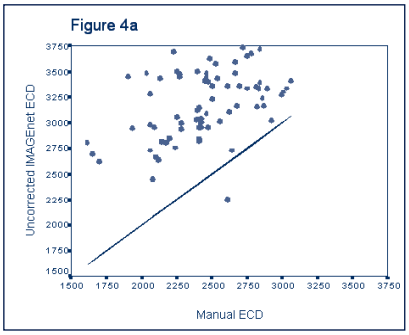


Figure 3.4 Scatter plot of the Uncorrected (fig.3.4a) and the Corrected (fig. 3.4b) IMAGEnet ECDs against the Manual ECD.



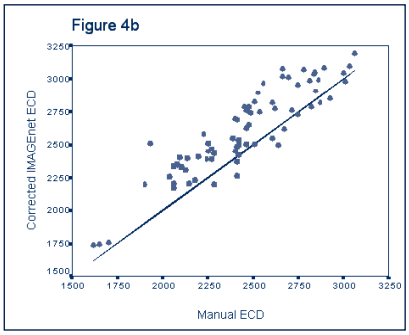
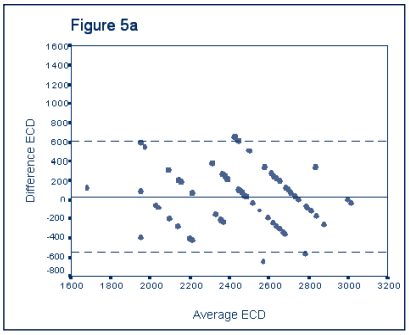
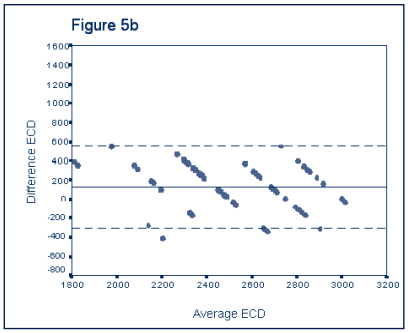




Figure 3.5 Bland Altman plots of the difference between the Estimated ECD and the Manual ECD, against the mean of the Estimated ECD and the Manual ECD, for each of the three observers (figs. 3.5a – c, respectively).



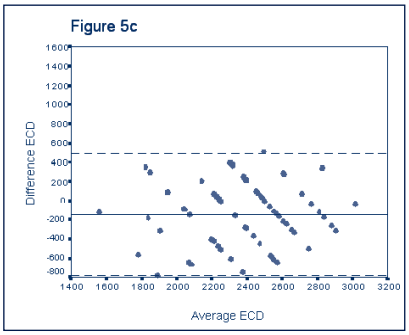



Figure 3.6 Bland Altman plot of the SP-2000P ECD and the Manual ECD.

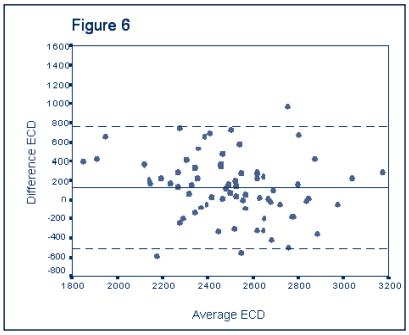
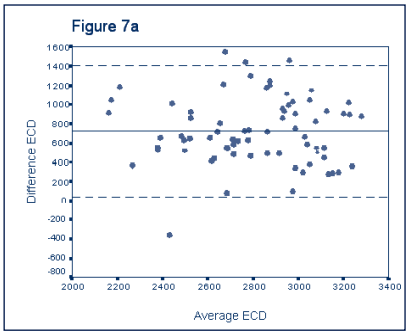
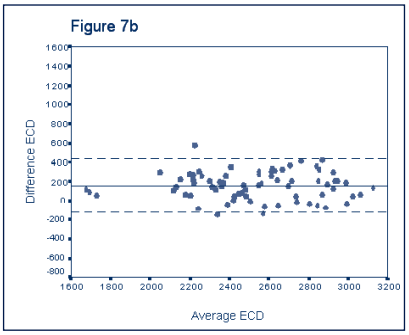



Figure 3.7 Bland Altman plots of the Uncorrected IMAGEnet ECD and the Manual ECD (fig. 3.7a), and of the Corrected IMAGEnet ECD and the Manual ECD (fig. 3.7b).



# chapter

# 4

# Reproducibility and biological variability of endothelial parameters

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## Abstract

### Purpose

To evaluate reproducibility and biological variability in the morphometric corneal endothelial parameters cell density (ECD), coefficient of variation of cell size (CV), and hexagonality; as well as in (optical) pachymetry.

### Methods

From the target eye of 25 "normal" volunteers, 17 bilateral contact-lens wearing volunteers, and 15 patients one year after bilateral phacoemulsification, endothelial images were obtained using a Topcon SP-2000P non-contact specular microscope. Nine central corneal images images were obtained in two same-day sessions and a third session 3 weeks later. Additional images were obtained from the paracentral target cornea, and from the central fellow eye. All images were analyzed using IMAGEnet 2000 semi-automated analysis, by one observer. Reproducibility of all parameters was evaluated using mixed-model ANOVA. Two additional observers analyzed 38 random images, and inter-observer reproducibility was calculated.

### Results

Time-related reproducibility was of much less importance than sampling error, which was low in pachymetry and in ECD, moderately low in CV, and high in hexagonality measurements. Inter-observer reproducibility was high. None of the parameters showed significant *systematic* differences between fellow and target eyes. ECDs and CVs from nasal and inferior paracentral areas were not significantly different from central corneal values.

### Conclusions

Although pachymetry, ECD, and CV measurements are reproducible, sampling error may influence the reliability of all endothelial measurements. This can be improved by calculating the mean from several replicate measurements. Values of contralateral eyes may substitute for one another. Nasal and inferior ECD and CV may substitute for central values.

## Introduction

Endothelial morphometric parameters such as the endothelial cell density (ECD), coefficient of variation of cell size (CV) as a measure for polymegathism, and the hexagonality of endothelial cells as a measure for pleomorphism, have been investigated extensively.[241] The measurement of the thickness of a cornea, the pachymetry, is one of the most important methods to evaluate corneal endothelial function *in vivo*.[84] All these parameters may be important in the planning of intra-ocular surgery, notably in assessing the risk for corneal decompensation. They can also be used to evaluate endothelial damage caused by surgery.

The Topcon SP-2000P is an easy-to-use, auto-focus specular microscope that combined with the Endothelial Cell Analysis Module in the IMAGEnet 2000 software package (both Topcon Corp., Tokyo, Japan) enables one to obtain ECD, CV, hexagonality, and optical pachymetry measurements *in vivo*. As the SP-2000P is a non-contact instrument, there is no risk of epithelial damage or microbiological (cross-)contamination, and examinations are easily tolerated. Therefore, this instrument seems very suitable for obtaining measurements before and after intra-ocular surgery.

In order to correctly interpret observed changes within patients and differences between patients in endothelial parameters, insight in the reliability of these measurements is essential. In a previous study the calibration of magnification of the SP-2000P and the IMAGEnet2000 software, and the validity of the available endothelial assessment methods were described. The semi-automated assessment method, in which software-defined cell borders are interactively corrected, was found to be the most valid method for obtaining ECD.[911]

In the present study other aspects of the reliability of measurements obtained with this instrument were investigated. We looked into the reproducibility of measurements in 'normal' and contact-lens wearing volunteers, as well as in patients one year after bilateral cataract surgery. In addition, the "biological variability", i.e. the reliability of paracentral or contralateral values of morphometric parameters as substitutes for central values, was studied. The methods we used are not restricted to the Topcon SP-2000P, but are also relevant to investigators using other specular or confocal microscopes, and other analysis software packages.

## Methods

Three groups of subjects were studied. In all subjects one eye was randomly designated 'target eye', the other eye 'fellow eye'. The first group consisted of

healthy volunteers (n = 25, mean age ± standard deviation: 38.2 ± 8.6 years; 14 females, 11 males), not wearing contact-lenses and without any known ocular disease. A second group of volunteers (n = 17, age: 32.4 ± 8.4 years, 11 females, 6 males) included long-term, i.e. longer than two years, bilateral contact-lens wearers (either rigid gas permeable or soft contact lenses) without any known ocular disease. The third and final group consisted of patients one year after uncomplicated bilateral phacoemulsification with implantation of an IOL (n = 15, age: 72.4 ± 10.0 years, 10 females, 5males). Both eyes of one patient were operated within 2 months by the same surgeon, using 5mm corneoscleral tunnel incisions, the same divide-and-conquer technique after continuous curvilinear capsulorhexis, and in-the-bag implantation of the same type of PMMA IOL. Phaco-times and –energies did not differ significantly between the eyes.

Endothelial images were obtained using a Topcon SP-2000P auto-focus non-contact specular microscope. Optical Pachymetry readings were directly provided on the microscope screen. The images were then imported into a personal computer and analysed with the Endothelial Cell Analysis Module of the IMAGEnet2000 software. Cell borders were interactively corrected by one of the authors (BTHvD). The images were masked for time-point and patient group. After the correction of cell borders a final analysis resulted in ECD, CV, and Hexagonality measurements. A more detailed description of this analysis method has been described previously.[911]

### Reproducibility

From the target eye, in a first session three successive endothelial images were obtained from the central cornea. In a second session a few minutes later, three more central endothelial images were taken. In groups 1 and 2, in a third session 3 weeks (mean ± SD: 23.7 ± 24.0 days) later three final central images were taken from the target eye. Statistical analyses were performed with the SAS Statistical Package (SAS Inc, Cary, Indiana). Using a mixed model Analysis of variance (ANOVA), total variance of all parameters was decomposed into a between-subjects and a within-subjects component. The latter component was further decomposed into three time-related within-subject components: within-session (three successive images), between-session same day, and between-session long term (three weeks). After expressing these components as percentages of total variance, we calculated various reliability indices as follows. The repeatability index was calculated as the sum of the between-subjects component, the within-subject between-session same day component, and the within-subject between-session long-term component. The short-term reproducibility index was calculated as the sum of the between-subjects component and the within-subject between-session long-term component. The long-term reproducibility index equalled the between-subjects component.

For the calculation of inter-observer variability, 38 randomly chosen images from the total pool of endothelial images were once more semi-automatically analyzed by two additional observers (AML,WvS). For 3 observers, inter-subject, inter-observer, and intra-observer variabilities were calculated using Components of Variance analysis with the software package SPSS, release 11.0 (SPSS Inc., Chicago, Ill., USA). The inter-observer reproducibility was expressed as the Intra-Class-Correlation-coefficient (ICC), which is defined as the inter-subject variance as proportion of the total variance.

### Biological variability

In the first session, 3 central images were obtained in both paired eyes in all groups. Endothelial measurements from target and fellow eyes were compared. Also, in groups 1 and 2 (n = 38) in the target eye endothelial images were taken from the other four paracentral "windows" in the SP2000P: superior, temporal, nasal, and inferior. These paracentral windows were located at a radius of 3 mm from the central axis at the 12, 10, 2, and 6 o'clock positions, as documented in the Topcon SP2000P manual. Paracentral measurements in group 3 (post-phacoemulsification patients) were not included in this study. Particularly measurements in the temporal and superior paracentral windows would be influenced by local effects of surgery on the endothelium (e.g. the sclerocorneal wound). This would have confounded the comparison to central measurements.

Differences between target and fellow eyes were statistically evaluated with a paired Student's t-test and Wilcoxon Rank Sign Test, using the software package SPSS, release 11.0 (SPSS Inc., Chicago, Ill., USA). A probability level of 0.01 was adopted for statistical significance because of the large number of tests that had to be performed. For the comparison of endothelial parameters in paracentral and in central corneal areas mean differences and limits-of-agreement according to Bland and Altman were calculated, [9,10] and Student's t-test was performed.



## Results

### Reproducibility

Table 4.1 shows for the four parameters ECD, CV, Hexagonality, and Pachymetry the various reliability indices (repeatability, short-term reproducibility and long-term reproducibility indices). Results are given for the aggregated group of subjects as well as separately per study group. For group 3, only the short-term reproducibility could be calculated, as there had been no third session.

Table 4.2 shows the inter-patient, intra-observer and inter-observer variabilities as obtained with Components of Variance analysis for the observer-influenced parameters ECD, CV, and hexagonality.

### Biological variability

Table 4.3 shows the mean differences between the target eye and the fellow-eye for all 4 dependent parameters.

Table 4.4 shows the values of all four parameters in the 4 paracentral windows, separate as well as aggregate, together with the p-values obtained when comparing with the values in the central window. Also the mean differences between the values in these paracentral windows compared to in the central window, as well as the Limits-of-Agreement are presented in this table.

## Discussion

### Reproducibility

The first part of the present study investigated the reliability of endothelial morphometric parameters and pachymetry, obtained with Topcon SP2000P specular microscopy and semi-automated endothelial analysis. The results in Table 4.1 show that for all endothelial parameters the reproducibility index is hardly worse than the repeatability index, and the long-term reproducibility index is hardly worse than the short-term reproducibility index. This implies that there is hardly any within-subjects, time-related, biological variability in any of these parameters. Furthermore, the within-subject variability is mainly caused by sampling error of the measurement process itself, rather than by within-subject variability of the parameter. The component of this sampling error in the total variance equals 100 minus the repeatability indices expressed in Table 4.1. By calculating the mean of a number of replicate measurements, the sampling error decreases inversely proportional to the number of measurements. This procedure may be advisable when very precise measurements are needed.

The very high repeatability index of pachymetry readings confirmed the capacity of the SP-2000P for discerning true intra- and inter-subject differences in pachymetry. Furthermore, true circadian and day-to-day biological fluctuations in corneal thickness may be expected to induce some variability in follow-up measurements and therefore accounted for most of the additional variability in the long- and short-term reproducibility indices. Our conclusion that SP2000P pachymetry readings are very reproducible confirmed the findings of Bovelle et al..[370] Our results were slightly better than those of Nichols et al. who used a different specular microscope (Konan SP-9000 LC).[288]

The capacity of the semi-automated assessment method to discern within- and between-subject ECD differences can be considered to be very good in normals and in post-cataract patients, and good in contact-lens wearers. Corneas of

contact-lens wearers usually demonstrate higher endothelial polymegathism, pleomorphism, and a more inhomogeneous ECD-distribution.[450] This may result in larger sampling errors in specular microscopy and more interpretation difficulties in (semi-) automated ECD-assessment. Hence a slightly lower repeatability index in this group of subjects may be explained.

Long- and short term reproducibility indices of the CV were only slightly less than the repeatability index in all the groups, indicating little or no time-related biological variability in this parameter. The repeatability in group 1 was remarkably low compared to the other two groups (and the aggregate group). This may be explained by the fact that in group 1 the CV itself and also the total variance of CV was lower than in the other groups. This allowed sampling errors in CV to be revealed more easily in group 1. In any case, for future reference a margin of error should be kept in mind when investigating within- and between-subject differences in CV.

At best, the repeatability index of Hexagonality was only moderate. In general it was much worse than for the other parameters. Therefore, the capacity to discern intra-subject differences in Hexagonality (e.g. in follow-up measurements) should be considered to be poor.

Inter-observer reproducibility was good to excellent for ECD and CV. For ECD an inter-patient component of variance of 98.5 % corresponding with an Intra-class Correlation Coefficient (ICC) of 0.985 was found. For CV, similarly high inter-patient and low inter-observer variances were found (ICC = 0.93). For Hexagonality there is a much higher intra-observer variability (30.6 %, ICC = 69.4).



With regards to ECD, CV, and Hexagonality, our findings on repeatability and reproducibility are slightly better than those reported by Cheung et al. who used the same instrument and comparable statistical methods. The same applies to the inter-observer reproducibility.[294] Other authors investigated other instruments and different endothelial assessment techniques, and used different statistical methods. Compared to their reports, sampling variances were relatively low and reproducibility indices were relatively high in our study.[286 288 293 301]

### Biological variability

For all parameters the mean differences between Target Eyes and Fellow Eyes were minute, and none of them were statistically significant. Differences in ECD between fellow eyes in our study, 0.7 %, were less than in Sperling's study on donor corneas, 4.0 – 4.5 %.[267]

Superior, temporal, and aggregate peripheral ECDs were significantly higher than central ECDs. For CV, superior and aggregate peripheral means were significantly higher than central. For Hexagonality no means were significantly different from central. As expected, all peripheral Pachymetries were significantly higher than central. The Bland-Altman calculations showed that the nasal ECD, and with a wider margin of error also the inferior ECD are similar to central ECD of the same cornea. In *individual* cases however the difference between nasal (or inferior) and central ECD may be considerable (wide Limits-of-Agreement). Our findings on paracentral differences in ECD compared to central ECD, 4.4%, were comparable to the 5.8 % reported in the study by Amann et al.. Differences between superior and central ECDs (9.9%) in our study was somewhat lower than their 15.9 %.[263] We found that CV follows ECD in this pattern. Both Amann et al. and Schimmelpfennig[262 263] report even larger differences in ECD when more peripheral corneal areas were compared to the central values. Far peripheral ECDs are therefore probably not representative for central ECDs.

### Conclusions

Semi-automated IMAGEnet 2000 endothelial cell analysis on SP2000P specular microscopic images was shown to have a very good capability for discerning true inter- and intra-subject differences in ECD. CV measurements were also reliable, whereas reliability of hexagonality measurements was much less. SP2000P pachymetry readings were very reliable. The capability for discerning between-subject and intra-subject differences in any parameter was not influenced by lack or reproducibility but mostly by sampling error. This may be reduced by obtaining the mean of several replicate measurements. Inter-observer variability of ECD and CV was proven to be small for trained observers.

Nasal (and inferior) paracentral ECD and CV may substitute for central values when the latter cannot be obtained. When the medical history of both eyes is identical, all parameters in the fellow eye may substitute for the target eye.

When using specular or confocal microscopes and endothelial analysis systems, reliability of measurements should be investigated in order to interpret intra- and inter-subject differences in endothelial parameters correctly. Our methods for reliability investigations are not restricted to our instruments, and can be used universally.

## References

See page 287.

Table 4.1 Reliability indices and total variance in endothelial parameters.

| Reliability index (between-subjects component in % of total variance) | ECD | CV | Hexagonality | Pachymetry |
|---|---|---|---|---|
| **All subjects (n = 57)** | | | | |
| (1) Repeatibility index | 96.9 | 92.0 | 60.8 | 98.0 |
| (2) Reproducibility short-term index | 96.9 | 92.0 | 60.8 | 97.2 |
| (3) Reproducibility long-term index | 96.8 | 90.6 | 57.2 | 97.0 |
| Total variance = $SD^2$ (= 100%) | $386^2$ | $6.71^2$ | $8.93^2$ | $0.040^2$ |
| **Group 1 (n = 25)** | | | | |
| (1) Repeatibility index | 96.0 | 76.4 | 57.2 | 97.6 |
| (2) Reproducibility short-term index | 95.7 | 75.3 | 57.2 | 97.4 |
| (3) Reproducibility long-term index | 95.6 | 68.2 | 45.4 | 96.7 |
| Total variance = $SD^2$ (= 100%) | $314^2$ | $3.45^2$ | $8.11^2$ | $0.034^2$ |
| **Group 2 (n = 17)** | | | | |
| (1) Repeatibility index | 92.4 | 90.7 | 74.2 | 97.7 |
| (2) Reproducibility short-term index | 92.4 | 90.7 | 74.2 | 97.4 |
| (3) Reproducibility long-term index | 92.3 | 90.6 | 74.2 | 96.1 |
| Total variance = $SD^2$ (= 100%) | $270^2$ | $6.10^2$ | $9.64^2$ | $0.035^2$ |
| **Group 3 (n = 15)** | | | | |
| (1) Repeatibility index | 97.9 | 93.9 | 47.8 | 98.5 |
| (2) Reproducibility short-term index | 97.9 | 93.9 | 47.8 | 97.0 |
| Total variance = SD2 (= 100%) | $462^2$ | $10.14^2$ | $9.65^2$ | $0.052^2$ |

(1) within-session
(2) between-sessions same day
(3) between-sessions long term (3 weeks)
Total variances are in the units of the investigated parameters (cells/$mm^2$, %, and %, for ECD, CV, and Hexagonality, respectively), squared. Reliability indices in %.

**Table 4.2** Inter-subject, Intra-observer, and inter-observer variances, expressed as components of the total variance.

| | ECD | CV | Hexagonality |
|---|---|---|---|
| Total variance = $SD^2$ (= 100%) | $450.4^2$ | $7.28^2$ | $9.61^2$ |
| Inter-subject variance = ICC (intra-class correlation coefficient) | 98.5 % | 93.0 % | 69.4 % |
| Intra-observer variance | 1.3 % | 4.1 % | 30.6 % |
| Inter-observer variance | 0.2 % | 2.9 % | 0.0 % |

Total variances are in the units of the investigated parameters (cells/$mm^2$, %, and %, for ECD, CV, and Hexagonality, respectively), squared. Standard deviations, i.e. the square root of the variances, are in the same unit as the parameters investigated. Components of variance are in %.

**Table 4.3** Mean differences between target- and fellow eyes in endothelial parameters.

| | ECD (cells/$mm^2$) | Relative ECD (%) | CV (%) | Hexagonality (%) | Pachymetry (mm) |
|---|---|---|---|---|---|
| Mean Difference | - 20.6* | - 0.7* | 0.55 | 0.04 | 0.003 |
| Standard Deviation | 175.5 | 7.0 | 3.14 | 6.00 | 0.016 |
| P-values | 0.379 | 0.446 | 0.193 | 0.959 | 0.172 |

*: Each mean difference is obtained by subtracting the values in the fellow eye from the values in the target eye. Negative mean differences therefore indicate that the values in the fellow eyes were higher than the values in the target eyes.

Table 4.4 Endothelial parameters in peripheral vs central windows.

| Parameter/ Window | Mean ± s.d. | Mean Difference (± s.d, comp. to Central) | P-value | Limits-of-Agreement |
|---|---|---|---|---|
| **ECD** | | | | |
| Superior | 3140 ± 442 | - 301 ± 367 | 0.000 | -1021 , 419 |
| Temporal | 3023 ± 317 | - 183 ± 143 | 0.000 | - 463 , 96 |
| Nasal | 2865 ± 337 | - 25 ± 197 | 0.440 | - 412 , 361 |
| Inferior | 2905 ± 303 | - 67 ± 202 | 0.050 | - 463 , 329 |
| Peripheral | 2984 ± 312 | - 144 ± 178 | 0.000 | - 492 , 204 |
| Central | 2858 ± 299 | n/a | N/a | N/a |
| **CV** | | | | |
| Superior | 34.9 ± 8.5 | - 5.1 ± 6.4 | 0.000 | -17.6 , 7.5 |
| Temporal | 31.8 ± 7.7 | - 2.0 ± 5.8 | 0.042 | -13.3 , 9.4 |
| Nasal | 31.5 ± 8.0 | - 1.8 ± 5.7 | 0.061 | - 13.1 , 9.4 |
| Inferior | 30.5 ± 5.4 | - 0.9 ± 3.3 | 0.103 | - 7.4 , 5.6 |
| Peripheral | 32.4 ± 7.1 | - 2.6 ± 4.7 | 0.002 | - 11.8 , 6.7 |
| Central | 30.1 ± 4.6 | n/a | N/a | n/a |
| **Hexagonality** | | | | |
| Superior | 56.5 ± 8.8 | 2.3 ± 8.4 | 0.096 | - 14.1 , 18.8 |
| Temporal | 58.5 ± 9.8 | 0.4 ± 9.6 | 0.808 | - 18.4 , 19.2 |
| Nasal | 61.7 ± 9.3 | - 2.6 ± 8.9 | 0.080 | - 20.0 , 14.7 |
| Inferior | 60.2 ± 8.5 | - 1.2 ± 8.5 | 0.415 | - 17.9 , 15.6 |
| Peripheral | 59.1 ± 6.9 | - 0.2 ± 6.4 | 0.844 | - 12.8 , 12.4 |
| Central | 58.3 ± 7.3 | n/a | N/a | n/a |
| **Pachymetry** | | | | |
| Superior | 0.616 ± 0.054 | - 0.087 ± 0.037 | 0.000 | - 0.159, 0.015 |
| Temporal | 0.583 ± 0.043 | - 0.054 ± 0.023 | 0.000 | - 0.100 , - 0.008 |
| Nasal | 0.616 ± 0.052 | - 0.087 ± 0.036 | 0.000 | - 0.157 , - 0.018 |
| Inferior | 0.565 ± 0.043 | - 0.037 ± 0.020 | 0.000 | - 0.076 , 0.001 |
| Peripheral | 0.594 ± 0.046 | -0.065 ± 0.027 | 0.000 | - 0.118 , - 0.013 |
| Central | 0.531 ± 0.034 | n/a | N/a | n/a |

ECDs are given in cells/mm$^2$, CVs and Hexagonalities in %, Pachymetries in mm.
All (mean) differences are calculated by subtracting the value at hand from the central value. Negative differences therefore indicate that the value in the specific window was higher than the central value. Limits-of-Agreement according to Bland and Altman are calculated by adding or subtracting 1.96 * standard deviation to/from the mean difference[910] and are presented as: lowest, highest Limit-of-agreement.

# chapter

# 5

# Comparison of in-vivo and in-vitro corneal endothelial cell-density-measurements

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## Abstract

### Aim

To investigate whether *in-vivo* specular microscopic endothelial cell densities (ECDs) systematically differ from ECDs obtained from the same corneas with an *in-vitro* technique in use in cornea banks.

### Methods

From 36 eyes planned to undergo penetrating keratoplasty, pre-operative in-vivo ECDs were obtained with a Topcon SP-2000P specular microscope and IMAGEnet 2000 semi-automated analysis. Pachymetries and corneal topographies were also measured. Post-operative in-vitro ECDs were obtained from the recipient buttons using endothelial-side light microscopy, after provoked intercellular swelling. Differences between in-vivo and in-vitro ECDs, and limits-of-agreement were calculated.

### Results

The mean difference between in-vivo and in-vitro ECDs was constant, 140 ± 219 cells/mm$^2$, and statistically significant (p= 0.004). Wide limits-of-agreement indicated that in *individual* cases differences were much larger. Compared to other eyes the mean difference in ECD was not significantly higher in keratoconic eyes, and there was no significant correlation between the mean ECD-difference, and pachymetries, mean sim-K values, or the time-interval between operation and in-vitro ECD counts.

### Conclusion

When comparing donor ECDs from the cornea bank to specular microscopic ECDs in grafted eyes, the possibility of a systematic difference between different ECD measurement methods should be considered. Due to its constant nature in our study, this difference could not be explained by optical factors alone.

## Introduction

Endothelial cell density (ECD) is the most frequently used quantitative parameter in the evaluation of the status of the endothelial cell layer. Clinically, ECD is paramount in the assessment of endothelial damage after surgery, especially after keratoplasty.[310 770] In the Cornea Bank Amsterdam, Amnitrans EyeBank, Rotterdam (both the Netherlands), and in other eye banks in Europe, a minimum ECD of between 2000 and 2500 cells/mm$^2$ is a major criterion for acceptance of a donor cornea for transplantation.[324 812]

In several eye banks in Europe light microscopy is used to determine the endothelial cell density on donor corneas in vitro. Images are taken from the endothelial side following vital staining with trypan blue and provoked swelling of the intercellular space.[324 332] This technique, also in use at the Cornea Bank Amsterdam, differs substantially from specular microscopy. In contrast, when using the specular microscopy principle,[3] an image of the endothelium is obtained from the external, epithelial side. Image analysis- and endothelial assessment methods for both techniques may differ substantially as well.[361]

In clinical follow-up studies on endothelial cell loss after keratoplasty, donor ECDs will be compared to post-operative in-vivo ECDs. In order to evaluate whether there was a systematic difference between ECD obtained by specular microscopy and light microscopy, in the present study we compared in-vivo ECDs from patients planned for penetrating keratoplasty (PK), to in-vitro ECDs obtained from these patients' recipient corneal buttons.



## Methods

### Clinical material

Thirty-six eyes of 36 patients planned for PK were included. In 24 patients, the diagnosis was keratoconus. Four corneas were transplanted for a corneal dystrophy, 3 had corneal scars after perforating trauma, 2 herpetic maculae, 1 had a leucoma of unknown aetiology, 1 a pseudophakic bullous keratopathy, and 1 operation was a re-PK.

### In-vivo endothelial cell densities

Specular microscopy was performed preoperatively using a non-contact autofocus SP-2000P specular microscope (Topcon Corporation, Tokyo, Japan) in the Rotterdam Eye Hospital, Rotterdam, and the Department of Ophthalmology of the Vrije Universiteit Medical Centre, Amsterdam, both The Netherlands. The magnification of both instruments had been calibrated as described previously.[911]

Primarily, endothelial images were obtained from the central cornea. When no good central images could be obtained, images were acquired from a paracentral area that was located within the dimensions of the future recipient corneal button (trephines used were all over 7.0 mm in diameter), by having the patient fixate slightly off-centre. When more images of one eye were available, one with acceptable quality was randomly chosen for analysis. All images were analysed using IMAGEnet2000 software (Topcon) with semi-automated correction of cell borders by one of the authors (BvD).

Whenever possible mean simulated keratometry readings (mean sim-K values, using an Alcon EH-290 Eyemap Corneal Topograph, Alcon Corp., Fort Worth, Tx.) and optical pachymetries (with the SP2000P) were obtained.

### In-vitro endothelial cell densities

PK was performed in the Rotterdam Eye Hospital, in the Department of Ophthalmology of the Erasmus Medical Centre, Rotterdam, and the Department of Ophthalmology of the Vrije Universiteit Medical Centre, Amsterdam. No intra-operative complications occurred. Recipient corneal buttons were trephinated and excised, and under sterile conditions placed in Eagle's Minimal Essential Medium with 2 % Fetal Bovine Serum and 5 % Dextran T500, the transport medium for corneas preserved by organ culture (The Cornea Bank Amsterdam, The Netherlands).[324 332]

The buttons were transported to the Cornea Bank Amsterdam for in-vitro analysis. The mean time between PK and analysis was 2 ± 1.5 days (min. 0.5, max. 8 days). ECD assessment of the recipient buttons was performed using the technique for evaluation of donor corneas described previously.[324 332] In summary, the buttons were stained with trypan blue (0.12 % in buffered saline), and swelling of the intercellular space was provoked with hypotonic sucrose in water (1.8%). Consecutively, with a light microscope at a magnification of 125 x and a calibrated graticule in the ocular, the number of cells was estimated in five different areas of the corneal button. The mean of these five estimates resulted in one in-vitro ECD.

Cell counts were performed in areas outside the trephination area. Dead or dying cells as indicated by trypan blue staining were included in the cell count. Indications for recent cell loss or restoration of damage to the cell mosaic, such as polygonal cells with sharp edges,[135 249] were not observed.

### Statistical evaluation

According to the method of Bland and Altman,[910] differences between in-vitro and in-vivo ECDs were calculated for each button and plotted against their mean. After establishing that the differences demonstrated a normal

distribution, the significance of the difference between the two ECDs was tested with a paired Student's t-test. The variability of the differences was evaluated by calculation of the limits of agreement, mean ± 1.96 standard deviations of the mean difference.

Possible influence of pachymetries, mean sim-K values, or the time between PK and in-vitro ECD assessment on the size of the difference was evaluated by calculating Pearson's correlation coefficients. For all analyses, SPSS software release 11.0 (SPSS Inc., Chicago, Ill., USA) was used.

## Results

In figure 5.1 in-vivo ECDs are plotted against the correspondent in-vitro densities. The identity line represents a hypothetical zero difference between the two ECDs. A relation between the difference and the absolute value of ECD was not demonstrated.

The mean (± standard deviation) in-vivo ECD was 2626 (± 730) cells/mm$^2$, and the mean in-vitro ECD was 2486 (± 716) cells/mm$^2$. The mean difference between in-vivo and in-vitro ECDs was 140 (± 219) cells/mm$^2$, which was statistically significant, p= 0.004. The difference between in-vivo and in-vitro ECD in patients with keratoconus (175 ± 280 cells/mm$^2$) was not significantly (p = 0.390) higher than the difference in ECD of patients with other indications for PKP (68 ± 238 cells/mm$^2$). There was no significant correlation of pachymetry, mean sim-K, both possible optical confounders, or the time interval between PK and in-vitro ECD assessment, with the mean difference (table 5.1).

The differences between the in-vivo- and in-vitro ECDs were plotted against the mean of in-vivo and in-vitro ECDs (figure 5.2). The observed mean difference (140 cells/mm$^2$) was shown as the middle horizontal line. The limits of agreement, represented by the upper and lower horizontal lines, were 644 (upper) and – 385 (lower) cells/mm$^2$, which indicated a considerable variability in *individual* measurements.

## Discussion

In our study a constant systematic difference of 140 cells/mm$^2$ that was not related to the absolute value of the ECD was observed between in-vitro and in-vivo ECDs. This absolute, constant systematic difference is remarkable. Several explanations, some of which optical, for possible systematic differences may be considered.

1. The oblique angle of observation in non-contact specular microscopy may lead to an underestimation in the horizontal (but not the vertical) dimension of the observed endothelial area, and hence an overestimation of the ECD.[274 287] However, this effect is corrected by a combination of adjustments in SP-2000P optics and in the ECD calculation software (P. Gelissen, Topcon Europe, personal communication).

2. Underestimation of observed area may be introduced by observing a curved surface instead of an assumed flat surface.[274] Aberrant corneal curvature and thickness such as in keratoconic eyes, which constituted the majority of our clinical material, may further influence ECD measurements.[311] For instance the difference between observed (real) curved area and assumed flat area may be increased. However, calculations using Olsen's formulas[274] with different measures for corneal thickness and radius demonstrated that only small additional errors were introduced (manuscript in preparation). In the present study the difference between in-vivo and in-vitro ECD was slightly, but not significantly, larger in conic corneas. No significant correlation between either pachymetry or mean sim-K values, and the mean difference between in-vivo and in-vitro ECD was found. Laing et al. confirmed that ECDs in conic corneas did not differ significantly from ECDs in normal corneas, although endothelial morphology sometimes differed substantially.[912]

3. When observing the endothelium from the epithelial side, a magnification[913 914] of the endothelial image is introduced at the anterior corneal curvature. This causes the observed area to be overestimated and the ECD underestimated. Recently, formulas were presented for the calculation of the change in the magnification of the endothelium after excimer laser refractive surgery.[913 914] When we applied these formulas in theoretical calculations (manuscript in preparation) with changes in corneal thickness, power, or radius as may occur in keratoconus, the induced change in two-dimensional magnification was only small.

4. When we had to resort to specular microscopic imaging of paracentral areas of the cornea in order to obtain a clear endothelial image, we may have introduced a sampling error. Amman et al. recently demonstrated that paracentral areas may have ECDs that are about 9 % higher than the central ECD.[263] In our study paracentral images were actually from areas much closer (i.e. within 1 mm) than Amann's paracentral areas of the corneal centre. The expected error would therefore be much smaller than 9 %.

Differences between in-vivo and in-vitro ECDs may also have been influenced by characteristics related to the in-vitro light microscopic technique.

5. Effects on ECD of swelling of the intercellular spaces just prior to in-vitro ECD-assessment might be supposed to influence ECD counts, but are not likely to occur. Cell counting was performed with the centre of the cells. In addition, regular comparison of the ECD obtained after swelling of the intercellular spaces and after staining the cell borders with Alizarine Red has not shown significant differences; the performance of the technicians and the interobserver variability is being monitored at set time intervals as prescribed by a quality management system (ISO 9001:2000). In porcine corneas Sperling found only very small differences in ECD attributable to osmotic intercellular swelling, compared to other ECD measurement techniques.[343]

6. To prevent interference of trephination or transport of the recipient button with the cell counting, precautions were taken during the assessment of the cell density as described in Materials and Methods. Mitosis of endothelial cells seldomly occurs in humans. Restoration of the wound area by sliding and stretching of the neighbouring cells, occurring *in vivo*, will hardly occur during transport of the disc at ambient temperature in vitro. In addition this phenomenon can be recognized by the presence of reformation figures in the cell mosaic.[249] In our study however, these morphologic characteristics were not observed.

7. When the endothelium is studied from the endothelial side in corneal buttons resting on their epithelium, gravity and the lack of normal biological support structures may cause the discs to flatten. As the observed area now better corresponds to a flat surface, the pre-existent overestimation of ECD by observation of a curved area diminishes. However, flattening may also increase the ECD artificially, as endothelial cells are squeezed together to fit on a smaller area. Hence, the net effect may still be overestimation in in-vitro ECD measurements.

8. The graticule used for the estimation of the in-vitro ECD is calibrated with the help of a nomogram: the number of cells counted on the lines of the graticule plotted against the cell density assessed with Gundersen's method.[279] In normal corneas and with ECDs ranging between 2000 and 3000, the used linear fit is reliable; however the best fitted line for the nomogram is exponential. See figure 5.3a. In the higher ranges up to 4000 cells/mm$^2$ the in-vitro ECD is underestimated, whereas in the lower ranges the in-vitro ECD is overestimated. This implicates that in the higher ECD ranges the observed difference between in-vivo and in-vitro ECDs was observed to be larger than it actually was, whereas in the lower ECD ranges the difference was observed to be smaller than it actually was (fig 5.3b) The data points in graphs 5.3a. and 5.3b. represent a sample from the database of the Cornea Bank Amsterdam. These were used for linear and exponential regression modelling, and with the linear model the counting nomogram was constructed.

9. A systematic difference between a fixed-frame manual ECD counting technique and a variable-frame semi-automated ECD assessment technique has been described before. In a previous study, we found a *relative* difference of 6.5 % between these two ECD assessment methods when we evaluated the validity of the semi-automated method.[911] An overestimation of ECD by variable-frame methods compared to a fixed-frame method was also reported by Olsen.[280]

The specular microscopic optical sources for a difference between in-vivo and in-vitro ECDs that are relevant in our study (2. and 3. above) were relatively small and opposed each other. Furthermore, a resulting error may explain a *relative* but not an absolute difference. The importance of the error introduced by biological variation (4. above) is difficult to estimate, as it was both inconstantly present and of undetermined magnitude. However, it would probably lead to a slight overestimation of in-vivo ECD, causing either a *relative* or an absolute difference. Overestimation of ECD in light microscopy (7. above) does not help in explaining a lower in-vitro ECD compared to *in vivo*. The most important possible source of error from light microscopy, (8. above) implied that the absolute, constant difference that we found between in-vivo and in in-vitro ECD actually was an inverse *relative* difference, i.e. a difference that becomes smaller with increasing ECD (fig. 5.3b). The systematic difference between manual fixed-frame counting and semi-automated variable-frame ECD assessment (9. above) that was found in our previous study, was a *relative* and not an absolute difference. These considerations impair an unequivocal explanation of our findings. Probably, our findings are the sum of several of the described factors.

To our knowledge no other study compared in-vivo non-contact specular microscopy to the in-vitro technique described in this study, which, with variations, is currently in use at the Cornea Bank Amsterdam, Amnitrans EyeBank Rotterdam (both The Netherlands), and other eye banks in Europe.[812] Binder et al. compared contact specular microscopy, both in vitro and *in vivo*, to scanning electron microscopy (SEM).[915] In some cases, significant differences were found between both in-vitro and in-vivo specular microscopy endothelial cell densities and SEM densities, even when taking into account tissue shrinkage due to preparation for SEM. In contrast, Williams et al. found a strong correlation between the in-vitro contact specular-microscopic ECDs and histological cell counts in human donor corneas.[916] However, calculating a correlation coefficient is not an adequate method for comparing two different assessment methods as Bland and Altman demonstrated. In spite of a strong correlation there may still be a significant systematic difference.[910] Geroski and Edelhauser found in 8 human donor corneas no significant difference in mean endothelial cell area between (contact) specular microscopic ECD measurements and light microscopic cell counts after alizarin red S-staining.[353] Wiffen et al. found no significant differences between specular microscopy pre-

enucleation (from the epithelial side) and in-vitro specular microscopy (from the endothelial side) post-enucleation in enucleated eyes. They used a diversity of specular microscopes.[917]

In conclusion, when comparing in-vitro donor endothelial cell densities obtained with light microscopic techniques such as used in many eye banks, to in-vivo ECDs, especially when obtained with non-contact specular microscopy, one should be aware of a possible difference. There is systematic variation related to the technique per se and intra-*individual* variation to consider.

## References

See page 287.

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Table 5.1 Correlation coefficients of possible optical factors with the difference between In-vivo and In-vitro ECDs.

| Possible optical factors | Pearson Correlation Coefficient | p = (2-tailed) |
|---|---|---|
| Pachymetries | - 0.067 | 0.699 |
| Mean K-values | 0.341 | 0.167 |
| Time Interval | 0.174 | 0.376 |

Mean sim-K-values were obtained by averaging the sim-K values obtained with an Alcon EH-290 Eyemap corneal topograph. Time Interval means the interval between surgery and the in-vitro ECD assessment.

Figure 5.1 Scatterplot of the in-vivo ECDs obtained by specular microscopy (y-axis) against the in-vitro ECDs obtained by light microscopy (x-axis).

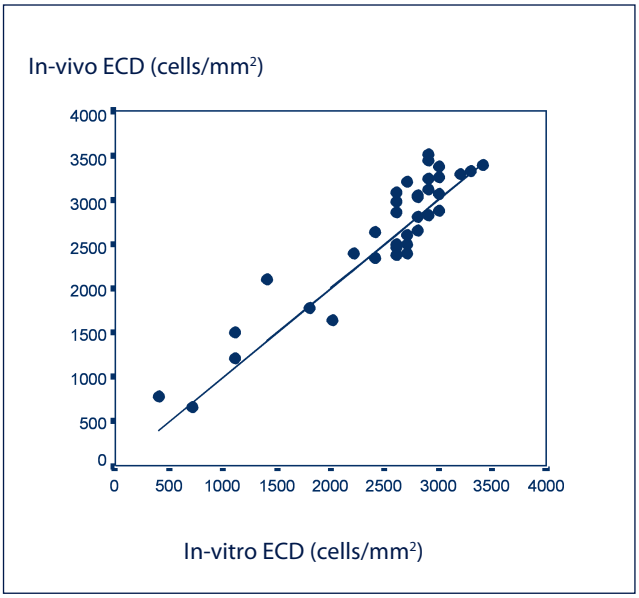


Figure 5.2 Bland-Altman plot: the difference between In-vivo ECDs and In-vitro ECDs (y-axis) is plotted against the average of In-vivo and In-vitro ECDs. The middle horizontal line represents the mean difference between In-vivo and In-vitro ECDs; the upper and lower horizontal lines represent the respective Limits-of-Agreement (mean difference ± 1.96 * standard deviation).

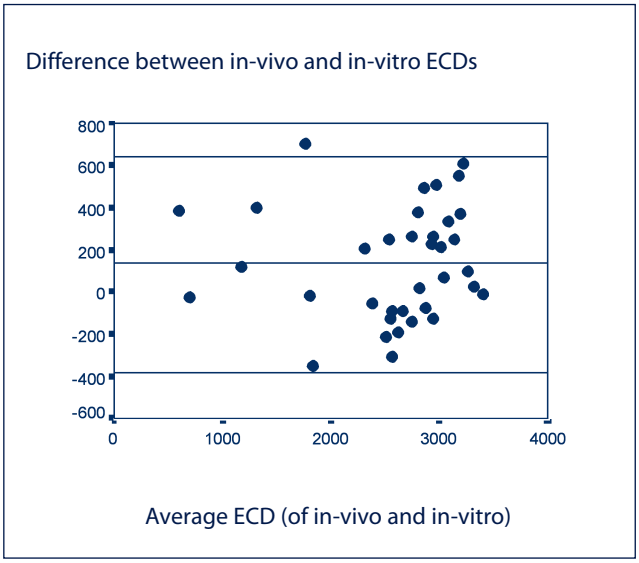


Figure 5.3 The data points in graphs 5.3a. and 5.3b. represent a sample from the database of the Cornea Bank Amsterdam and were used for linear and exponential regression.

a. Example of the fitted linear and exponential nomograms when assessing the in-vitro ECD using a graticule. The exponential nomogram has a better fit, which becomes more apparent in the in the lower and higher ECD regions.

b. The upper (dashed) straight line represents the observed systematic difference between the in-vivo ECD and the linear nomogram. In the lower ECD regions the difference is underestimated, whereas in the higher ECD regions up to 4000 cells/mm$^2$ the difference is overestimated. This implies an inverse *relative* difference instead of a constant difference.

Figure 5.3a.

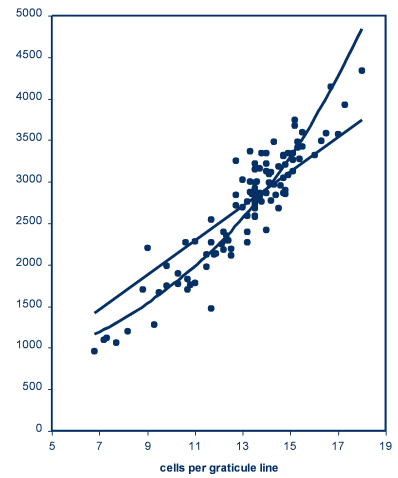
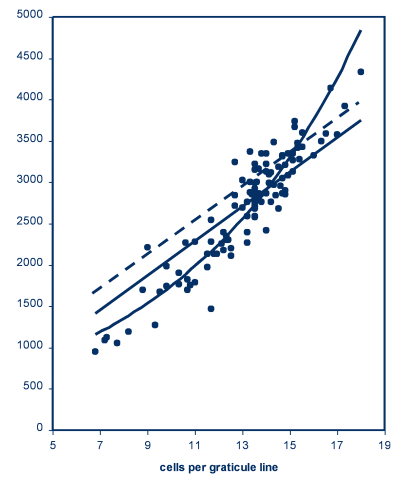



Figure 5.3b.



# chapter

# 6

# Optically induced errors in non-contact specular microscopic endothelial morphometry, and the influence of corneal curvature and thickness

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## Abstract

### Purpose

In non-contact specular microscopy, optical factors cause distortions that affect endothelial cell density (ECD) measurements. The purpose of this study was to review the existing theoretical formulas for each of these factors, and to calculate the amount of error in ECD they introduced, in relation to changes in corneal thickness and curvature.

### Methods

Optical distortions were caused by: oblique (horizontal) angle of observation, and observation of a curved area, both causing ECD overestimation; and magnification of the endothelial image by the anterior corneal curvature, causing ECD underestimation. Formulas were reviewed with which the amount of distortion in average corneas was calculated. Consecutively, the effect of different values of central corneal thickness, and corneal radius or power was calculated. The angle of observation of the Topcon SP2000P specular microscope (22.5°) was used.

### Results

The more oblique the angle of observation, the more substantial the overestimation of ECD, in this study 8.9 %. When this is corrected, as in the SP2000P, in most corneas the other optically induced errors are substantially smaller, except in thick and steep corneas where the underestimation of ECD due to anterior corneal magnification increases, in our examples up to 6.9 %.

### Discussion

Optical distortion needs to be taken into account in non-contact specular microscopic morphometry. Differences in the comparison of ECDs from non-contact specular microscopy with data obtained with contact-specular microscopy, confocal microscopy, or light microscopy, may in part be explained by differences in optical distortion.

## Introduction

Non-contact specular microscopy is an often used method for the evaluation of the corneal endothelium in patients. Both qualitative (morphologic) and quantitative (morphometric) aspects of the endothelium, such as the endothelial cell density (ECD) can be evaluated with this technique. Non-contact specular microscopy carries risk for neither epithelial damage nor microbiological contamination. Furthermore, non-contact examinations are well tolerated by the patient. Therefore, this method seems particularly suitable for follow-up of the corneal endothelium after surgical procedures.

The optical principles used in specular microscopy were first described by Vogt, who called this technique: "Spaltlampenuntersuchung im Spiegelbezirk" (i.e. slitlamp biomicroscopy in the specular plane)[3] In summary, specular reflection of light occurs from an interface between two materials with a difference in refractive index, such as the boundary between the corneal endothelium and the aqueous. Details from this interface can be observed at high magnification when it is sufficiently regular and when the incident angle of the illuminating light equals the angle of observation.[3 21] The first "clinical specular microscopes" that were developed from a prototype described by Maurice in 1968[8] were contact types,[13 14] although from early on also non-contact techniques were propagated.[12 21]

The optics of specular microscopy impose specific imaging characteristics and specific requirements regarding instrument settings. Both in non-contact and contact specular microscopy of the endothelium the incident (illumination) and observation angles with the normal onto the anterior cornea must be larger than zero, i.e. oblique, to avoid the dominant epithelial reflection in the endothelial image. See also figure 6.1. Furthermore, in non-contact specular microscopy the image is obtained through air and through a curved anterior cornea, from a curved instead of a flat plane. As the anterior cornea has a refractive index different from air, it acts as magnifying lens of the endothelium. See also figure 6.1. McCarey stated that in non-contact specular microscopy, the size of the image that is obtained is restricted.[21] Olsen showed that due to the dominant epithelial reflection, in non-contact specular microscopy the restriction of endothelial image size is governed by the angle of observation and the corneal thickness,[274] but not by the width of the illumination slit. In contrast, in contact specular microscopy the size of an image of the endothelium is for a large part determined by the width of the illumination slit. A wider slit allows acquisition of larger images with more endothelial cells. Unfortunately, wider slits also cause more scattering of light within the corneal stroma, and increased scatter in turn causes loss of contrast, rendering less useful images. [918] In both non-contact and contact specular microscopy

the incident (illumination) and observation angles with the normal may be increased, respectively to increase image size or to diminish scatter with wider slits. However, oblique angles of observation cause perspectivistic distortion, and the larger the angles are, the greater the distortion becomes. This is described in more detail below. Therefore, in any case a trade-off between image size, image contrast, and image distortion has to be found.[918]

In the current (semi-)automated autofocus contact and non-contact specular microscopes, fixed settings for the incident-and observation angles[287] and slit width are usually applied. The Topcon SP2000P (Topcon Corp, Tokyo, Japan) is such an often-used non-contact specular microscope. It has been shown to provide valid and reproducible ECDs when used with semi-automated endothelial analysis.[911, (Van Dooren et al, submitted for publication)] In the SP2000P, the slit width is around 0.2 mm - the exact value is fixed but differs in each *individual* instrument - and the incident and observation angles are 22.5°.[919] It is important to realise that in modern instruments such as the SP2000P, the requirements and restrictions of non-contact specular microscopy cause distortion of endothelial images as well. In turn, distortion introduces errors in the measurement of the ECD. Three factors can be discerned.

First, as mentioned before, the endothelium is observed from an oblique angle that cannot be negligibly small.[274 287] See figure 6.1a. Due to this oblique observation angle, an image of an endothelial area is perspectivistically distorted: the image actually depicts a larger area of the endothelium than it is assumed to do. Thus, perspectivistic distortion leads to an underestimation of the observed endothelial area. This in turn leads to overestimation of the measured ECD.

Second, the area that is imaged, the endothelium, is a curved rather than a flat plane. See figure 6.1b. A curved length is longer than the straight (dissective) length which is supposed to represent it. This fact also leads to underestimation of the observed area, and hence to overestimation of the ECD. Bron and Brown already mentioned this phenomenon but did not elaborate.[12] Olsen described it in more detail, with special attention to its relation to distortion caused by the oblique angle of observation.[274] The formulas deduced by Olsen for the combined distortions by oblique observation angle of a curved area will be reviewed in the "Methods" section.

Third, the observed endothelial area is magnified by the anterior corneal curvature. See figure 6.1c. Magnification of an image leads to overestimation of the observed area, which in turn causes underestimation of the ECD. Stefansson et al. reported in a normal eye a 2.5 % increase in the lateral image scale caused by this effect, but provided no calculations to substantiate this figure.[225] Bourne

and Enoch deduced formulas for the magnification effect in contact specular microscopy. Isager et al. and Nawa et al. published on altered magnification of the endothelium in non-contact specular microscopy after corneal refractive surgery.[913 914] Their fomulas will be reviewed in the "Methods" section.

In clinical practice often eyes are imaged in which the corneal curvature and thickness are substantially different from average, such as in keratoconus or after corneal transplantation. The formulas presented by Bourne and Enoch, Isager et al., Nawa et al., and Olsen, indicated that corneal curvature and corneal thickness affect all the effects of optical distortion mentioned above.[273 274 913 914] Hence, additional under- or overestimation of ECD may occur when non-contact specular microscopy is applied to corneas with non-average thickness and curvature.

The aim of the present study was to review the three errors that are caused by optical distortion in non-contact specular microscopic ECD measurements in general, and in Topcon SP2000P non-contact specular microscopy in particular. Furthermore, we wanted to investigate the effect of different values of corneal thickness and curvature on these errors. For this purpose we calculated the magnitude of the error that was introduced by each of the effects, for several different values of corneal thickness and curvature. We used the appropriate formulas, and took the SP2000P settings for observation angle and slit width as a starting point. The combined effect of these errors was also evaluated.

Furthermore, we compared whether these optical factors are applicable in other currently available imaging techniques of the endothelium. These include in-vivo techniques such as contact specular microscopy and in-vivo confocal microscopy. But also an in-vitro technique using light microscopy for evaluation of the endothelium of donor corneas from the endothelial side, which is performed in many eye banks,[812] is discussed.



## Methods

1. **Oblique horizontal angle of observation.**
As stated before, an oblique angle of observation is necessary in specular microscopy, to avoid the dominant epithelial reflex.[274 287 918] This applies only to the horizontal dimension. In the vertical dimension the eye is aligned with the specular microscope, and so there is no vertical angle of observation dictated by the technique.[274]
See figure 6.1.a. The magnitude of the error introduced by an oblique angle of observation is:

$$L_{assumed} = \cos \beta * L_{max}$$

with β as the (horizontal) incident angle to the orthogonal on the cornea, $L_{max}$ is the truly observed (maximal) horizontal area length, and $L_{assumed}$ is the resulting, underestimated horizontal length. In the settings of the Topcon SP2000P, the incident angle onto the anterior cornea is 22.5˚.[919] $L_{assumed}$ would therefore represent an underestimation of $L_{max}$ and also of the observed area, of cos(22.5˚ ) = 0.924. Hence an overestimation of the ECD of 8.2 % would be present.

It should be noted, that we used the angle **β** which is the angle with the normal *on to the anterior corneal curvature* for the incident and observation angles. In his paper, Olsen used as incident and observation angles the angle on to the endothelium *after anterior corneal deflection*.[274]. In figure 6.1.a. this latter angle is the angle **α.** However, Olsen appears to have made a mistake here, since the perspectivistic distortion of an image is not altered by deflection of the light. In the discussion section this issue is elaborated upon in more detail.

2. **Observation of a curved area.**

As discussed earlier, the area of the endothelium that can maximally be observed in non-contact specular microscopy, is restricted. See figure 6.1b. Olsen deduced formulas for this phenomenon and for the additional distortion by observation of a curved area in its relation to the distortion caused by the oblique angle of observation, in the horizontal dimension:[274]

1. $L_{max} = 2 * CT * \tan \alpha$ ,

in which $L_{max}$ is the maximally observed curve length of the endothelial reflection, **CT** = corneal thickness, and **tan α** is the tangent of the incident angle to the normal after anterior deflection of the incident light by the anterior cornea. See figure 6.1. *Please note that for this calculation the angle after deflection **α** must be used!*
If **α** = 16.1˚, and **CT** = 0.500mm (assumed average corneal thickness), then:
$L_{max}$ = 0.289 mm.

The maximal curve length $L_{max}$ can also be expressed as an angle of arc **φ** (in radians), using the corneal radius **r**:

2. $\varphi = L_{max} / r$ .

If **CT** = 0.500 mm, and **r** = 8.00 mm (assumed average corneal radius), then **φ** = 0.0361 rad.

The relation between the observed curve length $L_{observed}$ and $\varphi$ was derived by Olsen to be:

3. $L_{observed} = \cos(\frac{1}{2}\varphi + \beta) * \{ (r^2) * ( \sin^2 \varphi + (1 - \cos \varphi)^2 ) \}^{\frac{1}{2}}$ .

Substituting $\varphi$ = 0.0361 rad and $r$ = 8.0 mm results in:
$L_{observed} = \cos(0.0180 \text{ rad} + \beta) * 0.289$, which can be replaced by:
$L_{observed} = \cos(0.0180 \text{ rad} + \beta) * L_{max}$ .
Substituting $\beta = 22.5^{\circ} = 0.393$ rad results in:
$L_{observed} = \cos(0.411 \text{ rad}) * L_{max} = 0.917 * L_{max}$

*Please note that in formula 3. the undeflected angle* $\boldsymbol{\beta}$ *must be used!*
Thus, observing a curved plane causes an additional underestimation of 0.7 % of observed area, resulting in a 0.84 % additional overestimation of ECD compared to the error introduced by only the oblique observation angle. This therefore is only a relatively small additional error.

The source of error caused by the curvature in the vertical dimension was calculated by Olsen as well.[274] This error would be greatest at the uppermost and lowermost border of the image, because at these vertical image borders a slight vertical observation angle is present - at the horizontal plane the observation angle = 0. In slits of 1 mm high, the underestimation of the observed area at the upper- and lowermost borders (0.5 mm from the horizontal plane) would be according to the formula in part 1 and formula 2 in part 2 of this Methods section:

$L_{assumed} = \cos(0.5/8 \text{ rad}) * L_{max} = 0.998 *$ the vertical dimension.

Thus, an 0.2 % overestimation of ECD at the vertical borders of the image would result if the vertical slit length was 1mm. The SP2000P uses a slit illumination with a vertical dimension significantly shorter than 1 mm.[919] The distortion in the vertical dimension is therefore negligible.



**c. Magnification by the anterior corneal curvature.**

The following formulas were adapted from general optical formulas presented by Katz and Kruger in the handbook Duane's Clinical Ophthalmology, [920] and formulas by Bourne and Enoch,[273] who calculated this effect for contact specular microscopes. Especially valuable were the formulas by Isager et al.[913] and Nawa et al[914], who calculated changes in magnification after changes in corneal thickness and power/curvature had been induced by excimer corneal ablation. See figure 6.3.

If $CT$ = corneal thickness (mm), $r$ = anterior corneal radius (mm), and $P$ = anterior corneal refractive power (D), $n_{cornea}$ = the refractive index of the

cornea, assumed 1.376, and $n_{air}$ = the refractive index of air = 1.0, the following formulas apply for magnification:

4. **M** = magnification = **U** / **V**

5. **U** = reduced object vergence power
= ($n_{cornea}$ /object distance at the anterior corneal surface) = $n_{cornea}$ / **CT**, with because of divergence, the object distance **(CT)** defined as negative;

6. **V** = reduced image vergence power, and the relation between **U**, **V**, and **P** is: **U** + **P** =**V**;

7. $P = (n_{cornea} - n_{air}) / r$ ;

Then:

$M = (n_{cornea} / CT) / \{(n_{cornea} / CT) + P\}$
$= (n_{cornea} / CT) / \{(n_{cornea} / CT) + (n_{cornea} - n_{air}) / r\}$ or $= 1 / \{1 + (P * CT) / n_{cornea}\}$
$= (1.376 / CT) / \{(1.376 / CT) + (0.376 / r)\}$ or $= 1 / \{1 + (P * CT) / 1.376\}$

Remember to fill in a negative value for CT when calculating the magnification, and to enter CT in meters instead of millimetres when calculating with P in dioptres.

8. This applies for one dimension; $M^2 = U^2 / V^2$ etc. for 2 dimensions.

9. The normalized magnification **(NM)** is the magnification $M^2$ in a situation with a specific corneal thickness and radius c.q. power, divided by the magnification for the assumed average values ($M^2_{avg}$). In our case these assumed average values are **CT** = 0.500 mm, and respectively **r** = 8.0 or **P** = 43.0 D. In the settings of the Topcon SP2000P these also are the standard settings.[913] An increased (i.e. higher than 1.0) normalized magnification means that the observed endothelial area is additionally enlarged due to altered circumstances, whereas a normalized magnification lower than 1.0 implies less than normal magnification.



## Results

1 & 2. **Olsen's formulas for observation of a curved area under an oblique angle.**
In Table 6.1, the effects of optical distortion on the observed horizontal curve length in relation to the maximal curve length are presented for different values for corneal radius and thickness. For the calculations, Olsen's formulas for

oblique angle of observation and observation of curved areas were used, with a value of 22.5° for the observation angle, and assuming 0.500 mm as average corneal thickness, and 8.00 mm as average corneal radius.
From section 1 and 2 of Methods & formulas it was clear that the effect of an oblique angle of observation amounted to an overestimation error of ECD of 8.2 %, to which an error of 0.84 % was added due to observation of a curved area. From the calculations in Table 6.1 it follows that the effect of different values of corneal thickness and radius on these first two errors was relatively small. The largest effects were found in thick, steep corneae (e.g. r = 7.00 mm, CT = 0.600 mm), which caused in our example an extra overestimation of ECD of 0.3 %, or in thin, flat corneae (e.g. r = 9.00 mm, CT = 0.400 mm) in which the overestimation in ECD in an averagely curved cornea was diminished with 0.2 %. In clinically more common thin, steep corneae (such as in keratoconus, e.g. r = 7.00 mm and CT = 0.400 mm) the overestimation of ECD was not different than in the average cornea. For other combinations of radius and thickness, please see Table 6.1.

3. **Anterior corneal magnification.**

In Table 6.2, calculation examples for magnification **($M^2$)** and normalized magnification **(NM)** are given for several values of **CT**, **r**, and **P**. Some of these examples can also be found in the study by Isager et al. in their table 1.[913]. From Table 6.2 it appears that for the range of values provided for **CT**, **r**, and **P** (**CT** from 0.350 mm to 0.650 mm, **r** from 6.00 to 10.0 mm, **P** from 38 to 60 D), the image magnification ranges from 1.9 to 6.2 %. This causes an underestimation of ECD of 1.9 to 5,8 %. Thinner flatter corneas have the least magnification whereas thick steep corneas have the highest magnification. Thin steep corneas, such as often encountered in keratoconus patients, appear to have magnifications that are actually very close to those of normal corneas. (I.e. tenths of a percent point lower).

It should be noted that the assumed averages for corneal thickness (0.500 mm), radius (8.00 mm) and corneal Power (43.0 D) were chosen for convenient calculation in these examples, and do not represent the actual population average. This also explains why in our examples the assumed average corneal radius does not entirely correspond with the assumed average corneal power, although it physically should **($P = (n_{cornea} - n_{air})/ r$)**.

4. **Combined effects of optical distortions.**

In the preceding sections it was shown that the contribution of the oblique angle of observation to this overestimation of ECD is much more important in this overestimation than the effect of observing a curved area. In the SP2000P, for average values of **CT** and **r** these are 8.2 and 1.2 %, respectively. The third factor, the magnification of the image by the anterior corneal curvature leads

to an overestimation of the observed area and hence to an underestimation of the ECD. For average values of **CT** and **r (P)** this underestimation of ECD is 3.4 (3.1) %.

All these factors considered together result in an overestimation of ECD of 1.094 * 0.966 = 1.060, i.e. 6 %. However, the error introduced by the oblique angle of observation is supposedly corrected in the SP2000P, by a combination of adjustments in the camera optics and software. (P. Gelissen, Topcon Europe, personal communication). Hence, in an average cornea a remaining underestimation of ECD of 2.2 % would result from the effects of observing a curved area and the anterior corneal magnification (1.2 % overestimation vs. 3.4 % underestimation; 1.012 * 0.966 = 0.978).

## Discussion

In non-contact specular microscopy, three optical factors cause distortion and hence an error in ECD measurement. These are: an oblique angle of observation, observing a curved area, and magnification of the image by the anterior corneal curvature. Of the first two factors, the error caused by a horizontal oblique angle of observation is by far the more substantial. In the SP2000P this error is corrected (P. Gelissen, Topcon Europe, personal communication, 2004). In average corneas, the remaining two factors cause a slight underestimation of ECDs measured with the SP2000P, of 2 to 2.5 %.

Corneal factors such as corneal thickness and radius or power influenced these optically induced errors. Even with substantial alterations in these corneal characteristics, the overestimation of the ECD by observing a curved area from an oblique angle did hardly change (0.3 % or less). In contrast, the underestimation of the ECD by the anterior corneal curvature magnification ranged from 1.9 to 5.8 % with different values for corneal thickness and radius or power. The largest variation was caused by a substantially increased corneal thickness. Obviously, an altered corneal refractive index would also have an impact on the described optical factors. Both an altered refractive index and a severe thickening of the cornea may be present in corneal edema or other diseased states. However, in these circumstances it would be difficult, if not impossible, to obtain any specular microscopic images at all.

An essential point needs to be made regarding the literature on the effect of an oblique angle of observation in specular microscopy. Olsen used in his study not the incident angle onto the anterior cornea, but the angle that results after anterior corneal deflection (i.e. not 22.5˚, but 16.1˚). However, Olsen made a mistake here, since basic physics indicates that perspectivistic distortion

of an image is not altered by deflection of the incident and reflected light. A simple physical experiment in which a ruler attached to an glass surface is photographed under an oblique angle may demonstrate this. In the study by Ohno et al., also the incident angle onto the anterior corneal surface and not the angle after anterior corneal deflection is used.[287] Even Olsen's own experimental data indicate that he should have used the incident angle onto the anterior corneal surface (in his case 23°) instead of the angle after anterior corneal deflection (16.5°) in his calculations.[274]

Modern contact specular microscopes usually have smaller observation angles than non-contact specular microscopes.[287] Hence, in most contact specular microscopes, distortion by an oblique observation angle will be much less than in most non-contact instruments. So called "wide-field" contact specular microscopes use additional methods for image acquisition, and are left out of consideration.[25-27] In confocal microscopy a substantial angle of observation is not required, as the common focal point principle eliminates strong reflections from surrounding tissue. Furthermore, several images are sampled in three dimensions.[303] Hence, it may be assumed that there is not a significant effect of an oblique angle of observation. The distortion of an oblique angle of observation can also be neglected in eye banking endothelial light microscopy, where areas of the endothelium are observed from the endothelial side, under (close to) orthogonal angles.

The additional error in the overestimation of ECD introduced by observing a curved area instead of a flat area, was relatively small in non-contact specular microscopy. In contact specular microscopy, most instruments use a "dipping" applanation cone and a coupling medium to minimize corneal curvature deformation.[14 15 918] Yet some applanation of the cornea may still occur, which will diminish the curvature of the observed endothelial area. This in turn diminishes the difference between the truly observed and the assumedly observed area, causing less underestimation of the observed area, and hence less overestimation of the ECD. Similarly, in confocal microscopy applanatory effects may also occur. The sampling technique with which images in different dimensions are obtained will further diminish curvatural distortion.[303] In light microscopy curvatural distortion may be diminished by gravity. Excised donor corneoscleral buttons lying on their epithelial side in a petri-dish will become somewhat flattened. This again may diminish the overestimation of ECD. However, simultaneously the ECD may be increased by the flattening as the same amount of cells now have a smaller area on which they rest. Both effects may neutralize each other.

In contact specular microscopy and confocal microscopy, lack of a cornea-air interface due to a coupling medium, together with some applanation of the cornea, will diminish the effect of anterior corneal magnification.[273 303] When

observing the endothelium from the endothelial side, as in light microscopy, this factor is not relevant.

In conclusion, in the present study we have reviewed the optical distortions that may affect ECD counts in non-contact specular microscopy. An oblique (horizontal) angle of observation, and the observation of a curved area both cause ECD overestimation. Magnification of the endothelial image by the anterior corneal curvature causes ECD underestimation. Corneal thickness, and corneal radius or power affect the amount of distortion. The more oblique the angle of observation, the more substantial the overestimation of ECD. When this is corrected as in the SP2000P, in most corneas the other optically induced errors are rather small, and a slight underestimation of ECD remains. However, in thicker, steeper corneas, ECD underestimation due to anterior corneal magnification becomes more important. Differences that are found in the comparison of ECDs from non-contact specular microscopy to data obtained with contact-specular microscopy, confocal microscopy, or light-microscopy, may in part be explained by differences in optical distortion.

## References

See page 287.

**Figure 6.1** **Diagrammatic representation of the optic distortions in non-contact specular microscopy.**
See colour figure on page 328.

Table 6.1 Effects of observation under an oblique angle of a curved endothelial area, for different values of corneal thickness (CT) and corneal radius (r ).

| | $L_{max}$ (mm) | φ (rad) | $L_{observed}$ (mm) | $L_{max}$ (mm) | φ (rad) | $L_{observed}$ (mm) | $L_{max}$ (mm) | φ (rad) | $L_{observed}$ (mm) |
|---|---|---|---|---|---|---|---|---|---|
| | CT = 0.400 mm | | | CT = 0.500 mm | | | CT = 0.600 mm | | |
| r = 7.00 mm | 0.231 | 0.0330 | 0.917 * $L_{max}$ | 0.289 | 0.0412 | 0.916 * $L_{max}$ | 0.346 | 0.0495 | 0.914 * $L_{max}$ |
| r = 8.00 mm | 0.231 | 0.0289 | 0.918 * $L_{max}$ | 0.289 | 0.0361 | 0.917 * $L_{max}$ | 0.346 | 0.0433 | 0.915 * $L_{max}$ |
| r = 9.00 mm | 0.231 | 0.0257 | 0.919 * $L_{max}$ | 0.289 | 0.0321 | 0.918 * $L_{max}$ | 0.346 | 0.0385 | 0.916 * $L_{max}$ |

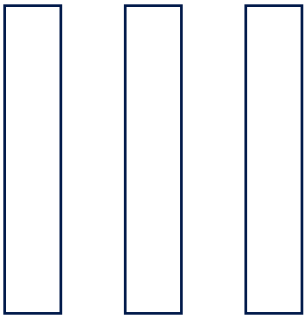
The meaning of $L_{max}$ , φ, and $L_{observed}$ is explained in the "Methods" section.

Table 6.2 Endothelial image magnification and normalized magnification for different corneal thicknesses, radii, and powers.

| R (in mm) or P (in D) | | CT (in mm) | | | | |
|---|---|---|---|---|---|---|
| | | 0.350 | 0.400 | 0.500 | 0.600 | 0.650 |
| 6.0 mm | $M^2$ | 1.033 | 1.037 | 1.047 | 1.057 | 1.062 |
| | NM | 0.998 | 1.002 | 1.012 | 1.021 | 1.026 |
| 7.0 mm | $M^2$ | 1.028 | 1.032 | 1.040 | 1.049 | 1.053 |
| | NM | 0.993 | 0.997 | 1.005 | 1.013 | 1.017 |
| 8.0 mm | $M^2$ | 1.024 | 1.028 | **1.035** | 1.042 | 1.046 |
| | NM | 0.990 | 0.993 | **1.000** | 1.007 | 1.011 |
| 9.0 mm | $M^2$ | 1.022 | 1.025 | 1.031 | 1.037 | 1.041 |
| | NM | 0.987 | 0.990 | 0.996 | 1.002 | 1.005 |
| 10.0 mm | $M^2$ | 1.019 | 1.022 | 1.028 | 1.034 | 1.037 |
| | NM | 0.985 | 0.988 | 0.993 | 0.999 | 1.001 |
| 60.0 D | $M^2$ | 1.031 | 1.036 | 1.045 | 1.054 | 1.059 |
| | NM | 0.999 | 1.004 | 1.013 | 1.022 | 1.026 |
| 43.0 D | $M^2$ | 1.022 | 1.025 | **1.032** | 1.039 | 1.042 |
| | NM | 0.990 | 0.994 | **1.000** | 1.006 | 1.010 |
| 38.0 D | $M^2$ | 1.020 | 1.022 | 1.028 | 1.034 | 1.037 |
| | NM | 0.988 | 0.991 | 0.996 | 1.002 | 1.005 |

The normalized magnification (**NM**) is the ratio of the magnification for a specific combination of corneal thickness (**CT**), and either radius (**r**) or power (**P**), divided by the magnification for assumed average corneal thickness (**CT** = 0.500 mm = 0.0005 m), and respectively radius (**r** = 8.0 mm) or power (**P** = 43.0 D).

part



# Effects on the corneal endothelium of the application of trypan blue

# chapter

# 7

# Characteristics, surgical applications, and toxicity of trypan blue and indocyanine green

# I. Introduction to vital stains in ophthalmology.

Early in the twentieth century the principle of vital staining was introduced in medicine. In this diagnostic technique, dyes are injected into the body of living animals or humans, or applied onto living cells. Non-vital staining techniques are more often applied and better known, e.g. histologic staining. In this technique dyes are mostly applied onto dead tissue, and furthermore, fixation (and sectioning) of the tissue is usually necessary before the staining itself is done, killing any cell that may still have been alive. Whether the dyes themselves in non-vital staining are or are not toxic is not really important, as the investigated tissues do not need to survive. In contrast, vital stains must not be lethal to the living cells, nor damage the organs, nor be harmful to the organisms, to respectively in which they are applied. Therefore, possible toxicity of vital stains is of great concern.[921-925]

The best known diagnostic vital stain used in ophthalmology is fluorescein, a yellow macromolecular hydroxyxanthene dye ($C_{20}H_{10}O_5Na_2$,[926]). It has seen a long and very frequent use and has a good safety record. Topical application of fluorescein aids in the detection of corneal epithelial lesions ("positive staining"). Coloration of the tear film aids in applanation tonometry, in evaluating tear film stability and lacrimal drainage, in contact lens fitting, and in testing of leakage of aqueous from corneal wounds (Seidel test). A so-called negative staining pattern refers to the highlighting of non-staining elevated lesions as they project through the stained tear film.[927-930] Systemic (intravenous or oral) application allows its use as a contrast dye in retinal angiography. Fluorescein can also be applied in a test of the endothelial barrier, as discussed in paragraph VIII c. Here, fluorescein concentration is quantitated with fluorophotometry.[1 40 84 85 364 377 379 381-384] Using the dye's fluorescence property enhances all of these tests, and in the case of fluorescein angiography fluorescence it is essential. To cause fluorescence, fluorescein molecules are excited with light from the blue wavelength part of the spectrum. In biomicroscopy for instance a cobalt blue filter is applied. In fluorescein's use as a tool to detect epithelial defects it stains areas where epithelial cells are completely absent or where their intercellular junctions are disrupted. It may stain living cells but less effectively than its alternatives, lissamine green and rose bengal. Rose bengal also is a halide derivate of the hydroxyxanthene dye family, akin to fluorescein. It stains devitalized corneal and conjunctival cells or cells that have lost their mucin surface, but is blocked by a healthy tear film. It is intrinsically toxic to the epithelium.[927-930]

Concern about toxicity of vital stains became more urgent when some of them were beginning to be used not only diagnostically but also during ophthalmic surgery. Currently, the most often used surgical applications of vital stains are

lens capsule staining in cataract surgery and membrane staining in vitreoretinal surgery. See figure 7.1. The most often used vital stains for both purposes are trypan blue (TB) and indocyanine green (ICG). The surgical use of both dyes implied a substantial increase in the scope their application. Furthermore, the staining was applied in the vicinity of very delicate living cells with critical functions, and without regenerative capacity: the corneal endothelium, the retinal photoreceptors and nerve cells, and the retinal pigment epithelium. Hence it is understandable that a lot of research on the toxicity of TB and ICG was stimulated by the fear for possibly widespread iatrogenic damage.

In the following paragraphs, a systematic review is presented of the literature on trypan blue and its counterpart indocyanine green, describing their history, characteristics, and toxicity; regarding both surgical and other applications. Other vital stains are touched upon only briefly, in the context of their use as an alternative adjunct for a specific surgical application. This review serves as a background to the studies described in Chapters 8 and 9. These studies present data on toxicity and safety regarding the corneal endothelium, concerning the use of trypan blue lens capsule staining in cataract surgery.

Fig. 7.1 **Trypan blue capsule staining to aid visualisation of the anterior lens capsule in mature cataract phacoemulsification.**
See colour figure on page 329.

## II. History, characteristics, and applications of trypan blue and indocyanine green.



### a. Trypan Blue (TB).

In 1914, Evans and Schulemann reviewed a category of vital stains that did not undergo chemical transformation (e.g., oxidation or reduction) inside the human body. An important characteristic of these stains was that they were not perceptibly toxic when injected into the bloodstream of living animals. These dyes were stored inside living cells, especially leukocytes (reticuloendothelial cells) by means of phagocytosis. Stain granules within cells could easily be detected with light microscopy. All dyes in this group contained benzidine-groups, and one of the foremost categories on which Evans and Schulemann reported was that of the di-azo dyes. Di-azo refers to the two N = N bonds between the three double hexagons (i.e. benzidine rings) that constitute the chemical backbone of the dyes in this group. The capacity of being a good vital stain was thought to depend on the presence, number, and/or position of sulphonic acid (radicle) groups, $NaSO_{3,}$ attached to the benzidine rings. Monosulphonic benzidine rings appeared to have lesser vital staining capacity than disulphonic benzidine rings.[921] Trypan blue was

one of the di-azo dyes and has the formula ($C_{34}H_{24}N_6O_{14}S_4Na_4$,[926]), with 4 sulphonic acid groups.[921] See figure 7.2.

Besides being phagocytized, diffusion of TB into dead or severely damaged cells is another way in which it stained cells. This type of staining is more uniform and diffuse than the granular staining after phagocytosis.[921 922 924] In Pappenheimer's study of 1917, experimentally damaged lymphocytes in animal thymus tissue were stained in this way.[922] Strictly speaking, this latter mechanism of TB staining should be not be called vital staining but supra-vital staining, as the dye does not stain living cells but dead or irreversibly damaged cells. For this type of staining substantial cell (or nuclear) membrane damage appears to be a prerequisite, and the nucleus more than the cytoplasm of the damaged cell is stained.[931]

Later a third way of staining with TB was discovered, when it was noted that TB stained basement membranes such as Descemet's membrane that had been denuded from their (endothelial) cells. (E. Pels, personal communication, 1998).

The first ophthalmic use of TB was described by Stocker et al., who, using the supra-vital staining principle, applied TB (0.25 %) to assess endothelial cell damage in donor corneas.[326-328] The technique of TB (supra-) vital staining of donor corneal endothelia has since then stayed in use in eye banks using the organ culture preservation method. Currently, usually TB in a concentration of 0.1 to 0.3 % is applied.[812] See Chapter 2 of this thesis, paragraph VIII c., for an extensive description of the development and the technique of TB staining of donor corneas; and paragraph XVI e. for an extensive discussion of the organ culture preservation method for donor corneas.

In 1967 Norn instilled TB in the conjunctival sac of patients to specifically stain pathological cornea processes. He judged a mixture of rose bengal and fluorescein to be more useful than TB.[931] In 1979, Norn described "postmortal vital staining" of the cornea and conjunctiva, in order to determine the nature of the processes that give dead eyes the "glazed" look.[932] As early as 1971, he reported on intra-operative endothelial staining of the endothelium with TB. He instilled TB 0.1 %- 0.25 % into the anterior chamber of 80 eyes during intra-capsular cataract extraction (ICCE). In this series he observed stained lines where the cornea had been bended, larger stained areas in cases of corneo-vitreous touch, and irregularly stained figures after alpha-chymotrypsin injection. He observed no postoperative complications related to the use of TB. In this paper he reported the observation that the lens capsule was also stained by TB.[933] In a follow-up study, he reported on 47 patients 6-12 months after ICCE with intraoperative TB instillation in the anterior chamber. They were compared to randomized control patients that had undergone ICCE without TB

staining. No significant differences in pachymetry or clinical outcomes such as visual acuity were found.[934] A second follow-up study, 8 years post-operatively, reported on 24 remaining patients from his cohort. In 15 patients from this group, the eye in which TB had been applied was compared with the operated but non-stained contralateral eye. No differences between stained and non-stained eyes were observed regarding endothelial cell density, pachymetry or clinical outcomes.[935] In both follow-up studies Norn concluded that the intra-operative use of TB was not (additionally) harmful for the corneal endothelium.

Completely different effects of TB have also since long been known. Among the earliest reports on the use of trypan blue was that it was the first agent used to treat bovine babesia, an intraerythrocytic parasite similar to malaria, in 1909.[936] Since 1948 it has been known that it can be used to create congenital malformations in animal models. Several animal species have been used for this purpose, including rabbits, chickens, rats and mice. Usually, TB was injected intraperitoneally in pregnant animals, in a species-specific teratogenic "window" early in gestation, in a species-specific teratogenic dose. (See also paragraph IV of this chapter). Especially closure defects of the neural tube, foremost spina bifida, have been encountered. There has been some discussion whether the abnormalities resulted from arrested embryological development, or from degeneration and necrosis from already formed "anlagen".[937 938] Schmidt et al. have demonstrated in a rat model that the development of virtually all important structures of the embryological eye can also be halted, directly or indirectly, by intraperitoneal TB injections in the mother animal.[939 940].
Besides teratogenic properties, TB also may have some carcinogenic capacities in animal experiments. In specific inbred strains of rats, reticuloendothelial tumours have been induced in the liver after regimens of repeated subcutaneous injections of small doses of a 1% TB solution. (For exact doses and regimens, see paragraph IV of this chapter). The tumours probably stem from macrophages (in the liver: Kupfer cells), which are indeed the cells expected to actively phagocytose TB.[941]



b. Indocyanine Green (ICG).

Indocyanine green is a tricarbonate green dye ($C_{43}H_{47}N_2NaO_6S_2$, [926]) with a molecular weight of 775 D. See figure 7.3. After intravenous injection, this dye is for more than 95 % bound to plasma albumin and it is completely excreted via the hepatic pathway. Since 1956 it has been used intravenously to determine cardiac output, hepatic function, and in liver blood flow studies.[923 925] In 1970 this dye was introduced in ophthalmology. It enabled choroidal angiography after intravenous injection. ICG angiography appears to be especially useful for evaluating retinal pigment epithelial detachments and choroidal neovascularisations.[942-944] These applications have seen a long and apparently safe use since.[945]

Similarly to trypan blue ICG has also been applied as a supra-vital stain in the evaluation of donor corneas, and also similarly did not appear to be harmful to living corneal cells. However, in contrast to trypan blue ICG has not seen widespread use in eye banks after the first publication.[946] To our knowledge, ICG was not used in intra-ocular surgery before 1998.[947]

Fig. 7.2 Structure formula of trypan blue. Note the two N = N (azo-)bonds, each connecting two double hexagonal rings. (Trypan blue is a di-azo dye).

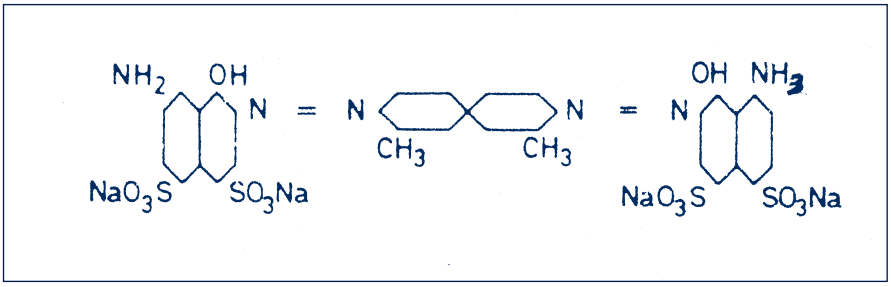
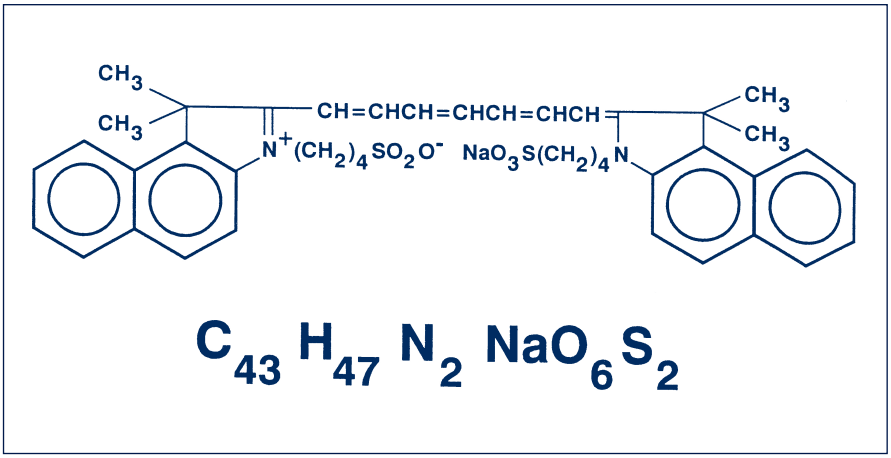



Fig. 7.3 Structure formula of indocyanine green.



## III. Intra-operative applications of TB and ICG: cataract surgery, vitreoretinal surgery, and keratoplasty.

### a. Lens capsule staining in cataract surgery.

An important objective of the extracapsular types of cataract surgery, which include the phacoemulsification techniques, is to preserve the lens capsule. The preservation of the capsule serves to ensure the stability of the eye, and to facilitate effective cataract extraction and placement of an IOL in its natural and logical position. An essential step was the development of a technique for providing an opening in the anterior lens capsule which simultaneously preserved the integrity of the remainder of the capsule. For this purpose, from the early 1980s onwards, the continuous circular (a.k.a. curvilinear) capsulorhexis or CCC was developed concurrently and independently by Gimbel in North America, Neuhann in Europe, and Shimizu in Japan.[948] However, in case of low contrast, e.g. in the absence of a red fundus reflex, performing an uncomplicated CCC may suffer from poor visualisation. This is often the case in eyes with a white, mature, hypermature, or intumescent cataract, and/or a heavily pigmented fundus, e.g. after panretinal lasercoagulation.

To overcome this problem, Gimbel proposed a two-stage CCC, in which first a small-diameter CCC is performed to allow for phacoemulsification, followed by a second, larger diameter CCC to facilitate IOL implantation.[949 950] Variations on this technique are currently still in use. Drawbacks are a longer procedure, incomplete CCC with or without anterior capsule tears or even posterior capsule tears (up to 28 % of cases), and the technically more demanding performance of phacoemulsification of a hard nucleus through a small capsulorhexis. Because of these drawbacks, sometimes conversion to ECCE with manual nuclear expression is necessary.[951 952] Instrumental approaches to the problem of poor visualisation of the capsulorhexis included anterior capsule diathermy using a straight, high-frequency bipolar diathermy probe,[953-955] and side illumination using a fiberoptic endoilluminator.[956]

Staining the anterior capsule was a third approach. Initially intracapsular injection of a Na-fluorescein solution, possibly combined with blue light,[957 958] and hemocoloration of the capsule with autologous blood were reported.[959] From 1998 onwards, the use of several other staining methods and dyes were reported.

Gentian violet (a.k.a. crystal violet; $C_{25}H_{30}ClN_{3,}$[926]) was among the first dyes to be investigated. Gentian violet in concentrations as low as 0.001 % (alone or in combination with methylene blue 1 %), provided adequate staining of the capsule. However it also resulted in temporary corneal edema, and

also in anterior chamber reaction.[960 961] Increased pachymetry, from 543.6 µm preoperatively to 578.1 µm 1 month postoperatively, which was not statistically significant, was measured after its use.[962] In rabbit experiments, all crystal violet concentrations were useful for staining purposes, but there was a concentration dependent toxicity. Concentrations of 0.25 % and lower did not result in (persistent) gross abnormalities on light or electron microscopy, and 0.05 % did not seem to result in any abnormalities.[963]

Horiguchi et al. reported on successful capsular staining with one or two drops of indocyanine green (ICG) in a concentration of 0.5 %. Furthermore, no toxic effects of ICG were seen with biomicroscopic, applanation tonometric, specular microscopic, and laser flare-cell photometric investigation in this study of 10 cases.[947] More reports on the successful use of ICG for capsule staining in several clinical and experimental settings followed. The dye, which is usually dissolved from the dry form into a 0.5 % solution in the operating room, is currently a popular choice for anterior capsule staining. [964-971] Studies on possible ocular toxicity of ICG are described more extensively in section IV of this chapter.

In 1999, Melles introduced the use of trypan blue 0.1 %, applied onto the anterior lens capsule under an air bubble. His results showed good capsule staining ability and no apparent toxic effects, in a series of 30 patients with mature cataracts. In this study Melles reported that he had studied staining properties in eye-bank globes of a great number of other dyes. However, except for TB the dyes either did not have good staining ability, or were suspected to be toxic, or had never been applied for intra-ocular use before.[32] TB, both generically made solutions and commercially available preparations (Vision Blue®, 0.1 % and 0.06 %, DORC, Zuidland, The Netherlands), is more economic in use than for instance ICG. This, added to its effective staining capacity and apparent safety, is probably what helped TB capsule staining to gain wide-spread popularity. TB has now become an often used tool in Europe, and Vision Blue® has recently been approved by the FDA for use in the United States as well. Especially in countries where white cataracts are still much more common, such as India and Turkey, it is used often. It were Indian and Turkish authors who were able to constitute reasonably large clinical series. These confirmed the effectivity and clinical safety of the use of TB.[962 972 973] Multiple minor modifications in the technique of application of the dye were reported. Modifications ranged from the shape of the cannula and the use of side to side movements during injection, to applying TB under a viscoelastic material instead of under an air bubble, to even mixing the dye with the viscoelastic Na-hyaluronate. Apparently all of these provided satisfactory capsule staining. [973-976] The effective concentration of TB for capsule staining under viscoelastics may be as low as 0.0125 %. [975] There are indications that TB does not only facilitate CCC by improved visualisation, but also by increasing the capsule stiffness due

to light-induced collagen cross-linking.[977] TB capsule staining may furthermore be useful for improved visualisation of the lens capsule during cataract extraction in patients with corneal opacities.[978] It has further been shown to be useful when applied to find the lost leading edge of a capsulorhexis that had already been started before staining.[979] Trypan blue has also been shown to be a useful aid to visualise inadvertently prolapsing vitreous into the anterior chamber.[980] Both ICG and TB were found to be useful for trainee surgeons in the performance of critical or additional steps in the phacoemulsification procedure, in experimental as well as clinical settings. [962 964 966-968]

In comparative experimental and clinical studies, both 0.5 % ICG and 0.1 % TB solutions were found to provide effective capsule staining, as did gentian violet 0.001 %. In contrast, autologous blood and subcapsular fluorescein 2 % were less effective stains, although digitally analysed colour saturation was comparable for fluorescein- and TB-stained capsular material. Moreover, subcapsular fluorescein was in some cases been found to inadvertently stain lenticular substance, or to leak into the vitreous. Both may decrease intra-operative CCC visualisation.[962 964 966-968 981 982] After long exposure (180 minutes) under experimental conditions, both TB 0.1% and fluorescein 2 % stained PMMA and especially hydrophilic acrylic IOLs.[982]

b. ICG and TB in vitreoretinal surgical membrane staining.

*1. Indocyanine Green for internal limiting membrane staining.*

An important goal in current vitreoretinal surgery for vitreomacular traction syndromes including macular holes, and for diabetic macular edema, is the removal of the internal limiting membrane (ILM) of the macular retina. Complete macular ILM removal has been shown to result in an improved macular hole closure rate and to support resolution of macular edema.[983-988] The ILM however is a poorly visible membrane, which makes complete removal difficult to perform and increases the risk on incomplete or inappropriate removal, leading to retinal damage, retinal edema, or pigment epithelium alterations.[985-987] Favourable reports on the intra-operative application of vital stains in cataract surgery (as discussed in the last section) encouraged intra-operative vital staining in macular surgery. From 2000 onwards experimental and clinical studies reported on effective and selective staining of the internal limiting membrane by ICG, resulting in anatomically and functionally successful macular hole surgery. Solvents to dilute the ICG solution included BSS and viscoelastic material. Usually applied concentrations of ICG ranged from 0.025 % to 0.5 %.[989-994] The first reported adverse finding after ICG-assisted ILM peeling was the case of a patient who showed persisting fluorescence at 795 nm, six weeks after macular hole surgery, however with good anatomical and functional improvement.[995] Soon thereafter more clinical and experimental studies reported a link between other troubling phenomena and ICG-assisted

peeling. These phenomena included the (unintended) finding of adjacent retinal structures on the peeled ILM; decreased mitochondrial activity in cultured retinal pigment epithelial (RPE) cells; ERG-changes in rats with and without morphological damage; clinically observed RPE changes in patients in concurrence with an unexpected high rate of poor visual acuity outcomes after macular hole surgery; and postoperative peripheral visual field defects in patients. After these reports appeared, the use of ICG in macular hole surgery has been controversial.[996-1001] The mechanisms of possible ICG-related toxicity are discussed in paragraph IV of this chapter.

2. *Trypan Blue for epiretinal and internal limiting membrane staining.*
In 2001 a study was published in which in a rabbit experiment trypan blue (TB) was injected into the vitreous cavity after gas-compression vitrectomy. TB in a concentration of 0.06 %, left in place for 4 weeks, was found not to result in any histopathological or functional (scotopic ERG) abnormalities. However, 4 weeks exposure to TB 0.2 % resulted in significant morphological aberrations in the inferior retina. [1002] TB 0.06 % has since then been applied to stain epiretinal membranes (ERM) in proliferative vitreoretinopathy (PVR), and to stain ILM and ERM in macular hole and macular pucker surgery.[1003-1005] In spite of the findings in the study of Veckeneer et al.,[1002] TB in a concentration of 0.2 % was also tried clinically in order to stain the ILM more intensely. This concentration provided adequate ILM and ERM staining in 50 patients with macular pucker, macular hole, a combination of both, PVR, or diabetic retinopathy. A commercial preparation of trypan blue 0.2% is now available (Membrane Blue, DORC International, Zuidland, The Netherlands).[1006] A clinical study using 0.15 % trypan blue intraoperatively in macular pucker surgery showed good surgical and clinical (visual acuity, visual fields) results in 10 patients compared to 10 matched controls. The same authors however reported in the same paper morphological retinal alterations in a study on post-mortem eyes with TB 0.15 and 0.25 %, but not with 0.02%.[1007] Studies on toxicity of trypan blue for retina and RPE cells will be discussed in more depth in paragraph IV of this chapter.

c. TB and ICG in keratoplasty.
After PK there sometimes may be inadvertently retained Descemets' membranes, especially in bullous keratopathy or in CHED. Trypan blue may assist in removing these.[1008]

During penetrating keratoplasty, deep anterior lamellar keratoplasty (deep LK) and posterior lamellar transplantation (PLK / DLEK), trypan blue and ICG have been applied. Descemet's membrane or its remainders can be stained with TB. This may be advantageous when preparing a nearly full-thickness donor lamella in deep LK by rubbing Descemet's membrane off,[1009] or to better visualise the "Descemet's roll" that is used in a variation of the posterior lamellar

transplantation technique.[1010] TB has also been used to stain wound edges and gauge dissection depth during PK and deep LK.[936 1011] The use of ICG has been reported to facilitate the visualisation of the posterior lamellar disk during PLK / DLEK, by staining its stromal side.[1012] In part IV, Chapter 10 of this thesis, techniques for lamellar keratoplasty will be more extensively reviewed.

## IV. Toxicity & biocompatibility of intra-operative TB and ICG.

### a. Systemic toxicity.

#### *1. ICG.*

Sporadic, moderate pseudo-allergic (i.e. non-specific release of histamine) to severe allergic (anaphylactic) reactions, as well as vasovagal reactions, have been reported after intravenous injection of ICG. No deaths have been reported. Risk factors for moderate to severe adverse reactions include liver disease and end-stage renal disease (uraemia), allergy to iodine (also: radio-opaque contrast agents, shellfish), and a cross allergy to sulfonamides and penicilline.[925 945] Experiments have shown that the dose for clinical intravenous applications is much lower than the lethal dose for 50 % of the mice (the LD50), which was 60/80 mg/kg.[1013] Systemic toxicity resulting from the intra-operative use of ICG, be it in cataract or in vitreoretinal surgery, is therefore not to be expected. Although ICG is bound strongly to plasma albumin, it has been observed to leak choroidally and intraretinally after intravenous injection.[1014]

#### *2. TB.*

As discussed in paragraph I of this chapter, trypan blue is known for its systemic toxicity, as it is a dye with mutagenic and carcinogenic capacities. A mechanism by which the mutagenesis and carcinogenesis of TB is brought about, has been postulated to be azo-reduction of trypan blue into the mutagenic product o-tolidine, by chemicals or by intestinal anaerobe bacteria.[937-940 1015]
It is interesting to determine whether intra-ocularly used doses might cause a possible hazard for teratogenicity or carcinogenicity. In the mentioned laboratory experiments, mice and rat strains were used which are inbred and therefore may be more susceptible to possible adverse effects. Assuming a mean weight of a laboratory mouse of 50 grams and of a rat of 200 grams, the intraperitoneally applied total dose of trypan blue to induce congenital malformations would be 50 – 100 mg/kg body weight.[937-940] The applied total dose in the carcinogenicity study of Ford en Becker amounted to at least 300 mg/kg body weight depending on the administration regimen, which required at least 6 subcutaneous administrations of trypan blue. [941]

In contrast, during cataract surgery maximally 0.5 ml of trypan blue 0.06 % is administered, i.e. a dose of ≤ 0.3mg (0.06 % = 0.6 mg/ml). Assuming for calculation purposes that the body weight of an adult patient is about 60 kg, then the dose administered amounts to 0.005 mg/kg body weight. For a body weight of 60 kg the administered dose is about 10,000 times lower than lowest known teratogenic dose and 60,000 times lower than the total carcinogenic dose – which needed to be administered repeatedly in at least 6 times. Moreover, the dose of 0.3 mg applied during cataract surgery does not remain completely in the eye. Excess dye is rinsed out immediately after application and a major part of the stained capsule is removed from the eye. All of the remaining dye is further diluted by irrigation during the operation and by aqueous circulation afterwards. Heavily diluted traces of dye could theoretically then reach the circulation via the aqueous drainage route.

In vitreoretinal surgery, 0.1 cc of 0.2 % is applied, resulting in a dose of 0.2 mg (0.2 % = 2 mg/ml), or 0.5 cc of 0.06 % resulting in a dose of 0.3 mg, or 0.5 cc of 0.15 % resulting in a dose of 0.75 mg. The latter dose is still 4,000 times lower than the lowest teratogenic dose. Doses in vitreoretinal surgery may even be more completely rinsed out of the eye and removed with the stained membranes than in cataract surgery.
In pregnant women and in young children it may however still be prudent not to use the entire provided dose of TB intraoperatively (or perhaps even not to use TB at all and switch to ICG if necessary), in order to minimize teratogenic or developmental side-effects.

b. Toxicity in vitreoretinal surgical applications.

*1. ICG*

As discussed in paragraph III of this chapter, clinical and experimental studies raised concerns that ICG applied directly onto the retina in macular hole surgery might be deleterious to RPE cells, and indirectly or directly, also to retinal receptor or nerve cells.[996-1001 1016] After ICG-assisted ILM peeling, Müller cell footplates and other retinal cell debris were found more than usually attached to ILM specimens. This led to the hypothesis that ICG staining led to either a different "cleavage plane" in the ILM peeling, or to more forceful traction on the ILM during peeling by the surgeon.[996 1000] ICG staining may also contribute to a higher susceptibility of RPE and retinal (ganglion- , Müller) cells to phototoxicity from endoillumination or (endo-)laser that is applied in vitrectomy surgery. However, this light-sensitization alone is not sufficient to explain all observed toxic effects.[997 998 1017-1021] Regarding the used ICG solutions, the factors pH and especially osmolarity of the solutions have been shown to be very important factors influencing the occurrence of toxic effects.[996 1021-1023] But again, effects of osmolarity (and pH) alone are not sufficient to explain all findings on toxicity caused by ICG. [1000 1001]

The concentration of ICG in its solution has in many experimental studies been shown to have an effect on the amount of ICG-caused toxicity. Exposure time has also sometimes been implicated. The toxicity threshold, i.e. the concentration at or above which toxic effects were observed morphologically, varied between 5 mg/ml (0.5 %) and 0.01 mg/ml (0.001 %). It should be noted that toxic effects were observed with different cell types and cell lines, and after different exposure times (3 minutes – 3 days).[1019-1021] Functional (ERG) abnormalities were observed in animal experiments at lower concentrations: 0.025 mg/ml in rats and from 0.5 mg/ml upwards in rabbits. These concentrations were not observed to cause consistent and persisting morphological alterations in the retina. However when a concentration of 2.5 mg/ml was used or when injected directly into the subretinal space structural damage was found. This experimental application may mimic subretinal diffusion of ICG when applied for macular hole surgery.[999 1023-1026]

In conclusion, indications exist that there is concentration- and exposure-time dependent toxicity of ICG to RPE and retinal cells. Possible mechanisms for additional toxicity may include light-sensitization. Part of the ICG-related toxicity however may stem from the toxic effects of hypo-osmolarity. The intraoperative application of ICG in concentrations that are currently used may have deleterious effects on the functional outcome of the patients.

*2. TB.*

The study by Veckeneer et al. of 2001 in rabbits showed that there was dose-related toxicity of TB to the retina. No toxicity was observed after 4 weeks exposure to TB 0.06 %, whereas 4 weeks exposure to TB 0.2 % led to significant morphologic abnormalities.[1002] These findings were more or less in line with study on post-mortem eyes, in which morphological alterations in the inner retina were observed after ILM staining with TB 0.15 % and 0.25 % (exposure: 1 minute, after which the excess dye was rinsed out), whereas no alterations were found after staining with TB 0.02 %.[1007] In this latter study however clinical ILM staining with TB 0.15 % in 10 patients led to good surgical, anatomical, and functional results.[1007] Studies on the effects of TB on RPE and Müller cell cultures did not find toxic effects, as measured with confocal microscopy and a live/dead fluorescent,[1027] or Mossmann's MMT assay [1028-1030] in two later studies.[1020 1021] In the first of these three studies however, TB concentration calculations were incorrect, so it is not clear whether their conclusions are justified.[1027] In the two MMT-assay studies, TB concentrations used varied from 0.0075 % to 0.2 %, with exposure times of 3, 5, 15, and 30 minutes, none of which led to any observed toxicity. Also, neither exposure of RPE cells to 0.0003% for 2, 24, or 72 hours, nor the use of direct illumination in addition to exposure to TB did lead to any observed toxicity.[1020 1021]

In contrast, other studies found indications for cell viability reduction with an MMT-assay, as well as apoptosis, in cultured RPE cells after exposure to TB 0.06 % and 0.4 % for 1 minute, 0.05 %, 0.1 %, and 0.5 % for 5 minutes, and more so after an exposure of 30 minutes. [1031 1032]

In conclusion, there are indications that TB application does show a concentration-dependent toxicity for RPE cells, retinal cells, and Müller cells. Whether such an effect is truly observed probably depends on the experimental circumstances, the assay that is used to observe toxic effects, and importantly, the exposure time. The safety of the current clinical intraoperative application of both TB 0.06 % and of TB 0.15 % therefore asks for more thorough investigation. Clinical, prospective, functional testing with methods such as multifocal ERG and (micro-) perimetry seems indicated in patients who were operated with intraoperative application of TB. Ultrastructural analysis of harvested ERM and ILM specimens and more extensive studies on the toxicity of TB in cell cultures and animals are also warranted.

### c. Toxicity in lens capsule staining in cataract surgery.

#### *1. ICG.*

In Horiguchi's well thought-out, randomized prospective study of 1998 on ICG capsule staining in cataract surgery, no postoperative visual, or tonometric, or biomicroscopic differences were found. This study compared 10 eyes in 10 patients in which ICG capsule staining was used to 10 eyes of 10 patients in which capsule staining was not used. Furthermore, there were also no significant differences in laser-flare photometry in the first postoperative week, nor in ECD-loss or endothelial morphometry 1 month postoperatively.[947] These safety results were confirmed recently in the randomized, clinical study by Dada et al. that compared ICG 0.5 %, TB 0.1 %, gentian violet 0.001 %, subcapsular fluorescein 2 %, and hemocoloration (autologous blood) capsule staining, each in 10 eyes of 10 patients with white cataracts who underwent phacoemulsification. No significant differences between the groups of differently stained eyes in tonometry, pachymetry, or endothelial cell loss were observed at 1 day or 1 month postoperatively.[962] A comparative experimental study on viability, function, and ultrastructure of human and rabbit endothelia after perfusion with ICG 0.5 % for 3 minutes did not find any significant differences between ICG perfused corneas and controls.[1033] In contrast to the observations on the RPE after ICG application in vitreoretinal surgery, ICG 0.5 % may be safe for the corneal endothelium in capsule staining during cataract surgery. However, clinical studies with more patients and longer follow-up, as well as more experimental studies are necessary to unequivocally demonstrate the safety for anterior segment toxicites structures. In addition, posterior segment may also be an issue, as recently a case was reported in which ICG 0.5 % that had entered the vitreous after attempted lens capsule staining, was the probable cause of retinal cell damage.[1034]

*2. TB.*

When we started our clinical and experimental studies in 1998, which are described in the next two chapters, no comparative clinical trials existed on the effects of TB on the corneal endothelium. The information that was known on possible toxicity for the corneal endothelium, were the data gathered by Norn in the 1960s and 1970s with regards to the topical and intra-operative application of TB,[931 933-935] and the experiments of Stocker in the 1960s and Sperling in the 1970s and 1980s regarding to the use of TB in donor corneal endothelial assessment.[326-328 343 344 762] All of these studies indicated that those applications and concentrations of TB were not toxic to the corneal endothelium. This confirmed the experience in the Cornea Bank Amsterdam, where since their start in 1983 more than 20000 corneas have been examined with TB 0.3 % (later 0.12 %).[323] In none of these endothelia an adverse effect caused by TB has been observed (E. Pels, personal communication, 1998). In the first study on TB capsule staining, no adverse effects were seen up to one year postoperatively.[32] With the aim to investigate possible toxicity of trypan blue for corneal endothelial cells more systematically, the studies described in chapters 9 and 10 were initiated.

## References

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# chapter

# 8

# Corneal endothelial cell density after trypan blue capsule staining in cataract surgery

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In 1999, we described the use of a vital dye, trypan blue, for staining the anterior lens capsule, to facilitate the visualization of the capsulorhexis in cataract surgery.[32] At one year, a first group of 30 patients showed no ocular or systemic side effects. (K.A. van Overdam, M.D. "One Year Follow-up After Trypan Blue Capsule Staining in Mature Cataract Surgery," presented at the XVIIIth Congress of the European Society of Cataract and Refractive Surgeons, Brussels, Belgium, September 2000). To our knowledge, no adverse effects related to the use of the dye have been reported.

Trypan blue 0.3% is widely used as an exclusion dye, to determine the viability of a donor endothelial cell layer, prior to corneal transplantation[344] It may therefore seem unlikely that trypan blue 0.06% would have a toxic effect on the corneal endothelium in the living eye, when used during surgery. However, to our knowledge, a quantitative evaluation of the biocompatibility of trypan blue with the corneal endothelium has not been performed.

The purpose of our study was to quantitate the endothelial cell damage induced by trypan blue capsule staining by comparing endothelial cell densities between both eyes of patients after bilateral phacoemulsification with or without trypan blue capsule staining, by the same surgeon, using the same surgical technique.

## Patients and Methods

Twenty-five patients with bilateral submature cataract in whom the use of capsule dye was relatively indicated were enrolled in the study after an institutional- review-board-approved informed consent was obtained. The mean age of the 8 men and 17 women was 72.7 ± 9.2 (S.D.) years. Exclusion criteria were uncontrolled glaucoma, uveitis, and diabetic retinopathy. Patients were scheduled for bilateral phacoemulsification by the same surgeon within four months. One eye of each patient was randomly selected for the intraoperative use of trypan blue.

In each eye, a scleral tunnel incision was made and aqueous was exchanged with air. Trypan blue 0.06 % (Vision Blue®) was applied onto the anterior lens capsule. After a few seconds, the anterior chamber was thoroughly irrigated with balanced salt solution to wash out the excess dye. In the contralateral eye, the anterior chamber was filled with air and then irrigated with balanced salt solution, without application of the dye. After irrigation, the air in the anterior chamber was exchanged with viscoelastic material, and the procedure was continued as routine phacoemulsification using a bimanual 'divide-and-conquer' technique, with the implantation of an intraocular lens.[1035]
Before and at twelve months after the second surgery, three images of the

endothelial cell layer were obtained from the central cornea in both eyes (Topcon SP2000p non-contact specular microscope) and analyzed (Imagenet 2000 software, Topcon Corp.) Digital images were manually corrected in masked fashion, to better define the cell border configurations. Outcome parameters were endothelial cell density, coefficient of variation of cell size, cell hexagonality, and pachymetry.

In each eye, the loss of endothelial cell density was calculated by subtracting the mean twelve months postoperative value from the mean preoperative value. The differences in the coefficient of variation, hexagonality, and pachymetry readings were calculated similarly. Statistical analysis was performed using the paired Student's t-test, repeated-measures analysis of variance, and the Wilcoxon signed rank test.

## Results

At twelve months after surgery, the postoperative endothelial cell loss averaged $167 \pm 188$ cells/mm$^2$ (- 7.3%) in eyes operated on with the use of trypan blue, and $266 \pm 278$ cells/mm$^2$ (- 10.2%) in the control eyes ($p \geq 0.1$). The preoperative endothelial cell densities were $2561 \pm 376$ cells/mm$^2$ and $2593 \pm 377$ cells/mm$^2$ respectively ($p \geq 0.1$). The difference in coefficient of variation of cell size, cell hexagonality, and pachymetry did not differ between both groups ($p \geq 0.1$).

At 1 year, the best corrected visual acuity averaged $0.8 \pm 0.2$ in experimental eyes, and $0.8 \pm 0.3$ in control eyes ($p \geq 0.1$). Three patients had mild atrophic age-related macular degeneration. All eyes were quiet, and the ocular pressure was normal.



## Discussion

Our study showed that no mid-term endothelial cell damage is induced by the intraoperative use of trypan blue for staining the anterior lens capsule. This finding may agree with the clinical observation that no corneal edema is induced by the intraoperative use of the dye.[32] The overall 7-11 % endothelial cell loss is similar to that reported previously after phacoemulsification.[500]

## References

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# chapter

# 9

# Biocompatibility of trypan blue with human corneal cells

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## Abstract

### Objective

To quantify the toxicity of trypan blue(TB) on human corneal cells according to exposure-time and concentration.

### Methods

Three in-vitro experiments were performed:

1. We exposed cultured human corneal fibroblasts to TB (0.0001% to 0.1 %) in Eagle modified minimum essential medium (EMEM) or phosphate-buffered saline (PBS) for 15 minutes to 24 hours. Cytotoxicity was evaluated by Mossman's colorimetric 3-(4,5-dimethylthiazol-2-yl)-2-5diphenyl tetrazolium bromide (MMT) assay.
2. We exposed human corneas in EMEM for 24 hours to TB (0.001% to 0.1%). Fellow donor corneas served as controls. Endothelial survival was evaluated morphologically and by cell density assessment.
3. We morphologically compared the endothelial viability of human donor corneas after exposure to 0.1% TB for 5 to 30 minutes with control corneas

### Results

In experiment 1, TB in EMEM was not significantly toxic at concentrations of 0.005% or lower. Higher concentrations were toxic only after exposure to TB for at least 6 hours. In PBS, significant toxicity was found after exposure to 0.1% TB for 30 minutes or longer. Lower concentrations were toxic after longer exposures. In experiment 2, exposure to 0.01% and 0.1% TB resulted in significant loss in cell density. At lower concentrations, the endothelium was affected only morphologically. In experiment 3, endothelial morphology changed in control corneas and after exposure to 0.1% TB for as little as 5 minutes. After 30-minute exposure, morphologic deterioration was more pronounced.

### Conclusions

Trypan blue was toxic in vitro to corneal endothelium and corneal fibroblasts at higher concentrations and at notably longer exposure-times. Toxicity was less in EMEM than in PBS.

### Clinical relevance

At commonly used concentrations, both during cataract surgery and in the cornea bank, trypan blue is safe for corneal cells. At higher concentrations or longer exposures, however, caution is warranted.

## Introduction

Trypan blue vital staining for the evaluation of the endothelium of donor corneas was first described by Stocker et al.[326] Trypan blue stains the nuclei of severely damaged and dead endothelial cells of donor corneas, as well as areas of Descemet's membrane denuded of endothelial cells. It does not stain viable endothelial cells with an intact cell membrane.[326 344 921 922] Sperling introduced trypan blue vital staining in combination with induced dilation of intercellular spaces (with NaCl 0.45 % and 0.9 %, or with sucrose 1.8 %), for visualization of endothelial cell borders, thus allowing light-microscopic assessment of both endothelial damage and endothelial cell density on potentially transplantable donor corneas.[343 762 822] Since 1982 this technique has been used by the Cornea Bank of the Netherlands Ophthalmic Research Institute (NORI) for the evaluation of the endothelium of human donor corneas after storage by organ culture preservation.[332 849] Variations of this technique, all using trypan blue, are applied in many cornea banks in Europe.

Norn[933-935] was the first to use trypan blue intra-operatively, during intracapsular cataract surgery, to evaluate endothelial status. Recently, intra-operative application of the dye has gained new interest: trypan blue anterior lens capsule staining aims at improving the visualization of the capsulorrhexis during phaco-emulsification in patients with absent red fundus reflex, eg., in mature cataracts.[32]

To expand the existing body of knowledge on the toxicity of trypan blue on human corneal cells, concentration- and exposure-time related effects of trypan blue were studied in 3 in vitro experiments on human donor corneas and human corneal fibroblasts.



## Materials and Methods

1. **Cytotoxicity of trypan blue to human corneal fibroblasts.**

***Trypan blue solutions***

Trypan Blue was obtained from Gurr, BDH Chemicals Ltd., Poole, England. Unless stated otherwise, trypan blue was dissolved in sterile phosphate buffered saline (PBS, Azua Pharmacy, Amsterdam, The Netherlands). The Cornea Bank NORI uses a 1.2 % solution from this stock for routine purposes since 1998. The photometric extinction value of this solution is 0.227 at 560 nanometer. This value is the same as for the 0.3 % trypan blue solution, obtained from another stock, and described and applied in previous years.[323 324 332]

***Human corneal fibroblast cultures and their experimental incubation.***

Corneal fibroblasts were obtained from normal human donor eyes as described

previously.[1036 1037] In short, corneal buttons were excised and fragmented. Three to 4 corneal pieces were implanted per well of a six well plate (Nunc, Roskilde, Denmark) and incubated with 1 ml of Eagle modified Minimum Essential Medium (EMEM) (ICN Biomedicals INC, Costa Mesa, Ca. , USA) supplemented with 10 % fetal bovine serum (FBS, Gibco Life Technologies Ltd, Paisley, Scotland), 100 units/ml penicillin (Gist Brocades, Leiderdorp, The Netherlands) and 50 µg/ml streptomycin (Biochemie GmbH, Vienna, Austria) at 37 °C. When the pieces adhered firmly to the bottom of the well by the outgrowing cells, 5 ml medium per well was added. Medium was renewed twice a week. Outgrowing fibroblasts were subcultured after 25-28 days and fibroblasts of the second passage were stored in liquid nitrogen. Cells were defrosted, subcultured for one passage, and used for experiments. At confluency the cells were removed form the culture flasks by incubation with a trypsin/EDTA solution, collected by centrifugation (200g), resuspended in medium, and plated out at 5 x $10^4$ cells per well in 96-well flat- bottomed tissue culture plates (Nunc, Roskilde, Denmark). The cells were cultured at 37°C in a humidified atmosphere with 5 % carbon dioxide before exposure to freshly prepared sterile trypan blue solutions, varying in concentrations of 0.0001 % to 0.1 %, in a vehicle of EMEM (half of the wells per tray) or sterile PBS. Wells were filled with 100 ml of study preparations, either with the trypan blue-solutions as described, or vehicle (EMEM or PBS) without added trypan blue as control.

*Quantitative cytotoxicity assessment with human corneal fibroblasts*

Cytotoxicity was determined with Mosmann's colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay directly after exposure to trypan blue.[1028] The MTT assay was performed as described earlier, [1029 1038]; it basically measures the capacity of mitochondrial enzymes to transform 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MMT) (Sigma Chemicals Co., St. Louis, Mo, USA) into a formazan product and thus reflects the mitochondrial activity of corneal fibroblasts. After exposure of the cells to the different concentrations of trypan blue during exposure times ranging from 15 minutes to 24 hours, the cells were carefully washed three times with PBS and 100 µl of a culture medium with 2 % FBS was added per well. Then 10 µl of a freshly prepared MMT solution (5 mg/ml MMT in PBS) was added to all wells and plates were incubated at 37 °C for 4 hours. The MMT-formazan reaction product was solubilized by the addition of 200 µl acid -isopropanol (0.04 N HCL in propanol-2) to the incubation medium without washing steps and overnight incubated at room temperature in the dark. The optical density of the solution was assessed at 560 nm in a spectrophotometer (IEMS Reader MF, Labsystems, Helsinki, Finland) taking medium with MTT and isopropanol as the blank. Each experiment was performed in 4-fold, and the experiments were repeated 3 times. Irreversibility of the effect of exposure to trypan blue was confirmed by repeating the MMT-assay 2-3 days and 7-8 days after exposure to trypan blue.

The cytotoxicity index CI of each culture was calculated using the formula:

$$CI = [(OD_{control} - OD_{test})/OD_{control}] \times 100\%$$

where $OD_{control}$ is the optical density in the control wells (cell incubated with vehicle only, PBS or EMEM), and $OD_{test}$ is the optical density in the wells with cells incubated with trypan blue in the vehicles PBS and EMEM.

A **CI** of higher than 20 %, was considered to be significant, thus compensating for the standard deviation of mean CI from the three experiments.[1029 1038]

2. **Endothelial cell survival after 24-hour exposure to trypan blue.**
Paired corneas from human donors judged unsuitable for transplantation for medical reasons or corneal scars were used. One randomly chosen cornea from each pair was used as a control and stored at 31 °C in EMEM with 2 % FBS and antibiotics, whereas the experimental cornea was preserved at the same temperature in the same medium, to which trypan blue was added. Concentrations of trypan blue that resulted were: 0.1 % (2 pairs), 0.01 % (2 pairs), 0.005 % (5 pairs), and 0.001 % (5 pairs). After exposure for 24 hours all corneas were rinsed and preserved in fresh EMEM at 31ºC for another 21 days. At 9 to10 and 20 to 21 days, corneal endothelium was evaluated by the technicians of the cornea bank. Storage of donor corneas in EMEM, as well as endothelial evaluation, was performed under sterile conditions using standard eye bank techniques, as described previously.[323 324 332] Endothelia of experimental and control corneas were compared on ECD and morphological status. Endothelial morphology was graded normal (score of 3) when a normal, "string-of-pearls"-like swelling pattern of the intercellular space indicative of a well functioning cell membrane, was present; dubious (score of 2) was assigned to endothelia with irregular or locally poor swelling of the intercellular space, and with the presence of many vacuoles within the endothelial cells; and abnormal morphology (score of 1), was used for endothelia with rounding-up of the cells, indicative of loss of intercellular contacts, and with generally poor swelling of the intercellular space (endothelia were called dead with a score of 0, when no recognizable endothelial cells could be discerned.[323 324]

Statistical evaluation of ECD was performed using a mixed model Analysis of Variance (ANOVA) with ECD as dependent variable, adjusted for its baseline measurements and time of investigation as covariate in the model. There was a within-subject factor (trypan blue vs. control) and a between-subject factor (concentration) in the model. Interest was in the interaction between these latter two factors. No structure was assumed for the within-subject correlations. Endothelial morphology scores were evaluated using a non-parametric Wilcoxon signed rank test. Experimental corneas, all taken together, were tested

against all control corneas, for both time-points. Testing by concentration group was considered not to be meaningful because of small group size.

3. **Endothelial viability by morphologic assessment after application of trypan blue.**

Human donor corneas were used that were judged unsuitable for transplantation because of corneal scars, low ECD (between 1800 and 2300 cells/mm$^2$), or signs of abnormal morphology such as significant polymegathism[323 324] These corneas were briefly (1-2 days) stored at the Cornea Bank under sterile conditions at 31 ° C in EMEM with 2 % FBS and supplemented with 5 % Dextran before shipment to the Rotterdam Eye Hospital for the experiment.

Both at the beginning and the end of the experiment, the endothelium of all corneas was evaluated using 0.1 % trypan blue vital staining and provoked intercellular swelling with sucrose 1.8 % solution (hospital pharmacy of The Rotterdam Eye Hospital), and photographed with an inverted light-microscope. Corneas with endothelia that showed severe degenerative changes (i.e. trypan blue stained nuclei, vacuoles in the cells, poor swelling of the intercellular space) before the experiment, were excluded from the experiment. The remaining corneas were randomly allotted to one of the treatment groups or the control group.

The control corneas (n = 8) were merely incubated for two hours in EMEM with 2 % FBS and 5 % dextran at room temperature. In three of the experimental groups, 0.1 % trypan blue solution was applied to the endothelium of the corneas directly after the preexperimental evaluation, for 30 (n = 7), 10 (n = 9), and 5 (n = 7) minutes. A fourth experimental group (n = 6) was exposed to 0.01% trypan blue for 10 minutes. Then, after trypan blue was thoroughly rinsed off with balanced saline solution, all experimental corneas were incubated in EMEM for two hours with 2 % FBS and 5 % Dextran at room temperature.

The pre- and post-experimental photomicrographs of the endothelium of control and experimental corneas were mixed and subjected to a masked assessment by five laboratory technicians of the Cornea Bank in Amsterdam. They used a four-tiered grading scale to estimate the percentage of dead endothelial cells (4, < 5 % dead cells; 3, < 5 % dead cells, but dead cells mainly over folds in Descemet's membrane; 2, 5-25 % dead cells, either diffusely or over folds, or both, and 1, > 25 % dead cells). The same morphology grading scale as in the second, "paired" experiment was used to assess morphology of areas without dead cells (3 = normal, 2 = dubious, and 1 = abnormal morphology). For each photomicrograph and for each of the observers, a product score ranging between 1 and 12 was calculated from the scores

on the two scales. Inter-observer correlation was evaluated by calculating Cronbach α. By averaging the product scores of the 5 observers an endothelial morphology assessment score was obtained for each cornea before and after the experiment. The product score was considered to be the best means to reflect the viability of the endothelium, irrespective of cell density or variation in cell size. Figure 9.1 demonstrates this grading system by showing four microphotographs of donor cornea endothelia used in this experiment with their resulting mean endothelial assessment scores.

The differences between pre- and postexperimental endothelial morphology assessment scores were calculated as change scores. Using these change scores, a linear regression model was constructed, and contributory effects of trypan blue exposure time were evaluated. The effects on the endothelium of the performance of the experiment per sé were also investigated.

## Results

1. **Cytotoxicity of trypan blue to human corneal fibroblasts.**
The relation between the mean cytotoxicity index CI, trypan blue concentration, and exposure time is shown in the three-dimensional graphs figure 9.2a and 9.2b, for EMEM and PBS respectively. Significant CIs (>20%) are represented by the gray bars in the graphs. Figure 9.2a shows that in EMEM, trypan blue concentrations of 0.01 % and higher resulted in significant toxicity after exposure for 6 hours or longer. At 24 hours' exposure, a trypan blue concentration of 0.005 % showed a threshold significant CI. Lower concentrations did not cause significant toxicity with exposures up to 24 hours. The graph in figure 9.2b shows that in PBS significant toxicity was observed at exposures of 30 minutes or longer to 0.1 % trypan blue. The exposure times needed to reach significant CIs increased with decreasing trypan blue concentrations. With 0.0001 % trypan blue in PBS, no significant CI was observed, regardless of exposure times.

Dose-response relations for trypan blue in the 2 vehicles, EMEM and PBS, were plotted for each of the different exposure times. Response was expressed as the mean CI obtained in the 3 (repeated) experiments. Figure 9.3 shows a representative dose-response curve, in this example after 18 hours exposure to trypan blue in EMEM. Using a sigmoidal fit program (Origin version 4.0, Microcall Software Inc., Northampton, Mass), the maximal toxicity (CI) levels and $LC_{50}$s (50 % lethal concentration, i.e. concentration resulting in half-maximal response) were calculated for all exposure times and for both of the vehicles. These data are summarized in Table 9.1. The $LC_{50}$ appeared to be independent from exposure time and vehicle, and was found at a concentration of 0.02-

0.04%. In contrast, the calculated maximal toxicity levels were strongly dependent on exposure time, and in PBS higher calculated maximal toxicities were found than in EMEM.

At days 2-3 and days 7-8 after exposure, the results (not shown) are essentially the same as the results directly after exposure, indicating that the observed damage to the fibroblasts was irreversible.

2. **Endothelial cell survival after 24-hour exposure to trypan blue.**
The results after 9-10 days and 20-21 days of preservation of the donor cornea pairs were expressed as mean endothelial cell densities (ECDs), endothelial morphology scores, and percentages ECD-loss, and are summarized in table 9.2. All corneas exposed to trypan blue 0.1 or 0.01 % suffered total endothelial cell loss at 20-21 days of preservation; the endothelium of one cornea exposed to 0.1% TB had already perished at day 9.

Analysis of variance outcomes showed a significant interaction between the within-subject factor (TB vs. control) and the between-subject factor (concentration) ($p < 0.001$), as well as separate significant main effects of the factor "concentration" ($p < 0.001$), and the factor "TB vs. control" ($p < 0.001$). These results indicate a significant concentration-dependent effect of trypan blue on ECD when experimental corneas were compared to their fellow controls. Estimated effects by this model of the interaction (concentration – tb vs. control) on ECD-loss, with 95 % confidence limits, were 60 cells/mm$^2$ for TB concentration 0.001% ($p = 0.57$), 93 cells/mm$^2$ for TB 0.005 % ($p = 0.38$), 2078 cells/mm$^2$ for TB 0.01 % ($p < 0.001$), and 1894 cells/mm$^2$ for TB 0.1% ($p < 0.001$) (Table 9.2).

On both observation points days 9-10 and days 20-21, the Wilcoxon signed ranks test showed a significant effect of trypan blue (all concentrations together) on endothelial cell morphology ($p = 0.03$ and $p = 0.02$, respectively) as compared to the endothelial cell morphology scores of all control corneas.

3. **Endothelial viability by morphologic scores after application of trypan blue.**
Endothelial morphology assesment (EMA) and change scores are summarized in Table 9.3. Cronbach α, a coefficient for interobserver reliability was 0.942; this confirms strongly the validity of the grading scales.

Morphologic change scores were normally distributed. Unpaired, s-tailed t-test revealed no significant difference ($p= 0.33$) between morphologic change scores in the corneas in the control group ($n = 8$) and the corneas in all the other groups together ($n = 29$), indicating an overall detrimental effect on the endothelium of manipulation of the donor corneas by merely performing this

experiment. A linear regression model was constructed of the morphologic change values for the 0.1 % trypan blue concentration. The overall contribution of exposure time was not significant ($p = 0.18$); however comparison of the different exposure times showed: controls vs. 5 minutes: $p = 0.88$, 5 minutes vs. 10 minutes: $p= 0.92$; controls vs. 30 minutes: $p = 0.08$; 5 minutes vs. 30 minutes: $p = 0.07$; and 10 minutes vs. 30 minutes: $p= 0.06$. This is an indication that exposure to trypan blue 0.1% for 30 minutes may cause additional deterioration of endothelial morphology.

The average morphologic change score of the group corneas exposed to trypan blue 0.01% appears to be lower than those of the groups exposed to 0.1 % trypan blue (table 9.3).

## Discussion

The results of our three experiments in which different methods were applied to assess toxic effects of trypan blue for corneal cells, endothelial cells in particular, demonstrated the following effects: (1) a concentration-related effect of trypan blue on corneal cell viability; (2) an effect of exposure time to trypan blue on cell survival; (3) an effect of the vehicle on the degree of toxicity that is caused by trypan blue; (4) damaging effects of (experimental) manipulation of cells or endothelia.

Each of the methods used in the 3 different experiments has its advantages and disadvantages. The first experiment assessing the cytotoxicity of trypan blue for corneal fibroblasts used an objective, quantitative evaluation method and homogeneous material of good quality, and had closely controlled experimental circumstances. However, no endothelial cells but human fibroblast cell cultures were used and therefore this was not a true assessment of toxicity of trypan blue on human corneal endothelium in situ. The second, paired experiment met this latter objection. Furthermore, its design, using organ culture preservation as a controlled condition "stress"-test for endothelial cell survival on paired experimental vs. control endothelia, provided for a powerful quantitative assessment of long-term endothelial cell viability after exposure to trypan blue, obviating the need of large amounts of tissue. On the downside, relatively subjective assessment methods were needed to obtain quantitative parameters, a dose-response relationship was much more difficult to evaluate, and effects of exposure time were not examined. In the third experiment several short exposure times were studied, and the design, application of trypan blue on to donor cornea endothelium, reflected the actual situation in eye banks rather well. However, statistical evaluation suffered from lack of power due to relatively small numbers of corneas per treatment

group, because of restricted supply of suitable donor material. Second, the assessment of endothelial morphometrics was subjective; however, the use of five independent masked experienced observers and standardized grading scales that provided for a very high interobserver reproducibility both improved on the reproducibility of this semiquantitative assessment. Third, the surprising outcome with exposure for 10 minutes to 0.01% trypan blue (table 9.3) indicates a limitation of the use of morphometric measures on viable endothelium. When the osmolarity of the solutions tested differs, e.g. by the use of a different concentration of trypan blue in PBS as was the case in this experiment, the endothelium may become temporarily edematous and more vulnerable to a subsequent swelling of the intercellular space necessary to visualize living endothelial cell borders. Therefore, concentration-dependent effects were not further tested in this experiment. Finally, preexperimental status of the endothelia was rather heterogeneous because of the selection criteria and preexperimental conditions. This was compensated for by carefully evaluating preexperiment morphometric status and discarding morphologically clearly abnormal endothelia before the experiment started. This preexperiment evaluation appeared to have succeeded, for table 9.3 shows that the preexperimental endothelial assessment scores were quite good, and similar in all experimental and control groups.

Although each of the experiments may have had its own flaws, each showed consistent results that furthermore were complementary to and in concordance with those from the other experiments. This study demonstrated a consistent relationship between concentration, exposure time, vehicle of trypan blue, and/or application circumstances. Under favourable conditions (the second experiment, and EMEM in first experiment) 24 hours of exposure to a trypan blue concentration of 0.005% was found to be a threshold at and below which there was no significant toxicity. Under unfavourable conditions (PBS in the first experiment, and the repeated manipulation in the third experiment) toxicity threshold was found at an exposure to trypan blue 0.1 % for 30 minutes; this threshold shifted towards longer exposure times as concentrations decreased and vice versa.

A recent experimental study[1002] confirms concentration-related toxicity and the existence of a threshold of toxicity related to trypan blue application. Interest had arisen in the application of the trypan blue as a surgical tool to facilitate visualization in epiretinal membrane surgery. Trypan blue application in a 0.06 % concentration in the vitreous cavity of rabbit eyes for up to 4 weeks did not cause any recognizable damage to the rabbit retina. In contrast, the application of 0.2 % trypan blue caused considerable damage to retinal cells and architecture in areas that were more exposed to the dye. It is however not feasible to compare in more detail the results of this study with our own

findings on endothelial cells, due to the different nature of the cells of interest, the different circumstances under which the exposure took place: in vivo vs. in vitro, and the far longer exposure times used, and with different techniques aplied for the determination of toxicity.

The results in our study seem to be in line with the literature, and with experience with the dye in eye banks. Sperling[344] and Stocker et al.[328] stained the endothelium of human donor corneas briefly (1 and 1.5 minutes, respectively) with 0.3 %, and 0.25 - 0.5 % trypan blue solutions respectively. Both authors found no indications for endothelial cell loss caused by the staining.[328 344] For many years the Cornea Bank NORI applied 0.3% trypan blue to the endothelium for 30-60 seconds for routine donor evaluation.[323 324] In 1998 colorimetric investigations of a new stock of trypan blue revealed higher extinction values of the 0.3 % solutions than before; the concentration of trypan blue solution for donor cornea evaluation was then changed to 0.12 % in order to use a trypan blue solution with the same extinction value as before. For almost 20 years and over 14,000 transplanted corneas, adverse effects to the endothelium have not been observed with application of 0.3 % or 0.12 % TB solutions. (E. Pels, unpublished results, 2000) Our present experimental findings may be considered to be congruous with our experience with trypan blue in the Cornea Bank NORI, and an extrapolation of the findings in the older studies. Although the reported concentrations of trypan blue were often higher than those used in our experiments, endothelia were exposed for shorter times, which most probably prevented possible toxic effects from taking place.

Observed toxicity limits in this study allow the intra-operative application of trypan blue. In trypan blue lens-capsule staining during cataract surgery, a concentration of 0.06 % is used to obtain adequate visualisation of the lens capsule in the absence of a red fundus reflex. The dye is applied on to the anterior lens capsule under an air bubble in the anterior chamber, which prevents dilution of the trypan blue by aqueous before staining the capsule and also impairs to some extent direct contact of the dye with the endothelium. Immediately after staining, the excess dye is washed out of the anterior chamber by copious irrigation, after which a viscoelastic substance is injected. The dye on the anterior lens capsule is partly removed with the anterior lens capsule after the capsulorrhexis is completed. The remaining dye is partly washed out with irrigation during the operative procedure, and remaining traces are thought to leave the anterior chamber by the aqueous route. The dye cannot be observed anymore on the first postoperative day.[32] Thus, an initial concentration of 0.06 % trypan blue may partly contact the endothelium for a few seconds. Thereafter, only diluted and steadily decreasing concentrations of trypan blue are thought to be present within the anterior chamber due to a continuous wash-out effect through aqueous

flow and resorption. The results of our in-vitro study support the view that concentrations of trypan blue in the anterior chamber after trypan blue anterior lens capsule staining in cataract surgery do not reach a toxic level. Confirmation of safety for the corneal endothelium of trypan blue capsule staining was published recently in a prospective, randomized clinical trial on endothelial cell loss. At 1 year postoperatively, no deleterious effects could be observed, neither on corneal endothelial cell density nor on endothelial morphometric parameters.[1039]

## References

See page 287.

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**Table 9.1 LC50 and maximum toxicity levels, as calculated from the sigmoidal fit program, for fibroblasts exposed to trypan blue**

| Exposure time | LC50 | | Max. toxicity level | |
|---|---|---|---|---|
| | (in mass %) | | (CI, in %) | |
| | EMEM | PBS | EMEM | PBS |
| 15 minutes | 0.04 | 0.02 | 8 | 30 |
| 30 minutes | 0.03 | 0.03 | 1 | 48 |
| 60 minutes | 0.02 | 0.04 | 13 | 60 |
| 3 hours | 0.04 | 0.02 | 30 | 57 |
| 6 hours | 0.03 | 0.02 | 60 | 125 |
| 12 hours | 0.02 | 0.03 | 31 | 85 |
| 18 hours | 0.03 | 0.02 | 55 | 103 |
| 24 hours | 0.02 | 0.03 | 84 | 147 |

The LC50s are presented as concentrations of trypan blue, i.e. mass-percentages. The CIs are percentages (dimensionless).

Table 9.2 ECD loss and morphological scores of donor corneal pairs, exposure to trypan blue for 24 hrs vs. controls

| TB conc. | Group | n= | Pre-experiment | | Day 9-10 | | Day 20-21 | | Estimated effects on ECD loss |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean ECD | Mean MS | Mean ECD | Mean MS | Mean ECD | Mean MS | |
| 0.1 % | Experim. | 2 | 2200 | 2.5 | 1100 | 0.5 | 0 | 0 | 1894 cells/mm$^2$, $p < 0.0001$ |
| | Controls | 2 | 2200 | 2.5 | 2200 | 2.3 | 2150 | 1.8 | |
| 0.01 % | Experim. | 2 | 2600 | 2.3 | 2150 | 1.3 | 0 | 0 | 2078 cells/mm$^2$, $p < 0.0001$ |
| | Controls | 2 | 2600 | 2.3 | 2550 | 2.8 | 2600 | 2.3 | |
| 0.005 % | Experim. | 5 | 2300 | 2.5 | 2280 | 2.5 | 2080 | 1.5 | 93 cells/mm$^2$, $p = 0.3779$ |
| | Controls | 5 | 2280 | 2.5 | 2300 | 2.8 | 2060 | 2.0 | |
| 0.001 % | Experim. | 5 | 2560 | 2.5 | 2460 | 2.7 | 2300 | 2.3 | 60 cells/ mm$^2$, $p = 0.5656$ |
| | Controls | 5 | 2500 | 2.5 | 2440 | 3.0 | 2400 | 2.9 | |

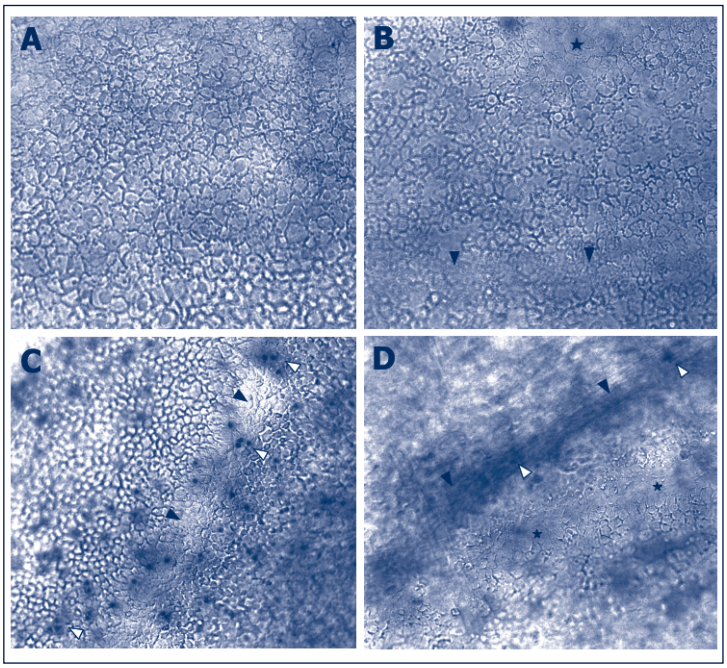
Legend: TB = trypan blue; ECD = endothelial cell density in cells/mm$^2$; MS = morphological scores (of endothelium, dimensionless).
The last column shows the estimated effects of exposure to TB on ECD-loss calculated by the ANOVA-model, as compared to the controls..

Table 9.3 Mean endothelial morphology assessment scores after direct exposure of donor corneal endothelia to trypan blue.

| | Controls | 30 minutes | 10 minutes | 5 minutes | 10 minutes |
|---|---|---|---|---|---|
| | | TB 0.1% | TB 0.1 % | TB 0.1 % | TB 0.01% |
| | (n = 8) | (n = 7) | (n = 9) | (n = 7) | (n = 6) |
| before experiment | 9.5 ± 2.6 | 9.6 ± 2.2 | 9.5 ± 2.3 | 9.5 ± 1.4 | 10.4 ± 1.5 |
| After 2 hours incubation in EMEM | 6.7 ± 3.3 | 4.7 ± 2.5 | 6.7 ± 2.9 | 6.9 ± 3.2 | 5.3 ± 2.8 |
| Morph change values* | - 2.7 ± 2.3 | - 4.8 ± 2.4 | - 2.7 ± 2.1 | - 2.6 ± 2.1 | - 5.1 ± 1.9 |

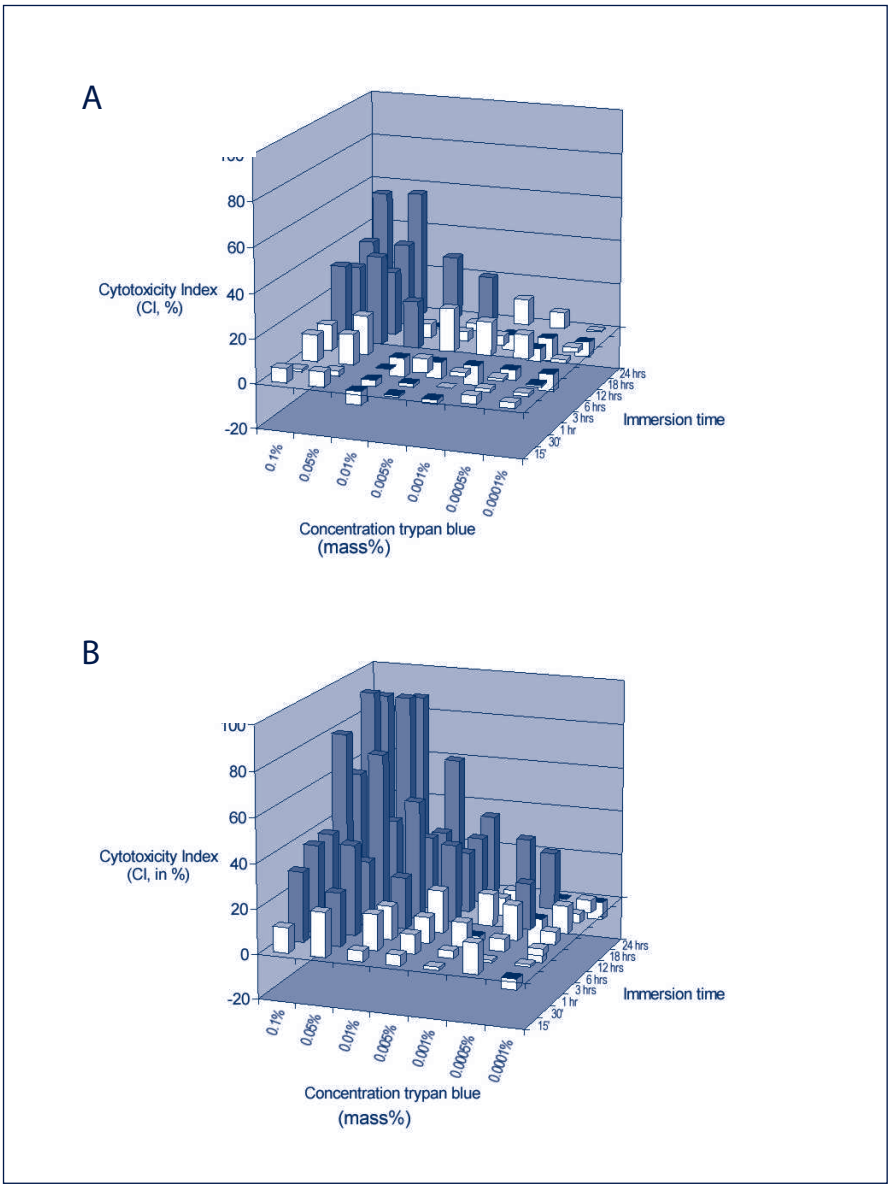
All scores (dimensionless) given in mean ± standard deviation.
*: From the linear regression model: 30' vs. controls: p = 0.08; 30' vs. 10' (0.1%): p = 0.064; and 30' vs. 5': p = 0.067

Fig. 9.1 Examples of endothelial morphometric assessment by grading scales.



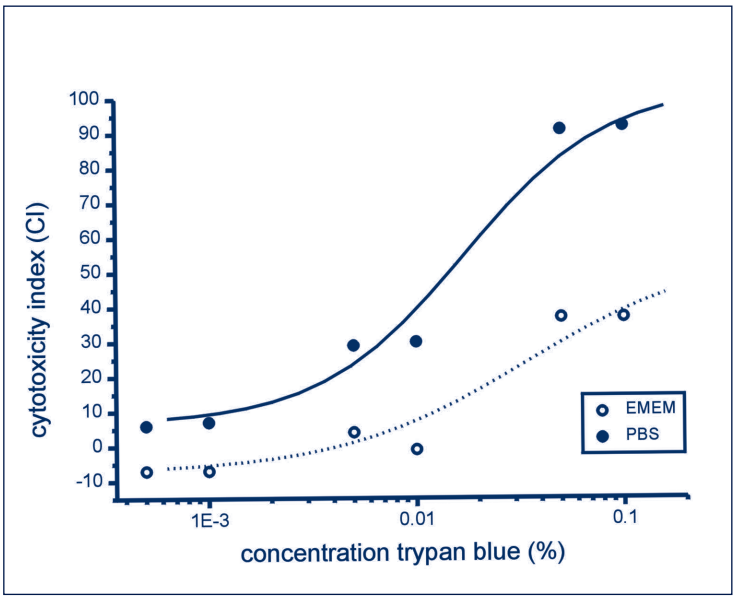

A: Mean product score of 11.2 : score 4 (<5% dead cells) x score 2.8 (completely normal morphology according to 4 of 5 observers). B: Mean product score of 6.4: score 4 (<5% dead cells) x score 1.6 (abnormal to dubious morphology, poor swelling [star] and Descemet's fold [arrowheads]). C: Mean product score of 6.2: score 2.6 (5-25 % dead cells [white arrowheads], over Descemet's folds and diffusely) x score 2.4 (Descemet fold [black arrowheads], relatively normal morphology in areas without dead cells). D: Mean product score of 2.6: score 1.6 (± 25 % dead cells [ white arrowheads], diffusely and over Descemet's fold) x score 1.6 (abnormal morphology: poor swelling [stars] and Descemet's fold [black arrowheads]).

Fig. 9.2 Cytotoxicity Indices of human corneal fibroblast cultures after exposure to Trypan Blue.




A: EMEM. B: PBS. The grey bars indicate significantly toxic CIs (Cut-off point: 20 %). Bars with black tops indicate CIs of less than 0 %.

Fig. 9.3 Example of a sigmoid concentration-response curve of human corneal fibroblasts exposed to Trypan Blue.




(In this case 18-hour-exposure, in EMEM vehicle [dashed curve] and PBS [solid curve]). The x-axis uses a logarithmical scale.

part

# IV

# Endothelial cell loss after lamellar keratoplasty

# chapter 10

# Development, indications, techniques, and results of lamellar keratoplasty

# I. Introduction.

From the earliest attempts in treatment of corneal opacification onwards, there has been a parallel development in full-thickness (i.e. penetrating) and partial-thickness (i.e. lamellar) corneal transplant techniques. The next paragraph provides an overview of the history of corneal transplantation, a.k.a. keratoplasty. They may illustrate how technical developments and new insights in transplantation biology guided both concepts separately to their current status, and also how developments lamellar keratoplasty (LK) and penetrating keratoplasty (PK) influenced each other's evolution. The development of the more recent concepts anterior lamellar keratoplasty and posterior lamellar keratoplasty are described. In paragraph III the background, rationale, and indications for anterior and posterior LK are described. In paragraph IV clinical results and effects on endothelial cell loss of anterior and posterior LK are described.

# II. The development of penetrating and lamellar keratoplasty.

## a. History of keratoplasty.

The first successful transplantation of living corneal tissue in a human patient was in fact a lamellar transplantation. A leucoma corneae was excised from a young girl's eye with Descemet's membrane and endothelium remaining, and a rabbit cornea was transplanted into the wound bed. This procedure was performed by von Hippel in 1886 and was described in 1888 as one of a series of 8 lamellar operations, of which in total 4 were succesful.[1040] It was not until 1905 that the first successful penetrating keratoplasty was performed in a human patient, by Zirm. A man suffering from corneal scars caused by a chemical injury, was transplanted with a donor cornea obtained from an enucleated eye of a young boy.[1041]

Leading up to these first successful keratoplasties in humans, in ancient times cosmetic treatment of corneal scars had been performed by means of a tattoo-like coloration of the scar. Lampblack or soot was used in Egyptian times (±1500 BC), and copper sulphate reduced with nutgall was applied to achieve reasonable cosmesis by Galenus (131-200 AD). In the England of 1761, an itinerant quack called Taylor was known to remove superficial corneal scars, by means of rudimentary what now would be called "superficial keratectomy" and abrasion. This practice was also widely performed by surgeons in France and Germany around that time.[1042] The idea of removing scars from the cornea using a trephine was first proposed by Erasmus Darwin (the grandfather of Charles Darwin) in 1796, but no one put this concept into practice.[1043] In 1789 Pellier De Quengsy introduced his ideas on treating corneal opacification with what now is called keratoprosthesis, i.e. to replace opaque corneal tissue by

man-made material. His concept entailed an artificial cornea made from glass framed in silver. No record exists if this idea was ever put into practice.[1044] Attempts in the second half of the 19th century to treat humans with artificial corneas, among others by von Hippel and by Nussbaum, were not succesful.[1040 1045] The artificial cornea concept was in fact not developed into a useful tech-nique until 1963, when among others Strampelli published on successful clinical applications, in his case the osteo-odonto-keratoprosthesis.[1042] In more recent times new keratoprostheses such as the Boston (Doane-Dohlman) and AlphaCor keratoprosthesis have been introduced. These new keratoprostheses and renewed attention for the OOKP have increased interest in the treatment of patients with corneal conditions that are very difficult to treat with keratoplasty. [1046-1048]

The first experiments with heterologous and homologous full-thickness corneal transplantations in animals are attributed to Reisinger in 1818, who also introduced the term "keratoplasty" for corneal transplantation.[1049] Homologous transplantations are transplantations within the same species, whereas heterologous transplantations are transplantations with tissue from one species into another species. Bigger performed the first successful transplantation in animals, a homologous keratoplasty in a gazelle, in 1837.[1050] Heterologous transplantations of animal tissue to humans were then attempted. Already in 1838 Kissam transplanted a pig's cornea into a human patient, but this opacified very soon after the operation.[1051] The experiments on corneal transplantation in humans and animals conducted by Power, described in 1872, suffered the same fate.[1052] Success remained elusive until the successful (lamellar) heterograft by Von Hippel, described above. After the first successful homologous penetrating corneal transplantation by Zirm, also the concept of auto-keratoplasty or homograft was initiated. In this concept the donor cornea was harvested from the patient itself: from the fellow, blind eye, as described by Plange,[1042] or a rotation graft in the diseased eye with which a small corneal scar can be rotated out of the visual axis, as described by Kraupa.[1045] This is in contrast to allografting, in which the donor cornea is harvested from another individual of the same species.

It took quite some time before reproducible results with corneal allografting were obtained, as the operative technique and donor tissue preservation and preparation had to be further developed and standardized. Much work in this respect was done and published in the 1930s and 1940s by Elschnig from Tsjechia, Filatov from Russia, Tudor Thomas in the UK, and Castroviejo in the U.S..[1053-1059] Improvements in lamellar transplants, leading to a temporary renewed popularity of this treatment modality, were re-initiated by the French and Swiss ophthalmologists Paufique, Sourdille, and Franceschetti, from the 1930s through the 1950s.[1060 1061] Paufique also described the concept of "maladie du greffon", i.e. opacification of a previously clear cornea, which he attribut-

ed to sensitization of the donor by the recipient.[1060] This seminal concept of immunological rejection of the donor graft was proven by Maumenee in 1951.[1062] Much important work in the field of corneal allograft rejection was done by Khodadoust and Silverstein.[695] The use of corticosteroids realised a breakthrough in the treatment of rejection and in the prevention of corneal opacification. This concurred with the introduction of antibiotics, and the introduction of the operation microscope and the development of microsurgical techniques and of newer suture materials that ensued. Other important developments included the better understanding of endothelial physiology and of donor preservation, as described in chapter 2. U.S.-based ophthalmologists and scientists such as Paton, Troutman, McCarey, and Kaufman played important roles in these developments.[1042 1063-1066] All these developments led to a great improvement in the popularity of PK and in the number of cases operated with this technique. PK was performed for the first time in the Netherlands in 1939 by A. Deutman.[1067] Further pioneering work regarding technique, tissue, and transplant survival was among others done by Kok-Van Alphen.[1045]

b. **Anterior lamellar keratoplasty.**

The frequency with which lamellar keratoplasty (LK) was performed sank inversely with PK's increasing popularity. Until recently, only 3-8 % of all corneal grafts were LKs.[1066 1068] Obviously, indications for LK are more restricted than for PK (see also paragraph II of this chapter). Endothelial disorders and full thickness corneal opacifications cannot be treated by anterior LK.[1069] But this alone cannot explain the unpopularity of LK. The fact that LK was technically more demanding and more time consuming than PK may explain better why it was so little used. And most importantly, LK's visual results were dissapointing. Much of this last problem was caused by irregular scattering of light (diffraction) at the recipient-donor wound interface. The need for a very smooth recipient and host surface at the wound interface was recognized early on, but to attain this goal would require even more technical skills and time investment.[1069] Yet, the advantages of LK over PK in suitable indications remained tempting. There were less complications to be expected, as LK was not truly intra-ocular surgery, and there was no risk for postoperative endothelial rejection, which would prolong graft survival. Lower postoperative astigmatism and hence shorter postoperative rehabilitation time were other probable advantages.[1065 1069] In the 1960s and 70s several attempts were made to improve on the visual results of LK and to simplify its technique. These include the use of a microkeratome by Barraquer,[1070] the use of pre-prepared dried autologous donor material by Urrets-Zavalía,[1071] and the proposal of standardized dissection techniques as described by Malbran, Polack, and Anwar.[1069 1072] However, studies from the late 1970s still show for keratoconus, one of the most suitable indications for LK, visual results were better after PK (Visual acuities (VA) ranging from 20/30-20/20) than after LK (VA ≤ 20/30), in spite of lower postoperative astigmatism after LK.[1073-1075]

Gasset (re-)emphasized the need for very deep lamellar dissection, at or just above the level of Descemet's membrane, where he found a natural, smooth line of cleavage that provided an opportunity to obtain an optical interface as smooth as possible.[1074 1075] During the 1980s and early 1990s several authors elaborated on the principle of deep lamellar dissection of the donor bed by injection of air into the corneal stroma just anterior to Descemet's membrane. This was found to alleviate deep lamellar dissection substantially.[1076-1079] After several years of relative quiet, renewed interest in deep lamellar dissection arose in the late 1990s. Manual deep dissection as described by Gasset and other authors remained a popular technique and some variations on this technique were developed. [1068 1075 1080-1083] But also variations on the air-assisted deep lamellar dissection technique were introduced, including "big bubble" (of air)-, hydro- and visco-dissection or -delamination approaches, and even the use of trypan blue injected intrastromally. Visualisation of the endothelial surface with an endoscope in the anterior chamber may be of use when the air injection clouds the vision.[1011 1065 1080 1084-1088] See figure 10.1.

The technique introduced by Melles in 1998 and 1999 for deep anterior lamellar keratoplasty (DALK) differed from the previously mentioned techniques, in that the deep lamellar dissection was not performed after an anterior corneal trephination had been made. Instead, a tunnelled corneoscleral incision was used through which a deep lamellar dissection was performed across the cornea, using slightly curved, custom-made spatulas. Thus, a deep stromal pocket was created. Dissection depth was gauged with an air bubble in the anterior chamber that functioned as an optical interface. Consecutively, trephination was performed to the depth of the deep stromal pocket, and subsequently the recipient's anterior corneal disc was removed "in toto". In a later developed variation on this technique, the manual deep lamellar dissection was replaced by deep visco-elastic dissection. The excised disc was replaced with a near full-thickness donor corneal button, from which the endothelium and Descemet's membrane had been removed.[34 1009 1089 1090]

Besides deep lamellar dissection a different approach developed, concurrent with the emergence of new or renewed refractive surgical techniques. In this approach automated microkeratomes, developed from the types as originally proposed by Barraquer, were used for very smooth and regular cutting of both donor and recipient lamellar surfaces, at various depths. These LK techniques using an automated microkeratome, carry similarities with the refractive surgical procedure called "anterior lamellar keratoplasty", in which however no donor corneal tissue is transplanted. Some authors even used the 193 nm excimer laser itself for lamellar dissection. [1070 1091-1098] The excimer laser can also be used for phototherapeutic keratectomy (PTK), in which a superficial layer of the cornea is removed, in the case of superficial anterior corneal disorders that cause

superficial stromal opacities and/or recurrent corneal erosions.[1099 1100]
With regards to the donor lamella that is transplanted into the deep lamellar dissection bed, it was recognized that retaining Descemet's membrane and endothelium on donor grafts predisposed to insufficient healing. A pseudo-anterior chamber was often formed. Furthermore, retaining Descemet's and endothelium did not lead to an optically clearer recipient-donor interface.[1101] From then on the donor endothelium was usually removed, when nearly full-thickness buttons were used in deep LK. Often, Descemet's membrane is removed as well. Staining with trypan blue may be helpful in identifying remaining (damaged) endothelial cells or rests of Descemet's membrane, as this dye stains bare Descemet's membrane and damaged endothelial cells, but not the corneal stroma.[34 1009 1080 1081 1084 1085 1087 1102 1103] Preparing the donor tissue before the operation by means of cutting lamellae or lenticules of desired dimensions from frozen tissue by means of a cryolathe is a different approach. This aimed at simplifying the procedure and saving operation time, as the need for the preparation of the donor lamella in the operating room was obviated. Moreover, the preservation techniques for lamellar donor buttons could now be standardized. However, this technique is currently not widely used, although good visual results have been reported.[1079 1104 1105]

Other, not yet mentioned lamellar keratoplasty techniques are currently used for tectonic purposes, i.e. to restore corneal integrity and/or contour. Sometimes this is an emergency measure to gain time before a PK can be performed. Also, lamellar keratoplasty techniques are often performed in ocular surface disease. Among these lamellar techniques are:

- annular lamellar keratoplasty for peripheral corneal thinning disorders;
- large-diameter corneoscleral grafts for global corneal thinning such as in keratoglobus, peripheral corneal thinning disorders, and ocular surface disease;
- amnion membrane transplantation, with or without limbal stem cell transplantation, for ulcerative and melting corneal defects and ocular surface disease;
- tectonic lamellar onlay procedures using donor tissue for corneal thinning disorders;
- use of combined penetrating and lamellar techniques, especially in the peripheral wound configuration, for several indications;
- and combinations of the above.[1061 1066 1097 1098 1100 1106-1108]

### c. Posterior lamellar keratoplasty.

The visionary ophthalmic surgeon Dr José Barraquer is to be credited with the development of the concept of selective transplantation of an endothelium-containing posterior corneal lamella, for the treatment of corneal edema.[1109-1111] In 1951 he reported for the first time on such a design, which involved the (manual) cutting of a hinged anterior lamellar corneal flap, followed by the excision and

replacement of a deep corneal stroma lamella including the endothelium.[1109] In 1964 he reported on the first results obtained with this technique in two human patients, who obtained clear grafts and good visual acuities. In 1983 he introduced the electronically driven microkeratome for the cutting of the anterior flap in both donor and recipient, and reported a good result in one patient.[1112]

Apparently unaware of Barraquer's work, Tillet published a report in 1956 on the selective transplantation of a posterior donor corneal lamella with endothelium, that he had performed successfully in a patient with Fuchs' endothelial dystrophy in 1954. The posterior recipient disc had been excised after a manual lamellar dissection through a 180° superior corneal incision. The half-thickness donor posterior disc was positioned onto the posterior surface of the recipient's anterior cornea, and fixated with silk sutures. The graft remained clear for at least one year, however the visual results were dissapointing because of poorly controlled glaucoma.[1113]

In the late 1970s the concept of selective endothelial transplantation regained a lot of interest, when experimental models were developed for the transplantation of cultured human and heterologous corneal endothelial cells. Experiments were performed with seeding the endothelial cells on animal and human donor corneas, Descemet's membranes, amnion membranes, and artificial carrier devices.[1114-1120] Experiments on corneal constructs with cultured human corneal endothelial cells have continued into the present time and appear promising. However, none of these techniques has reached the clinical phase yet. [1121 1122 132 1123-1126 1126-1128] See also chapter 14.



In 1993, a U.S. patent was obtained by White for a microkeratome-assisted posterior lamellar keratoplasty technique. Results of this procedure in eye bank eyes were presented in 1995 at the ASCRS meeting, with no patients treated at that time.[1110] In 1998 Jones and Culbertson reported, unaware of White's work, on the lamellar transplantation in eye bank eyes of a 250 µm thick posterior corneal lamella, after a 480 µm hinged flap had been made. Both the posterior lamella and the hinged flap were sutured with 10.0 nylon. They called this procedure endothelial lamellar keratoplasty (ELK).[1129] Their first clinical results, obtained in co-operation with Battle from the Dominican Republic, were presented in 1999.[1110] In 2000 Ehlers et al.[1130] and Busin et al.,[1131] and in 2001 Azar et al. (on a case operated in 1996),[1132 1133] reported clinical results with their own, similar techniques. Flap and button thickness and diameter varied among the techniques. Later, more cadaver eye studies were done by the group of Chuck and Behrens et al.,[1134-1136] and other clinical case series were reported by Silk et al,[1137] Guëll et al.[1138] and Culbertson.[1110]
A quite different approach for posterior lamellar transplantation, more in line with the technique described by Tillet, was put forward by Ko et al. They used

a technique of posterior lamellar transplantation in rabbits though a superior limbal incision, after chemical destruction of the recipient endothelium. The posterior lamella was sutured against the recipient corneal surface.[1139] In 1997 and 1998 Melles reported on an eye bank model for posterior corneal transplantation or posterior lamellar keratoplasty (PLK) based on Ko's report: the transplantation of a posterior corneal lamella through a 9 mm corneoscleral tunnel incision.[33 1140] Lamellar dissection across the cornea was performed (a stromal pocket was created) through this incision, using custom-made, curved spatulas. This was similar to Melles'technique for deep anterior lamellar keratoplasty (DALK) described earlier in this paragraph.[34 1090] The posterior recipient disk was excised using an especially designed intrastromal posterior disc trephine and custom-made curved scissors.[33 1140] Remarkable in this technique is that posterior donor disc is not held in place by sutures. The mechanism that is supposed to maintain good donor disc apposition, is the mere pumping action of the endothelial cells. The pressure of an air bubble in the anterior chamber helped to keep the disc in place in the first postoperative hours during which period the endothelial pump is supposed to start functioning.[33] However, the unsutured posterior disc was also observed to stay in place under non-physiological circumstances, e.g. in cadaver eye models. Therefore other explanations were also postulated. These include the inherent adhesive quality of bare stromal surfaces, assisted by the intraocular pressure.[1066 1111] In 1999 the first, encouraging result in a patient with pseudophakic bullous keratopathy was reported with this technique.[1141] In 2000 the one-year postoperative results in the first 7 patients were presented. All transplants were clear and in situ.[1142] Postoperative visual acuities, astigmatism and ECDs are discussed in paragraph in paragraph IV of this chapter.

In the next years, Melles introduced 2 variations on his first PLK technique. In his second technique, the recipient donor disc was completely excised with curved scissors after the deep lamellar cross-stromal dissection had been completed, obviating the intrastromal trephine. In this second technique there was no need for a 9 mm incision to allow entrance for the intrastromal trephine, so that the incision could be redesigned to a self-sealing 5 mm incision. This also obviated the need for suturing the tunnel incision, rendering a completely sutureless technique. The donor disc was now introduced folded like a taco, with the endothelial side inwards, through the 5 mm incision. In contrast, in the first, 9 mm-incision technique the donor posterior disc had been introduced into the recipient eye unfolded on a spoon-shaped glide covered with visco-elastic, with the endothelium facing down. The visual results in the first patient operated with this "5-mm incision" method were good: at one year postoperatively, BCVA was 0.8 and astigmatism was 2.0 D.[1143]

The third technical variation entailed the use of a 5 mm tunnelled corneoscleral incision, through which from the posterior side a 9 mm diameter "descemeto-

rhexis" was performed with a blunt cannula or a custom-made "scraper". Accidentally, the term descemetorhexis has also been used to describe an inadvertent removal of Descemet's membrane during phacoemulsification,[148] and for the secondary, trypan-blue-assisted, removal of inadvertently retained Descemets' membranes after PK.[1008] The descemetorhexis technique for PLK eliminated the need for the deep lamellar cross-corneal dissection. This technique further allowed for a larger diameter transplant, enabling the recipient cornea to be replenished with a larger number of donor endothelial cells. This may be beneficial in disorders such as bullous keratopathy in which more graft failures are expected because of greater and more rapid chronic endothelial cell loss.[1144 1145] The donor Descemet's membrane (DM) with endothelium was stripped from donor corneoscleral buttons. The donor material spontaneously formed a roll with the endothelium on the outside (as identified by trypan blue staining of DM), although other authors debated that the endothelium may have been on the inside. The donor roll was introduced into the recipient eye using a custom-made inserter that carried some similarity to an injector that can be used to implant a foldable IOL in cataract surgery. The inner portion of the roll was manipulated, so that the endothelial side spread out over the recipient iris. Alternatively, the donor disc may also be folded into a "taco" and introduced into the eye, in the same way as in Melles' second technique. After injecting an air bubble between the donor disc and the iris, the donor disc was positioned in the recipient wound bed. [1010 1097 1146-1149] In all Melles' techniques, initial apposition of the posterior donor disc to the recipient posterior bed is aided by an air bubble in the anterior chamber. See fig. 12.1 for a schematic representation of Melles' first 2 techniques.

Price has reported on cataract surgery following PLK in 6 phakic patients out of a series of 47 patients. He used the 9-mm and 5-mm incision deep dissection PLK techniques as proposed by Melles.[1150] Price later adopted Melles' third technique, which involved making a Descemetorhexis. He named this technique Descemet's stripping with endothelial keratoplasty (DSEK). He reported very promising results regarding visual acuity, astigmatism, and refraction, and, although there had been some initial problems, a high primary donor disc attachment rate.[1151 1152] Melles' first two techniques, that applied the 9 mm and 5 mm incisions, were adopted by Terry et al. and introduced in the US with slight modifications in the US under the name Deep lamellar endothelial keratoplasty (DLEK).[1153 1154] The two different names have led to a disagreement between both authors.[1155] Terry et al.'s results are similar to those by Melles et al. with excellent postoperative astigmatism and a rapid visual rehabilitation, although only one of Terry's patients has reached 20/20 BCVA postoperatively. In some instances, Terry combined DLEK with cataract surgery in one operation session.[1154 1156-1162] John has reported on two cases in which he used the DLEK procedure combined with ICG staining of the stromal side of the donor corneal disc. This increased visualization and helped donor disc alignment in the recipient lamellar bed. The ICG was

not observed anymore after 24 hours. He did not report on visual or refractive outcomes, but the grafts were reported to be clear and non-edematous.[1012]

For donor preparation in all techniques designed by Melles, fresh whole donor globes were used, in which a deep lamellar dissection was performed, followed by the excision of a corneoscleral button and a punch-trephination of a posterior disc from the endothelial side. However, using a donor posterior disc that is prepared from a donor corneoscleral button using an artificial anterior chamber is also a feasible concept in PLK, as has been proven by Terry and Price. It should be kept in mind however that Terry and Price use donor corneas preserved with cold storage in Optisol, whereas donor corneas in The Netherlands are stored in organ culture preservation at 31 ° C. This difference in storage technique may have consequences for posterior donor disc preparation, if an artificial anterior chamber were to be used. Moreover, donor disc adherence may not be

**Fig. 10.1** **Diagrammatic representation of the deep anterior lamellar keratoplasty technique.**

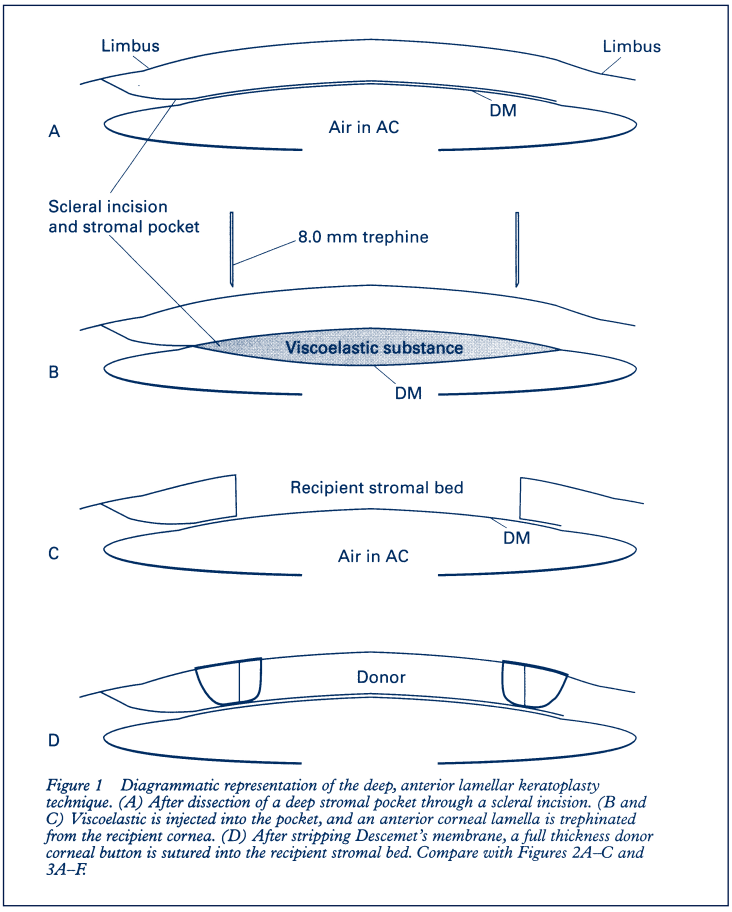



*Figure 1 Diagrammatic representation of the deep, anterior lamellar keratoplasty technique. (A) After dissection of a deep stromal pocket through a scleral incision. (B and C) Viscoelastic is injected into the pocket, and an anterior corneal lamella is trephinated from the recipient cornea. (D) After stripping Descemet's membrane, a full thickness donor corneal button is sutured into the recipient stromal bed. Compare with Figures 2A–C and 3A–F.*

comparable between posterior discs obtained from cold stored donor corneas and from organ-cultured corneas.[1150 1153 1154] An attempt of vitrification of pre-prepared posterior corneal lamellae, i.e. cryopreservation of posterior discs, resulted in tremendous endothelial cell loss after thawing and is currently not an option.[1163]

## III. Background, rationale, and indications for anterior and posterior lamellar keratoplasty.

### a. General background and epidemiology.

In 2004, 844 corneal transplants were performed in The Netherlands, of which between 24 and 73 (2.8 – 8.6 %) were anterior or posterior lamellar transplants. Between April 1 2004 and March 31 2005, 2378 corneal transplants were performed in the UK, of which between 42 and 76 cases (1.8 – 3.2 %) were lamellar keratoplasties. In 2004, more than 46000 transplants were performed in the US, of which an unknown number were anterior and posterior lamellar transplants.[1164-1167] Lamellar keratoplasty techniques have increasingly become a focus of interest in the last few years, judging by the rapidly increasing number of studies that have been published on this subject. However, from the figures on performed number of keratoplasties it is obvious that at this point in times full-thickness PK still is the mainstay in the surgical treatment of corneal opacification.

In the next sections of this paragraph it will be brought forward that in many, even up to 50% or more of PK cases, either anterior or posterior LK techniques could have been chosen as treatment modality. That is, as a treatment for optical improvement. Tectonic lamellar keratoplasties will be left out of consideration. The choice for tectonic treatment modalities is often more determined by their ability to save the integrity of the globe, than by their direct postoperative visual outcome. Furthermore, they may provide just the first step in the treatment plan. Additional treatment for improving optical results, including new keratoplasty, may be delayed until the primary disease process has quietened down.[1066 1097 1098 1100 1106-1108]

### b. Rationale.

#### *1. Anterior lamellar keratoplasty.*

Theoretical advantages of lamellar keratoplasty concepts over PK are manifold. In (anterior, deep) LK, the endothelium, which in Chapter 2, paragraph XVI of this thesis was shown to be crucial in graft survival after PK, is not transplanted. Rather, the recipient's endothelium is retained. This implies that the possibility for endothelial graft rejection is absent.[1065 1066] Furthermore, since LK is essentially extraocular surgery, the chronic ongoing endothelial cell loss seen after PK

may be less. Therefore, performing LK and thus preserving the patients's own endothelium are expected to diminish the risk for retransplantation importantly, as compared to performing a PK.

Suture and wound-healing related complications, such as suture-abscesses and (traumatic) wound dehiscence or wound dehiscence after suture removal, are important and relatively frequent complications occurring after PK. This is probably in part related to the relatively weak wound strength after PK wound healing.[1168-1180] Because of frequently occurring suture-related problems, studies on different suture materials in PK were conducted. Alternatives to the commonly used nylon, such as mersilene (polyester) and stainless steel, have been proposed and investigated.[1181-1184] However, a material such as polyester with experimental and theoretical advantages did not always result in less complications in clinical series.[716 1180] However in LK, by conserving the recipient's Descemet's membrane, a stronger wound configuration and a larger area for wound healing is ascertained. Also, less serious consequences may follow if nevertheless wound dehiscence occurs. Earlier suture removal is possible, which may be expected to result in a lesser risk for these suture- and wound-healing related complications.
Perhaps the most important problem after PK is postoperative astigmatism, which is importantly related to both the presence and the removal of sutures.[1177 1185-1190] Postoperative astigmatism is also expected to stabilize earlier in LK because of early suture removal. Early suture removal may also improve patient comfort, especially when for instance RGP contact lens wearing is necessary for optimization of postoperative visual acuity. Both factors will aid in faster and better visual rehabilitation.[1065 1066] Moreover, transplantation of a normal-thickness and normally structured anterior lamella in LK, in a central corneal thinning disorder such as keratoconus, may reinforce the cornea and decrease ectasia, comparable to reported effects of epikeratoplasty. Hence, high preoperative spherical equivalent and astigmatism may be improved postoperatively. [1066 1191 1192] This may also provide for better postoperative visual acuity, both corrected and uncorrected. In case of a very steep conus however, the stretched Descemet's membrane and posterior stroma that remain after lamellar dissection may also dimple and fold during the LK procedure. This would cause bad visual acuity postoperativily.

As for disadvantages of LK, the higher demands regarding technical skills and time investment of the surgeon may especially still be present in the deep dissection approaches. In this aspect the microkeratome-assisted approaches may have an advantage, as probably more ophthalmologists are experienced in using a microkeratome due to refractive surgery practice, than in performing deep lamellar dissection. The problem of poorer visual results after LK compared to PK is currently addressed by efforts to improve the optical quality

of the recipient-donor interface. The indication for LK should be evaluated carefully. Keratoconus that is very steep, and stromal opacities that reach very deep, may better be treated with PK. Technically, 2 principles can be applied to obtain an optically good interface. These are either very deep dissection at or just above the level of Descemet's membrane, where a natural smooth cleavage plain seems to exist, or using a microkeratome to make a very regular and smooth wound configuration.[1065 1066 1096 1097]

At this time it is appropriate to point out that virtually all of the advantages mentioned above for LK hold true for epikeratophakia or epikeratoplasty when applied for keratoconus. Epikeratophakia was one of the surgical fields pioneered by Barraquer. Early in the 1980s, the original purpose was to correct aphakia by suturing onto the anterior surface of the cornea (and in the periphery tucked into the stroma) a lyophilised and (cryo-)lathed lamellar piece of corneal tissue. As this lyophilised tissue can be lathed into desired optical properties, in this way spectacle or contact lens correction of aphakia could be lessened or even obviated. Lathed lamellar corneal tissue was commercially available in the 1980s. Soon this procedure was also applied to keratoconus where it was called epikeratoplasty, and where it had the additional advantage of flattening the cone and reducing myopia, and being reversible. Results on the short and long term were very encouraging, very much comparable to what has been found after lamellar keratoplasty. Among the disadvantages of this procedure was that central opacities (e.g. after hydrops) were not removed. Moreover, quality of vision after PK was in some studies reported to be superior. In the 1990s epikeratoplasty became less popular as a treatment option of keratoconus, but may still be a very valid treatment alternative to PK (or LK), if lathed lamellae are still available.[1191-1196]



*2. Posterior lamellar keratoplasty.*

In anterior lamellar keratoplasty only opacified anterior layers of the cornea are replaced. Posterior lamellar keratoplasty is a treatment concept to replace only the diseased posterior layers, which have caused corneal opacification through edema. Disorders that may be treated with PLK involve endothelial dystrophies (mainly Fuchs' Endothelial Dystrophy), and aphakic and pseudophakic bullous keratopathy, which are types of endothelial failure after cataract surgery (without or with IOL implantation).[1066 1096 1097 1100 1110 1111 1157] Results of PK performed for bullous keratopathy are quite varying: at times they are quite satisfactory, but in some series they are outright discouraging, especially after longer follow-up.[1144 1145 1197-1200] PK for Fuchs endothelial dystrophy is usually rather succesfull.[1197 1200 1201] In Fuchs, there are still more good endothelial cells left in the periphery of the recipient's cornea, so that less cells need to be replenished with a transplantation. (See also Chapter 2, paragraph XVI.d.) New surgical techniques for these indications need to have clear and substantial benefits before they will be widely accepted, more so for Fuchs than for bullous keratopathy.

This seems to be the case however, as with the posterior corneal transplantation concept some important problems may be addressed that occur frequently after PK. High and/or irregular astigmatism may be prevented when the preoperative corneal contours are not altered as is the case after PK. Both the tunnel-incision concept and the flap concept for PLK aim at influencing the corneal contour only minimally, and if so, more predictably and regularly. Also, unaltered corneal contour will give better predictability to IOL power calculation, whether cataract surgery is performed before, simultaneously with, or consecutively to PLK. Better postoperative astigmatism after PLK will enable more rapid visual rehabilitation. After PLK, the need for selective suture removal is obviated, and the frequency of additional surgical procedures to address high astigmatism will diminish. All these factors together are thought to diminish the amount of necessary postoperative consultations which benefits both patients and clinic logistics. Additionally, the suture-related complications described before may be diminished in frequency or even completely prevented by performing posterior lamellar keratoplasty techniques. This may be all the more a benefit in patient's with dry eyes.[33 1066 1096 1097 1100 1110 1111 1142 1143 1153 1154 1156-1160 1202] Especially of interest is that wound dehiscence was also observed to occcur relatively frequently after PK for bullous keratopathy and perhaps also after Fuchs' Endothelial dystrophy, both important indications for posterior lamellar keratoplasty.[1180] Tunnel-incision approaches probably have an advantage in avoiding suture-related complications over flap-approaches, in which sutures are still necessary.[33 1066 1096 1097 1100 1110 1111 1142 1143 1153 1154 1156-1160 1202]

Disadvantages of posterior lamellar keratoplasty techniques include the fact that these techniques may seem quite difficult to learn. Again, the micro-keratome-based techniques might be learned more easily by ophthalmologists experienced in refractive surgery. Regarding the tunnel-incision techniques of PLK, pseudophakic status is much preferable to phakia from a technical standpoint. Furthermore, cataract has been found to rapidly develop after PLK. Phacoemulsification and PLK may be performed in one surgical session or following each other shortly, however. Interface opacities after PLK might compromise visual outcomes. After longstanding bullous keratopathy, only transplanting the endothelium may not suffice to clarify the cornea, due to secondary fibrotic changes. Also, the transplanted donor endothelium is a target for endothelial rejection. [1096 1097 1110 1150 1157 1158 1202 1111] And as it is not yet known whether the procedure itself is more traumatic to the endothelium than PK, scrutiny of pre- and postoperative endothelial cell density is advisable. Evaluation of the rate of ongoing, accelerated postoperative cell loss is also warranted, since the evolution of endothelial cell density is crucial to transplant survival.

c. Indications

*1. Anterior lamellar keratoplasty*

Indications for LK include many but not all cases of :

- keratoconus,
- epithelial and (anterior) stromal corneal dystrophies,
- post-infectious (i.e. non-active) corneal scars, herpetic and non-herpetic.
- non-infective corneal scars, which do not extend too deep into the cornea;
- as well as some of the active infectious and non-infectious ulcerative corneal conditions.

Over the last 25 years, PK for keratoconus has constituted 15 to 29 % - depending on the series - of the total number of PKs.[1203 679 729 1200 1204-1207] Keratoconus is either the most frequent, second, or third indication for PK in most reported series, alternating with pseudophakic bullous keratopathy and regraft. The majority of cases with keratoconus may be treated with LK. However, in eyes with corneas that are too steep, have thinned too much, or in which Descemet membrane rupture and hydrops have occurred, LK is usually not a valid treatment option. Furthermore, PK for keratoconus has been reported to be very successful and the advantages of LK need to be pronounced in order to replace PK as treatment of choice for keratoconus.[1074 1191 1192 1208 1209]

In some studies Fuchs endothelial dystrophy and other, more anterior dystrophies are grouped together into one category. In the studies in which the dystrophies are subdivided, non-Fuchs dystrophies appear to contribute 1.5 to 3 % to the total number of PKs. Some, but not all of these may be treated with LK.[1200 1203 1210]

In some studies the share of PKs for infectious keratitis (herpetic and non-herpetic) reported includes both quiescent and active disease, whereas in other studies these conditions are separately reported. Reported ulcerative corneal conditions may include non-infectious melting, infectious ulcers, or both. Corneal scarring may in some studies have both traumatic and infectious origins, whereas in other studies these indications are counted separately, and in some instances this category includes other causes as well. This non-uniformity in categorization may explain the wide variation in reported proportion of PKs , 11 - 33 % for these indications. Certainly not all cases with these indications will be open for treatment with LK.[679 729 1203 1210]

LK may therefore be an alternative treatment modality in roughly between 25 and 65 % of all cases for which PK is considered. Other currently available alternative surgical treatment modalities for some of the indications for which either LK or PK are considered are discussed in chapter 14 (Summary and Perspective).

*2. Posterior lamellar keratoplasty.*

As mentioned before, the most import indications for PLK are Fuchs' endothelial dystrophy, pseudophakic, and aphakic bullous keratopathy. Reports from most studies indicate that between 20 and 35 % of all PKs were performed for (pseudo- and aphakic) bullous keratopathy; in one meta-analysis the range extended from 12 to 37 % of all PKs. This indication may be expected to decrease in importance, as cataract surgery and IOLs have become less deleterious to the endothelium. (See chapter 2, paragraph XIII.) Fuchs' endothelial dystrophy constituted between 4 and 16 % of all PKs. Estimatedly, in 25 - 50 % of PK indications, posterior corneal transplantation techniques can be a therapeutic consideration.[679 729 1200 1203-1207 1210]

## IV. Results of anterior and posterior lamellar keratoplasty.

### a. Clinical results.

*1. Anterior LK.*

After LK, some studies reported poor to moderate visual acuity.[1082 1102] Reasons for reduction of VA were interface opacification, blood-vessel ingrowth in the interface, and high astigmatism.[1082] In most studies on LK however, mean astigmatism after LK is between 2.5 and 4.5 diopters, and certainly not higher than after PK.[1105 1211 1081 1083 1102 1103 1212 1213] The visual results of the newer, deep LK techniques are encouraging in several studies. These include the comparative and prospective studies that could be retrieved. Equal or better results were found after LK compared to after PK. Results on the speed of visual rehabilitation compared to after PK varied.[1080 1083 1103 1212-1214] Sugita found no difference in visual acuity comparing cryolathed grafts to full-thickness grafts.[1080] A number of studies report on the results of microkeratome assisted LK (also known as ALTK – anterior lamellar therapeutic keratoplasty, in contrast to an older refractive surgical procedure called ALK). These results were varying in success, but some satisfying results have been reported, including an older study from the Netherlands.[1092 1215 1216]

Larger systematic comparative prospective studies are needed to establish whether the theoretical advantages of LK over PK as described in part III of this chapter are truly accomplished in practice, and whether the disadvantages of LK are diminished with the current technical developments. In The Netherlands, a multicentered randomized prospective clinical trial has started (the Dutch Lamellar Corneal Transplant Study –DLCTS). Visual and other outcomes of several contemporary techniques of PLK and ALK are evaluated and compared to the results of PK in the control group.

*2. Posterior LK.*

At one year postoperatively, best corrected visual acuity in the 6 patients operated with Melles 9-mm incision technique was 20/80 – 20/20, and in the patients without pre-existent maculopathy or amblyopia 20/40 – 20/20. Post-operative astigmatism was 1.5 D. At 3 years postoperatively, BCVA in 9 patients operated with the 9-mm or 5 –mm incision, deep dissection techniques, without concomitant eye disease ranged from 0.7 - 1.0. Mean astigmatism was 2.1 D.[1142 1148 1217] Price reported on cataract development within one year after PLK in his only 4 phakic patients in his total series of 47 PLK patients. He had operated these patients with techniques similar to Melles' 9-mm and 5-mm incision, deep dissection techniques. These 4 phakic PLK patients with cataract subsequently underwent phacoemulsification. One year post cataract-surgery, BCVAs ranged from 20/50 to 20/25, with manifest refraction astigmatism of 0- 1.5 D.[1150] Price also reported on his results in 50 eyes in 47 patients in which he used Melles' third PLK technique (with the 'descemetorhexis'). Price called this technique Descemet's Stripping with endothelial keratoplasty (DSEK). At 6 months follow-up, 62 % of operated eyes saw ≥ 20/40 and 76 % saw ≥ 20/50, with significant improvement from preoperative BCVA, and with unchanged astigmatism or spherical equivalent.[1151] Terry reported in his first series of DLEK patients BCVAs of 20/70 –20/30 in 8 patients at 6 months postoperatively, and 20/60-20/30 in 4 patients at 12 months postoperatively, with induced astigmatisms of 1.1 and 0.8 D at 6 and 12 months respectively.[1154] In later, larger series (30 eyes) he reported mean BCVAs (from logMAR visual acuities) of 20/56 at 6 months, 20/51 at 12 months, and 20/48 at 18 months postoperatively. In this series, at 6 months postoperatively, 33 % of patients had BCVAs ≥ 20/40, 53 % ≥ 20/50, and 97 % ≥ 20/200. At 12 months all these figures had improved slightly. Mean topographic astigmatism was 2.1 D at 6 months, and 2.2 D at 12 months.[1160] A later, larger series followed for 6 months postoperatively had similar results, with mean induced astigmatism of 0.28 D, and with a mean BSCVA of 20/46 and only one patient reaching 20/20.[1162] Induced astigmatism and spherical equivalent changes in his clinical and cadaver eye series were similarly low.[1153 1158]

With the microkeratome assisted posterior keratoplasty a.k.a. ELK a.k.a. endokeratoplasty, no postoperative BCVA's higher than 20/30 were reported, except by Culbertson.[1110] In most studies on the microkeratome assisted PLK techniques, including those on experimental cadaver eye models, postoperative keratometric astigmatism is more unpredictable than in the tunnel incision techniques.[1110 1130-1132 1134 1138 1157]

Reported complications during and after PLK (DLEK) include:

- microperforation during lamellar dissection necessitating conversion of the technique to PK,
- early postoperative iridocorneal adhesions that required reintervention,

- primary graft failure,
- sequestered viscoelastic material in the donor-recipient interface that required secondary PK after 1 month,
- visually impairing increasing donor-recipient interface haze that necessitated secondary PK at one year post-operatively,
- endothelial rejection episodes that were usually mild and reversible, and sometimes caused by patient noncompliance,
- occasional development of glaucoma, early or later after PLK/DLEK, sometimes induced by topical steroid medication, and occasionally requiring trabeculectomy,
- visually disturbing posterior graft folds,
- the rapid development of visually disturbing cataract in phakic PLK patients,
- and probably the most often occurring complication: incomplete or no initial graft adherence to the posterior stroma.[1142 1148 1150 1152 1154 1157 1160 1218 1219]

For ELK/endokeratoplasty/MAPK, reported complications include:
- flap melt, requiring flap removal and resuturing the posterior disc,
- epithelium downgrowth, requiring flap lift and aspiration,
- extreme hyperopic shift (+ 16 D) due to unknown causes,
- necessary suture adjustments,
- wound leaks,
- primary endothelial failure, requiring retransplantation,
- and Mycobacterium chelonae interface infection, requiring PK after failure of conservative antibiotic therapy.[1110 1130-1133 1137 1220]

Larger systematic comparative prospective studies are needed to establish whether the theoretical advantages of PLK over PK as described in part III of this chapter are truly accomplished in practice, and whether the disadvantages of PLK techniques do not outweigh their advantages. In The Netherlands, a multicentered randomized prospective clinical trial has started (the Dutch Lamellar Corneal Transplant Study –DLCTS). Visual and other outcomes of several contemporary techniques of PLK and ALK are evaluated and compared to the results of PK in the control group.



### b. Endothelial cell loss.

#### 1. *ECD after anterior LK.*

Only a few studies reported on endothelial cell loss after lamellar keratoplasty. In the study by Bodereau et al, the ECD after LK was twice that after PK, both performed in 11 eyes for herpetic keratitis. However, not clear was if there was a difference in post-operative time, and whether the study had been performed in a randomized fashion of that historic controls had been used.[1221] Sugita reported in his series of 113 eyes that had undergone deep LK, ECDs of 2225 cells/mm$^2$ at 1 months postoperatively, and 1937 cells/mm$^2$ at 24 months post-

operatively, representing a loss of 13 %. In 74 eyes after deep LK for keratoconus, the ECD almost stabilized after 6 months postoperatively, in contrast to the continuing drop in ECD observed in a nonrandomized control group of 48 eyes after PK for keratoconus.[1080] In the study by Morris et al. no pre-operative ECDs had been obtained in 20 eyes that had undergone deep LK with lyophilized donor tissue. At one to eight years postoperatively (mean: 3 years) the mean ECD was 2417 cells/mm$^2$. In 13 eyes with corneal disease thought to afflict the endothelium such as keratoconus and lattice dystrophy, the mean ECD was even 2837 cells/mm$^2$; however, there was a substantial spread, with individual corneas having densities around 1000 cells/mm$^2$. In 4 patients a comparison was made to the unoperated fellow eye; ECD was on average 10 % (range 0.25-20 %) lower in the LK eyes. In 4 patients LK eyes were compared to the fellow eye that had undergone PK; here the ECD was on average 50 % lower (32-71%) in the PK eyes. It should be noted however, the PKs had a longer follow-up time of 0.6 to 7,1 years compared to the LKs.[1068] Trimarchi et al. retrospectively compared 150 patients after deep LK, to 150 age-, sex-, and indication-matched patients who had undergone PK. The general follow-up time for the LK patients was 5 months, whereas for PK patients it was 13 months. It is not clear from their paper whether in all 300 patients ECD measurements had been obtained. The mean ECD after LK was 2235 cells/mm$^2$, whereas after PK is was 979 cells/mm$^2$.[1213] Panda et al conducted a prospective clinical trial in which they compared results after LK to after PK, performed on 24 eyes each. Mean ECDs after LK were 2233 cells/mm$^2$ at six months, and 2220 cells/mm$^2$ at 12 months postoperatively; differences with ECD after PK were highly significant at both time points ($p < 0.001$; 1903 cells/mm$^2$ at 6 months, and 1579 cells/mm$^2$ at 12 months, respectively).[1083] Shimazaki et al. conducted a randomized prospective clinical trial comparing deep LK to PK. They investigated 26 eyes in 24 patients. They found ongoing endothelial cell loss until 6 to 12 months with stabilization at 24 months postoperatively, when also the difference with PK became statistically significant (2183 cells/mm$^2$ vs. 1868 cells/mm$^2$, $p= 0.044$).[1103]

In order to investigate the amount and pattern of endothelial cell loss after deep anterior lamellar keratoplasty (DALK) performed with the manual deep lamellar dissection technique described by Melles et al., [34 1100] the prospective clinical study described in chapter 11 of this thesis was initiated. The multicentered randomized prospective Dutch Lamellar Corneal Transplantation Study that has started will also look into endothelial cell loss after anterior lamellar keratoplasty techniques.

*2. ECD after posterior LK.*

Endothelial cell loss is essential in corneal transplantation follow-up, and all the more in posterior lamellar transplant survival. Therefore it is remarkable that only two authors reported on endothelial cell densities after ELK and similar

micro-keratome based procedures. In Culbertons's series ECD was1693 cells/mm$^2$ at 6 months postoperatively (range 994 – 2168 cells/mm$^2$), and in Ehlers' series of 3 patients ECD ranged from 1200 to 2300 cells/mm$^2$ at 12 months postoperatively.[1110 1130] No figures on relative endothelial cell loss compared to preoperative donor ECDs were provided.

In Melles' initial report of his first 6 PLK patients (9 mm incision technique), mean ECD at 12 months postoperatively was 2520 cells/mm$^2$.[1142] In his report on 14 PLK patients with the 9-mm and 5-mm incision deep dissection techniques which also included these first 6 patients, the ECDs were 2126 cells/mm$^2$ at 6 months, 1839 cells/mm$^2$ at 12 months, 1418 cells/mm$^2$ at 24 months, and 1137 cells/mm$^2$ at 36 months postoperatively. Price reported on 3 PLK eyes out of a much larger series of 47 PLK eyes. Postoperative ECDs were 1680 cells/mm$^2$ in one eye at 21 months after PLK followed by phacoemulsification 5 months later; 540 cells/mm$^2$ in an eye 21 months after PLK followed by phacoemulsification 6 months later; both of these two eyes furthermore experienced reversible rejection episodes; and 1810 cells/mm$^2$ in a third, pseudophakic eye at 9 months after PLK. The second and third PLK eye were of the same patient. He did not report on ECDs of his entire PLK series nor in his DSEK series, however.[1150 1151] Terry's reports from 2003 are all on the same cohort of patients, with different numbers of patients and varying follow-up lengths reported. In his initial report, the mean 12-month post-operative ECD in 8 patients was 2409 cells/mm$^2$. In later reports with more patients, ECDs at 6, 12, and 24 months were all measured around 2200 cells/mm$^2$, which implied a loss of 17 – 25 % from preoperatively. Remarkably, at 24 months postoperatively, only slight additional endothelial cell loss was observed compared to 12 and 6 months, respectively.[1154 1156 1157 1159 1161 1162]

The multicentered randomized prospective Dutch Lamellar Corneal Transplantation Study that has started will also look into endothelial cell loss after posterior lamellar keratoplasty techniques. In order to investigate the amount and pattern of endothelial cell loss after PLK performed with the first two techniques described by Melles et al. (9- and 5- mm incision techniques), the prospective clinical study described in chapters 12 and 13 of this thesis was initiated.

## References

See page 287.

# chapter 11

# Endothelial cell density after deep anterior lamellar keratoplasty (Melles technique)

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## Abstract

### Purpose

To measure the recipient endothelial cell loss after the Melles technique for deep anterior lamellar keratoplasty.

### Methods

In 21 eyes of 21 patients a deep anterior lamellar keratoplasty procedure was performed. Before surgery, and at 6, 12 and 24 months after surgery, specular microscopy was performed to evaluate the endothelial cell density. For each postoperative time interval, the mean endothelial cell loss relative to the preoperative value was calculated.

### Results

Mean postoperative endothelial cell loss averaged 283 cells/mm$^2$ (± 293) at six months, 335 cells/mm$^2$ (± 309) at 12 months, and 421 cells/mm$^2$ (± 316) at 24 months. Estimate relative endothelial cell density losses obtained by mixed model ANOVA were 11.1%, 2.0%, and 1.2% respectively, each time compared to its previous measurement point. Second order comparisons showed that the loss within the first six months was significantly higher than after six months.

### Conclusion

In deep anterior lamellar keratoplasty, the recipient corneal endothelium showed a small initial drop in endothelial cell density followed by a physiological rate of cell loss. Cell survival after lamellar keratoplasty may be expected to be better when compared to that following penetrating keratoplasty.

## Introduction

Corneal endothelial cell loss after penetrating keratoplasty occurs at a higher than physiological rate (0.6% per year),[310] to a cumulative cell loss of 50% or more within the first ten years. This suggests that after the initial surgical trauma, donor endothelial cell survival is compromised in the host ocular environment.[310 779]

In the past years, several techniques for deep anterior lamellar keratoplasty have been described, including deep stromal manual dissection,[34 1080] as well as dissection by injecting air or visco elastic just anterior to Descemet's membrane, to separate the Descemet's membrane from the posterior stroma.[1009 1084 1087] In contrast to penetrating keratoplasty, the recipient Descemet's membrane and endothelium are left in-situ in deep anterior lamellar keratoplasty, i.e. only the anterior cornea is replaced by donor tissue. However, to our knowledge, no reports exist on the pattern of the recipient endothelial cell loss following deep anterior lamellar keratoplasty.

Since the condition of the recipient endothelium is one of the major criteria to determine long-term transplant survival, we evaluated the pattern of corneal endothelial cell loss within the first two years after deep anterior lamellar keratoplasty.

## Materials and methods

From a larger group of patients who underwent deep anterior lamellar keratoplasty for various indications, 21 eyes of 21 patients were selected by the availability of pre- and postoperative specular microscopy examinations (Table 11.1). The average patient age was 43.2 (± 15.2 ); ten patients were male and eleven female. Twelve patients were operated on for keratoconus, three for a quiescent Herpes Simplex stromal scar, three for a postinfectious quiescent stromal scar, and three for a hereditary stromal dystrophy. Each patient signed an informed consent as part of an Institutional Review Board approved study.

All patients had the same surgical procedure performed by the same surgeon (GM).The surgical technique has been previously described in detail.[34] In short, a half-depth scleral incision, 5.0 mm in width, was made 1-2 mm from the limbus at the 12 o'clock surgical position. A sclero-corneal tunnel was dissected extending 1.0 mm into the clear cornea. Through a side-port at 3 or 9 o'clock, the anterior chamber was completely filled with air, to create an air-to-endothelium interface, i.e. a reference plane to optically visualize the depth of the dissection within the cornea during surgery.[1090] With dissection spatulas (DALK/PLK dissection spatulas, D.O.R.C. International, Zuidland, The Netherlands), a manual stromal dissection

was made at approximately 95% corneal depth,[1090] using the air-to-endothelium reflex to monitor dissection depth through the operating microscope. The corneal pocket was extended up to the limbus over 360 degrees.

Then the pocket was filled with a visco elastic (hydroxypropylmethylcellulose, Ocucoat, Bausch & Lomb, Clearwater, USA) to displace the posterior corneal layers toward the iris to avoid damaging these layers during trephination. A Hessburg-Barron suction trephine was positioned onto the anterior corneal surface, and the blade was turned downward until visco elastic was seen to escape from the pocket. Remaining attachments of the anterior button were then cut with microscissors, and the recipient stromal bed was thoroughly irrigated to remove all visco elastic.

With a dry sponge, the endothelial side of the donor button was gently 'touched' to damage or remove the endothelial cells. Trypan blue 0.06% (VisionBlue, D.O.R.C. International) was then applied onto the donor posterior corneal surface, to stain the dead cells or the denuded Descemet's membrane. After removing all excess dye, the donor Descemet's membrane with the endothelium was gently swapped from the button with a dry sponge.[1009] The donor button was then positioned onto the recipient stromal bed, and fixated with two double-running 10-0 sutures.

Before the procedure, and 6, 12, and 24 months after surgery, the endothelium was photographed and evaluated using a Topcon SP2000p non-contact autofocus specular microscope (Topcon Corp, Tokyo, Japan). Images of the central corneal window were analyzed using Imagenet 2000 software (Topcon Corp) by the same observer (B.v.D.); manual correction of the cell borders was performed prior to final analysis of the endothelium. For each postoperative time interval, three measurements of endothelial cell density, coefficient of variation of cell size (polymegathism) and hexagonality (pleomorphism) were averaged.

Statistical analysis was performed using a mixed-model ANOVA (analysis of variance), with which (1) estimates of mean relative endothelial cell density losses per time point, (2) differences between time points, and (3) second order comparisons between the intervals were calculated. The within-subject correlation structure was assumed to be first order auto-regressive.[310]

## Results

The postoperative course was uneventful in fifteen patients. Two patients operated on for a quiescent Herpes Simplex scar had mild recurrent attacks under oral acyclovir treatment throughout the study period. Two patients de-

veloped glaucoma that could be managed with topical medication. One of the latter patients also suffered from a temporary epithelial defect 14 months after surgery with secundary keratic precipitates that resolved after treatment with antibiotics and topical steroids. One patient underwent a phacoemulsification 11 months after surgery that was complicated by a retinal detachment in the presence of high myopia (-18 diopters).

The overall endothelial cell density averaged 2823 (± 549) cells/mm$^2$ before surgery, and after surgery 2481 (± 506) cells/mm$^2$ at six months, 2514 (± 506) cells/mm$^2$ at 12 months, and 2476 (± 432) cells/mm$^2$ at 24 months (Figure 11.1). Mean postoperative endothelial cell loss averaged 283 (± 293) cells/mm$^2$ at six months, 335 (± 309) cells/mm$^2$ at 12 months, and 421 (± 316) cells/mm$^2$ at 24 months (Table 11.1). Cell morphology as measured by endothelial cell polymorphism and hexagonality, did not change significantly over time.

Statistical analysis by mixed model ANOVA resulted in estimated endothelial cell density losses; at 6 months postoperatively 11.1% ($p<0.0001$), whereas the estimate cell loss at 12 months compared to 6 months decreased to 2.0% ($p = 0.3$) and the estimate cell loss at 24 months compared to 12 months was 1.2% ($p = 0.5$) (Figure 11.2). Additional second order comparisons indicated that endothelial cell loss in the first postoperative period (0-6 months), was significantly higher than the cell loss in the subsequent time intervals (6-12 months: $p = 0.0019$, and 12-24 months: $p = 0.0008$).

## Discussion



Sufficient corneal endothelial cell density is required for long-term functional success of any type of keratoplasty. With penetrating keratoplasty, an overall endothelial cell loss has been reported of approximately 33% within the first two postoperative years, and the cell density continues to decrease at an accelerated rate up to 20 years after surgery.[310 1068 1080 1083 1103] To our knowledge, it is unknown if the decrease in recipient endothelial cell density mimics that of the donor endothelium in penetrating keratoplasty, or whether it shows a physiological cell loss as in unoperated corneas. This is important in order to assess the risk of long-term lamellar transplant failure, and to determine if the risk varies between lamellar and penetrating grafts.

In the current study, corneal endothelial cell loss was measured at intervals of 6, 12 and 24 months after deep anterior lamellar keratoplasty. Compared to the preoperative values, the transplanted corneas showed a drop in endothelial cell density of about 400 cells per sq. mm (~ 11%) at the first postoperative measurement at six months. Thereafter the rate of cell loss diminished to about

100 cells per sq. mm per year (~ 1-2%), thus approaching a physiological cell loss as found in virgin corneas.[229 310] These findings suggest that an initial drop in cell density is induced by surgical trauma and/or topical treatment, but no continued elevated cell loss is present thereafter. This interpretation of the postoperative cell loss is supported by second order comparisons: in the first postoperative period (0-6 months) the cell loss was found to be significantly higher than in the two later periods (6-12 and 12-24 months).

In contrast, endothelial cell loss occurs at continuous accelerated rate following penetrating keratoplasty.[310 778] Although its exact cause is unknown, cell loss after penetrating keratoplasty may result from the surgical trauma, endothelial cell redistribution, and allograft rejection periods.[310 777] Although we did observe stromal allograft rejections with formation of keratic precipitates onto the endothelium after deep anterior lamellar keratoplasty, the recipient endothelium in lamellar keratoplasty may not be compromised in the long-term like the donor endothelium in penetrating keratoplasty.

If the recipient endothelium underneath a lamellar graft shows a rate of cell loss similar to that in virgin corneas, deep anterior lamellar keratoplasty would have an important advantage over penetrating keratoplasty in the management of anterior corneal disorders. The majority of these disorders, like keratoconus and corneal scars, occur in young people in whom long-term endothelial cell survival is important. If the recipient endothelium following deep anterior lamellar keratoplasty is not subjected to significant surgical trauma and shows a physiological cell loss thereafter, the likelihood of a clear corneal graft throughout life may be higher than with a penetrating keratoplasty. Even more so when cataract surgery at old age may further compromise the condition of the corneal endothelium.[310]

## References

See page 287.



**Table 11.1 Endothelial cell density and cell loss after deep anterior lamellar keratoplasty.**

| Postoperative time interval | Measured endothelial cell loss (cells/mm$^2$) | Estimated endothelial cell loss (%) |
|---|---|---|
| 6 months | 283 (± 293) | 11.1 |
| 12 months | 335 (± 309 ) | 2.0 |
| 24 months | 421 (± 316) | 1.2 |

Fig. 11.1 Measured ECD-loss after Deep Anterior Lamellar Keratoplasty

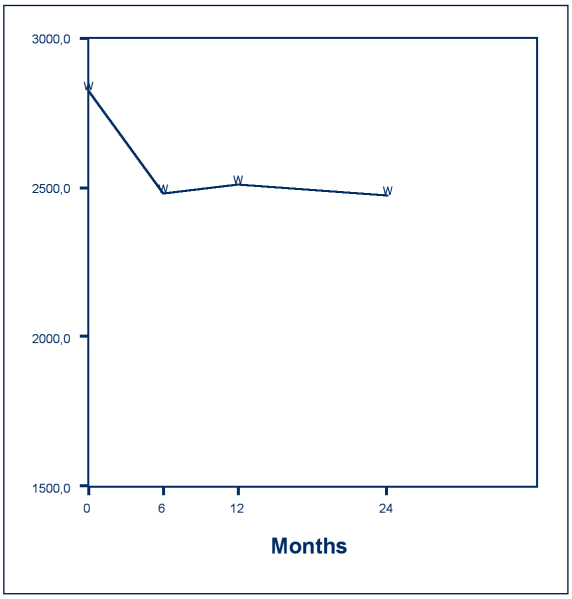
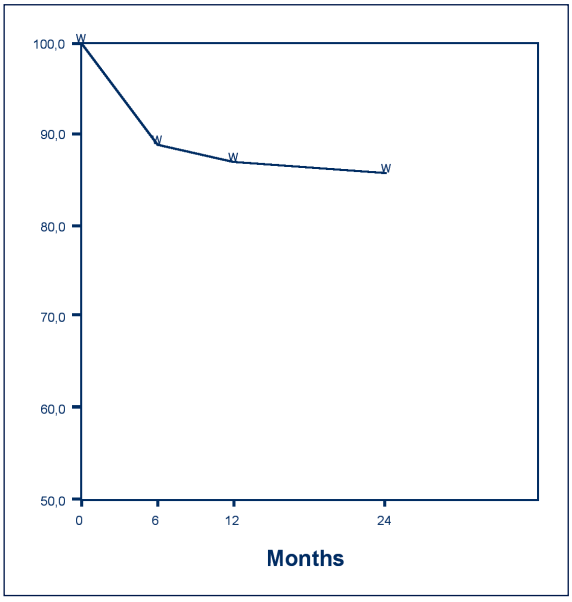



Fig. 11.2 Estimated percentual ECD-loss after Deep Anterior Lamellar Keratoplasty



# chapter 12

# Endothelial cell density after posterior lamellar keratoplasty (Melles techniques); 3 years follow-up

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## Abstract

### Purpose

To report the mid-term endothelial cell density measurements after posterior lamellar keratoplasty (Melles techniques).

### Design

Cohort study.

### Methods

Fifteen consecutive eyes of 15 patients in whom a posterior lamellar keratoplasty procedure was performed for pseudophakic bullous keratopathy or Fuchs' endothelial dystrophy were evaluated. In eleven corneas the donor tissue was inserted through a 9.0 mm sclerocorneal pocket incision (Technique A); in four cases the donor was folded and inserted through a 5.0 mm incision (Technique B). Specular microscopy was performed at 6, 12, 24 and 36 months after surgery, to measure the endothelial cell density.

### Results

Mean postoperative endothelial cell density averaged 2126 cells/mm$^2$ (± 548) at six months, 1859 cells/mm$^2$ (± 477) at 12 months, 1385 cells/mm$^2$ (± 451) at 24 months, and 1047 cells/mm$^2$ (± 425) at 36 months.

### Conclusion

In posterior lamellar keratoplasty, the donor corneal endothelium showed a decrease in cell density similar to that following conventional full-thickness penetrating keratoplasty.

## Introduction

In 1998, we described a new surgical technique, posterior lamellar keratoplasty, for the management of corneal endothelial disorders.[33] Instead of excising a full-thickness corneal button, we evalutated the transplantation of a posterior lamellar disc, 7.0 to 8.0 mm in diameter, through a 9.0 mm scleral pocket incision. In 1999 and 2000 we reported on the preliminary results of this technique (Technique A; Figure 12.1).[1141 1142]
In 2000, we also introduced a second technique, in which a larger 8.5 to 9.0 mm diameter posterior lamellar disc was folded and transplanted through a 5.0 mm scleral tunnel incision (Technique B; Figure 12.1).[1100 1143] Both techniques eliminated the need of suture fixation of the donor tissue, so that the posterior cornea could be transplanted without the use of corneal surface incisions or -sutures. We expected that the advantages of either technique were (1) lower with the rule astigmatism, (2) elimination of suture related complications, and (3) a lower incidence of wound deshiscence, as compared with penetrating keratoplasty.
Because corneal endothelial disease is the main indication for performing these procedures, mid-term and long-term evaluation of the grafted endothelial cell density seems mandatory. The purpose of the current study was to determine the pattern of endothelial cell loss in the first 3 years after posterior lamellar keratoplasty.

## Materials and methods

Fifteen consecutive patients who underwent posterior lamellar keratoplasty for pseudophakic bullous keratopathy and Fuchs' endothelial dystrophy prior to August 2000, were enrolled in the study (Table 12.1). The average patient age was 74.3 (± 8.8) years; seven patients were male and eight were female. Each patient signed a informed consent as part of an institutional review board approved study.

All patients were operated on by the same surgeon (GRJM); eleven patients underwent 9.0-mm incision surgical technique (Technique A; Figure 12.1),[33 1141 1142] and four with the 5.0 mm incision surgical technique (Technique B; Figure 12.1) (Table 12.2).[1100 1143] Postoperative endothelial cell density measurements were performed by the same observer (BTHvD) using the same specular microscope and software for processing of the digital images in each patient at each postoperative time interval.[1222]
The surgical techniques have been previously described. )[33 1100 1141-1143] In short, a deep stromal pocket was dissected across the recipient's cornea through a scleral tunnel incision using an air bubble in the anterior chamber to monitor

dissection depth during surgery.[1090] In patients operated on with Technique A, an intrastromal trephine was inserted into the stromal pocket to excise a recipient posterior lamellar disc. The remaining stromal strands were cut with microscissors. In patients operated on with Technique B, a 9.0 mm mark was made into the corneal epithelium, and only the microscissors were used to excise a (9.0 mm) posterior disc.
After preparation of a donor posterior lamellar disc, implantation of the disc was performed via the scleral incision. In Technique A, the disc was placed endothelial side down on a spoon shaped glide covered with visco elastic to protect the donor endothelium.[33 1141 1142] The scleral incision was then sutured with 10.0 nylon. In Technique B, an 8.5 mm posterior disc was folded prior to insertion into the anterior chamber, with visco elastic covering the endothelium for protection.[1100 1143] The scleral incision was left unsutured. No corneal sutures were used in any case for fixation of the donor tissue.

At 6, 12, 24 and 36 months after surgery, the endothelium was photographed and evaluated using a Topcon SP2000p non-contact autofocus specular microscope (Topcon Corp, Tokyo, Japan). Images of the central corneal window were analyzed using Imagenet 2000 software (Topcon Corp); manual correction of the cell borders was performed prior to final analysis of the endothelium (Table 12.1).[1222] For each postoperative time interval, three measurements of endothelial cell density were averaged.

A donor posterior lamellar disc was excised from a whole globe during surgery, so that preoperative endothelial cell counts could not be obtained. Since the endothelial cell density has been reported to be within 10% between mate eyes,[267] for the surgeries whole globes were selected that had a contralateral cornea with a cell density of at least 2500 cells/mm$^2$.[1142]

For statistical analysis of the relatively small number of patients, subdivided by two surgical techniques and with maximally five measurements per subject in time, a parsimonious exponential decay model for endothelial cell loss had to be constructed that was still flexible enough to fit to the data satisfactorily. This also had to be done in such a way that statistical inference could be made for both surgical techniques separately and that differences between both techniques could be easily tested (see Appendix). For estimating the coefficients of this model, analysis-of-variance for repeated measures was used (PROC MIXED of SAS, release 8.2 (2001), SAS Institute Inc, Cary, USA). The model was also used to make predictions, e.g., at which time point the endothelial cell loss in the two different techniques would be the same.

## Results

At the three year postoperative time interval, all patients had a clear corneal graft without corneal or epithelial edema. For Technique A (Figure 12.1), the overall endothelial cell density averaged 2366 (± 387) cells/mm$^2$ at 6 months (n = 10); 2062 (± 321) cells/mm$^2$ at 12 months (n = 9); 1538 (± 484) cells/mm$^2$ at 24 months (n = 9); and 1126 (± 461) cells/mm$^2$ at 36 months (n = 9) after surgery (Figure 12.2 a; Table 12.1). For Technique B (Figure 12.1), mean cell density averaged 1535 cells/mm$^2$ (± 322) at 6 months (n =4); 1216 cells/mm$^2$ (± 156) at 12 months (n = 3); 1040 cells/mm$^2$ (± 128) at 24 months (n = 4); and 869 cells/mm$^2$ (± 134) at 36 months (n = 4) after surgery (Figure 12.2a; Table 12.1). The overall endothelial cell density as measured in the contralateral donor cornea before the surgery averaged 2865 cells/mm$^2$ (± 158) for Technique A (n = 10) and 2700 cells/mm$^2$ (± 158) for Technique B (n = 4) (Figure 12.2b; Table 12.1). For each of the surgical techniques, we first tried to apply the non-linear bi-phasic exponential decay model proposed by Armitage and associates.[231] However, with our data-set covering only maximally five measurement points per subject during three years of follow-up, no good fit of this model containing four coefficients to be estimated could be achieved, for neither technique. After trying to fit several more parsimonious linear (one-phasic) exponential decay models, the models that showed the best fit to our data were:

Technique A: Predicted ECD = exp (7.9410 – 0.3034 t – 0.03876 t $^{1/3}$),
Technique B: Predicted ECD = exp (7.9410 – 0.02672 t – 0.7659 t $^{1/3}$);

in which t = time after the operation measured in years. The Appendix provides more details on these decay models.

There was a highly significant difference between the two Techniques ($p<0.0001$) considering the coefficients simultaneously. With these models, the extrapolated, predicted time after which the endothelial cell density would reach 500 cells/mm$^2$ was 5.5 years for Technique A and 7.8 years for Technique B; and it could be predicted that after 4.25 years ECDs would be the same for both operation techniques. (Figure 12.3)

## Discussion

To manage corneal endothelial disorders like pseudophakic bullous keratopathy and Fuchs' endothelial dystrophy, a posterior lamellar keratoplasty may be performed as an alternative procedure to penetrating keratoplasty.[33 1100 1141-1143] With penetrating keratoplasty, an overall endothelial cell loss has been

reported of approximately 33% after the first year after surgery (Table 12.2),[764] and the cell density has been found to continue to decrease at an accelerated rate up to 20 years after surgery.[310 779] This suggests that after the initial surgical trauma, donor endothelial cell survival is compromised in the host ocular environment.[755]

In the current study, the mid-term endothelial cell density was evaluated after posterior lamellar keratoplasty. Fourteen consecutive eyes with at least three years of follow-up were measured, to determine the rate and the pattern of cell loss after this new type of corneal surgery. The evaluation may be particularly important, because a posterior donor disc was implanted into the recipient eye using a spoon shaped glide covered with visco elastic (Technique A, Figure 12.1), or by folding the donor for implantation through a small tunnel incision (Technique B, Figure 12.1). Both implantation techniques could potentially harm the donor endothelial cell layer.

In the first group of patients, in whom the donor tissue was implanted using a spoon shaped glide (Technique A, Figure 12.1), the endothelial cell counts averaged 2062 cells/mm$^2$ (27.6% cell loss) at one year postoperative. Except two, all eyes had an endothelial cell density of 2000 cells/mm$^2$ or more. A similar result was found by Terry et al. who performed our procedure with some minor modifications in eight patients.[1154 1155] These findings suggests that the surgery itself, i.e. the preparation of the donor posterior lamellar disc, positioning of the corneal disc endothelial side down onto the spoon-shaped glide, and the implantation of the disc through the scleral incision, did not cause an unacceptable cell loss.

However, endothelial cell density measurements in the following years showed a rapid decline of the cell density, to 1126 cells/mm$^2$ at three years (cumulative cell loss 61%). This rate of cell loss may still be similar to that after penetrating keratoplasty (Table 12.2).[216 228 229 310 480 492 497 500 678 770]

The result may have been biased due to two additional factors. Initially patients were selected with a low visual potential due to a concomitant ocular disorder, that in some cases may have had an effect on postoperative endothelial cell survival, for example, the iris-fixated anterior chamber lens in case 1, or the uveitis glaucoma hyphema syndrome in case 2. Postoperative events may also have affected the endothelial cell counts: severe herpes keratitis 12 months after surgery, with poor compliance to topical treatment in case 8, and uveitis in the early postoperative period in Case 11.

In the second group of patients, in whom the donor tissue was implanted by folding the donor (Technique B, Figure 12.1), the endothelial cell counts

averaged 1215 cells/mm$^2$ at one year postoperative. We are unaware of previous reports on the endothelial condition with this technique so far. Although no statistical difference in endothelial cell density was found between Techniques A and B at the three years postoperative time interval, there may be two possible explanations for the lower endothelial cell density in Technique B than Technique A (2062 cells/mm$^2$ at one year). First the implantation of folded donor tissue may be more traumatic. Second, more endothelial cell redistribution may have occurred, because the recipient opening was intentionally made larger than the diameter of the donor disc to avoid inadvertent wrinkling of the donor tissue. As a result, a small stromal gap was present along the entire circumference of the donor disc. After surgery, some stromal edema was present over the area of the gap, disappearing within the first months probably because of donor endothelial cell migration covering the area of the stromal denuded of endothelial cells adjacent to the donor tissue.

It should be noted, that the models were fit on small groups with relatively short follow-up periods. This may have resulted in overfitting of the models and may explain that we could not fit a biphasic model (yet). According to extrapolated calculations, both Techniques would have the same endothelial cell densities after 4.25 years, with a slower decrease in cell density in Technique B. A possible explanation for the slower cell loss in Technique B may be that in Technique B an 8.5-9.0 mm donor was implanted, instead of a 7.0-7.5 mm donor in Technique A (Figure 12.1). As a result, the donor disc in Technique B is carrying more endothelial cells, and cell re-distribution toward the host peripheral rim may occur at a different rate or with a different pattern in each of the Techniques.[777]



In conclusion, the one year measurements in our study suggests that either an implantation technique using a spoon-shaped glide (unfolded donor) (Technique A) or a technique in which the donor is folded to allow implantation through a small incision (Technique B), does not cause unacceptable damage to the donor endothelial cell layer. The three year measurements suggest that the donor endothelial cell density after posterior lamellar keratoplasty may be similar to that following conventional full-thickness penetrating keratoplasty.

## References

See page 287.

Table 12.1 Endothelial cell density (cells / mm²) after posterior lamellar keratoplasty

| Case # | Age / sex | Indication surgery | Preoperative endothelial cell density (contralateral cornea) (cells / mm²) | Postoperative (months) endothelial cell density (cells / mm²) | | | | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | 6 | 12 | 24 | 36 | |
| **Technique A – 9.0 mm incision technique (unfolded donor)** | | | | | | | | |
| 1 | 79F | PPBK | 2700 | 2120 | 2077 | 973 | 582 | Anterior chamber lens in situ |
| 2 | 63M | PPBK | 3000 | 2842 | n.a. | 1566 | 842 | Preoperative: UGH syndrome |
| 3 | 86M | FED + PPBK | 2900 | 2255 | 2094 | 2033 | 2064 | |
| 4 | 74F | FED + PPBK | 3100 | 2969 | 2102 | 1407 | 1088 | |
| 5 | 74F | FED | 2900 | 2796 | 2508 | 1905 | 1141 | |
| 6 | 72F | FED + PPBK | 3100 | 2332 | 2247 | 1932 | 1423 | |
| 7 | 85M | ABK | 2700 | n.a. | n.a. | n.a. | n.a. | Lost to follow up 4 months postoperative |
| 8 | 90F | FED + PPBK | 2800 | 1896 | 1738 | 1355 | 814 | 12 months postoperative severe HSV keratitis |
| 9 | 77M | FED | 2750 | 2412 | 2287 | 1928 | 1574 | |
| 10 | 61F | FED + PPBK | 2600 | 2325 | 2177 | n.a. | n.a. | 13 months post-operative PKP for interface scar |
| 11 | 81M | FED | 2800 | 1681 | 1338 | 741 | 605 | Postoperative uveitis |
| **Average 9.0 mm incision technique** | | | **2850** | **2366** | **2062** | **1538** | **1126** | |
| SD | | | 158 | 357 | 320 | 434 | 461 | |
| **Technique B – 5.0 mm incision technique (folded donor)** | | | | | | | | |
| 12 | 77F | FED | 2600 | 1787 | 1465 | 1122 | 1077 | |
| 13 | 64M | FED | 2500 | 1916 | n.a. | 1084 | 750 | |
| 14 | 66F | FED + PPBK | 2800 | 1168 | 1053 | 821 | 751 | |
| 15 | 65M | FED | 2900 | 1268 | 1227 | 1133 | 899 | |
| **Average 5.0 mm incision technique** | | | **2700** | **1535** | **1216** | **1040** | **869** | |
| SD | | | 183 | 322 | 156 | 128 | 134 | |
| **Overall average** | | | **2818** | **2126** | **1859** | **1385** | **1047** | |
| SD | | | 181 | 548 | 477 | 451 | 425 | |

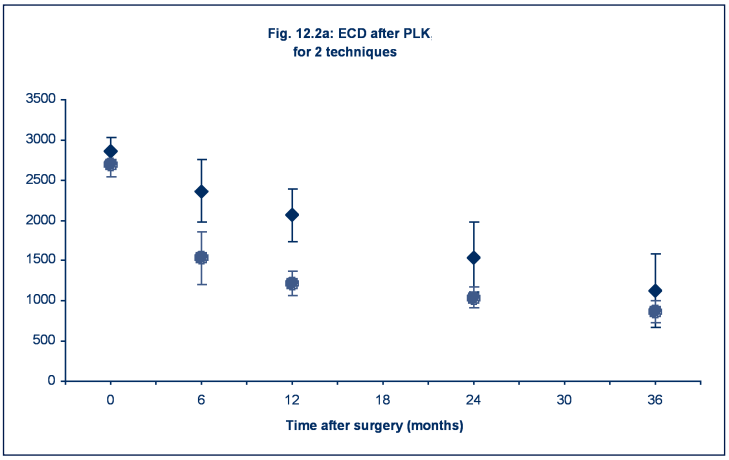
PPBK = Pseudophakic bullous keratopathy, FED = Fuchs' endothelial dystrophy
UGH = uveitis, glaucoma, hyphaema sydrome , PKP = penetrating keratoplasty

Table 12.2 Literature review on endothelial cell loss

| | Per year | >1 year | >3 yrs | >4 yrs | >5 yrs | >10 years | References |
|---|---|---|---|---|---|---|---|
| Unoperated | 0.6 (0.3-1.0) % | | | | | | 228,229 |
| | 2.9%(<14 y) – 0.3%(>14 y) | | | | | | 216 |
| CE + I | 2.5 % (after 10 years) | | | | | | 492 |
| | | | 11.6 – 25.6 % | | | | 480 |
| | | 9.6-10.9 % | | | | | 497 |
| | | 16 % | | | | | 500 |
| PKP | 7.8 % (after 5 years), 4.2 % (after 10 years) | 33% | 52% | | 59% | 67 % | 310 678 |
| | | | | 46% | | | 769 |
| | | 34% | | | | | 763 |
| | 14-29 % (dependent on diagnosis, after 2-3 years) | | | | | | 776 |

Fig.12.1 Diagrammatic representation of the techniques for posterior lamellar keratoplasty.
See colour figure on page 330.

Fig. 12.2a Measured decrease in endothelial cell density following posterior lamellar keratoplasty in (a) patients with the 9.0 mm incision technique and unfolded donor (Technique A) and (b) patients with the 5.0 mm incision technique and folded donor (Technique B).




◆ = Technique A, ● = Technique B

Fig. 12.2b Measured overall decrease in endothelial cell density following posterior lamellar keratoplasty in all patients (Technique A + B).

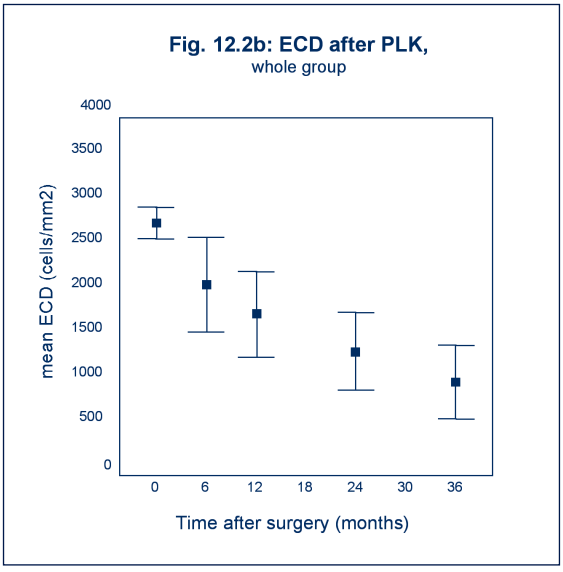
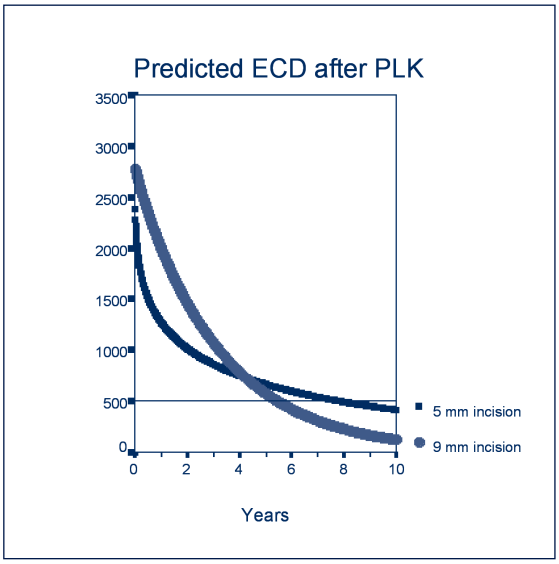


Fig. 12.3 Estimated endothelial cell density following posterior lamellar keratoplasty for (a) 9.0 mm incision technique and unfolded donor (Technique A) and (b) the 5.0 mm incision technique and folded donor (Technique B). The horizontal line represents the 500 cells/mm$^2$ level..Compare to Figure 12.2a.




**Appendix: Exponential decay models of endothelial cell density after posterior lamellar keratoplasty**

The specified model that showed a plausible fit to our data for ECD loss in time was an exponential decay model with a linear term and a cubic root term of time, of which the coefficients are assumed to be different between the two surgical techniques. Differences in the coefficients between the two techniques are represented in the model by interaction terms between time and technique. The intercept is assumed to be the same for both techniques. The residuals (differences between observed and predicted ECD) are assumed to have a spatial (i.e. distance in time) correlation structure, i.e. the correlation between repeated measurements decreases as the time interval between the measurements increases, apart from a separate component in the residual variance due to measurement error.
The estimates are given below, with time t measured in years.

**Technique A:**

| | | | | |
|---|---|---|---|---|
| Predicted ECD = exp | (7.9410 | - 0.3034 t | - 0.03876 $t^{1/3}$) |
| SE's: | (0.0672) | (0.0496) | (0.0793) |
| p-values: | | $p < 0.001$ | $p = 0.627$ |

**Technique B:**

| | | | | |
|---|---|---|---|---|
| Predicted ECD = exp | (7.9410 | - 0.02672 t | - 0.7659 $t^{1/3}$) |
| SE's: | (0.0672) | (0.0753) | (0.1224) |
| p-values: | | $p = 0.725$ | $p < 0.001$ |

There is a highly significant difference between the two techniques ($p < 0.001$) considering the two coefficients of the linear and the cubic root term of time simultaneously. The predicted time after which ECD will reach 500 cells/mm$^2$ was calculated to be 5.5 years for technique A and 7.8 years for technique B (figure 3, the horizontal line represents 500 cells/mm$^2$.) The predicted time at which both techniques have the same ECD, that is the time where the two curves cross, is 4.25 years.

# chapter 13

# Endothelial cell density after posterior lamellar keratoplasty: 5-7 years follow-up

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## Abstract

### Purpose

To report the 5-7 year endothelial cell density (ECD) measurements following posterior lamellar keratoplasty (PLK, Melles techniques).

### Methods

Fifteen consecutive eyes of 15 patients in whom a PLK procedure was performed for pseudophakic bullous keratopathy or Fuchs' endothelial dystrophy were evaluated. In eleven corneas the donor tissue was inserted through a 9.0 mm sclerocorneal pocket incision (Technique A); in four cases the donor was folded and inserted through a 5.0 mm incision (Technique B). Specular microscopy for ECD measurements was performed longitudinally until 3 years post-operatively, and then cross-sectionally at 54 to 84 months post-operatively.

### Results

Three patients were not available or lost for follow-up. One graft had decompensated. In the remaining 11 PLK eyes, mean postoperative ECD was 741 cells/mm$^2$ (± 305) cells/mm$^2$, ranging from 368 – 1576 cells/mm$^2$. Mono- and bi-phasic regression models of ECD against time were fitted on the data, for each group separately.

### Conclusion

After PLK the ECD continued to decline in a rate higher than physiological, and probably also higher than after conventional full-thickness penetrating keratoplasty. Both early- and longer-term endothelial cell loss rates may be related to the surgical technique.

Since 1998, we have described various new surgical techniques for posterior lamellar keratoplasty (PLK). These techniques were popularized in the United States as deep lamellar endothelial keratoplasty (DLEK), small incision DLEK, and Descemet stripping endothelial keratoplasty (DSEK), respectively.[1010 1096 1097 1100 1142 1143 1149 1151-1153] From 1998 thru 2000, we operated on a series of patients using the two preliminary techniques. Fifteen patients were followed longitudinally, until the three year follow-up interval. Our previous study on prospective central corneal endothelial cell density measurements (ECDs) in these patients involved monophasic regression models of ECD against time.[1223] Using extrapolation of these models, predictions were made that ECD-loss would reach the 500-cells/mm$^2$-level at 5.5 to 7.8 years postoperatively. [1223] In the current study, we report on the cross-sectional ECDs obtained in 2005 and 2006, at 54 to 80 months follow-up (mFU), for the same series of patients. This was the first group of PLK patients ever operated.

## Methods

Eleven patients *(Group A)* were operated on with a PLK technique in which a 7.5 mm diameter, unfolded donor posterior disk was transplanted through a 9.0 mm scleral incision.[1096 1100 1142 1153 1223] In four patients *(Group B)* a PLK technique was performed in which an 8.5 mm diameter, folded donor posterior disk was transplanted through a 5.0 mm scleral incision. [1010 1143 1223] (Surgery by GRJM) Central endothelial photographs were obtained with Topcon SP2000p non-contact specular microscopy. Semi-automated endothelial analysis was performed with Topcon IMAGEnet 2000 software, and involved manual correction (by BTHvD) of incorrect automatedly drawn cell borders (Topcon Corp, Tokyo, Japan).[911] (Specular microscopy and analysis by BTHvD.) Donor ECDs were the ECDs of the contralateral donor cornea, obtained at the eye bank using light microscopy counting with a calibrated graticule after trypan blue 1.2 % and sucrose 1.8 % staining.[332]

## Results

Table 13.1 shows the ECDs of the patients at the different FU points. In *Group A,* one patient (case 7) was altogether lost to FU 4 months postoperatively. One patient (case 10) was re-transplanted using penetrating keratoplasty (PK) at 13 months postoperatively for interface scarring. One patient (case 8) was not available for FU for the present study. One graft in an eye with UGH syndrome (case 2) was found to have undergone late endothelial decompensation, and the patient was scheduled for penetrating keratoplasty (PK). In a fifth eye, the graft showed focal decompensation over one of the 'claws' of an aphakic anterior chamber IOL apparently due to 'endothelial touch'. In this eye the central ECD measured 368 cells/mm$^2$ (80 mFU). In the remaining six eyes in this group in the

present study, the central ECD ranged from 592 - 1576 cells/mm$^2$ (60–84 mFU). In *Group B,* central ECDs ranged from 595 – 805 cells/mm$^2$ (54 mFU). One patient in this group had experienced a mild, reversible endothelial allograft rejection. With these additional data, the existing mono-phasic regression models of ECD loss over time for both groups from our previous study[1223] were adjusted slightly, and extrapolations now predicted that both Groups would reach the 500-cells/mm$^2$-level almost simultaneously between 7 and 8 years postoperatively. See figure 13.1. ECD-loss was also evaluated using bi-exponential regression models as proposed by Armitage et al..[231] The model that was fitted for Group A was:

ECD(time)= 353*exp[-0.5830*(time)] + 2498*exp[-0.2412*(time)]

which revealed fast-phase ECD loss with a half-time of 1.2 hours regarding a pre-operative proportion of 353 cells/mm$^2$ of ECD , and a slow-phase half-time of 2.9 years regarding a proportion of 2498 cells/mm$^2$. For Group B the bi-exponential regression model was:

ECD(time)= 1229*exp[-0.2280*(time)] + 1494*exp[-0.2054*(time)]

with a fast-phase half-time of 3.0 hours which was related to a proportion of 1229 cells/mm$^2$ , and a slow-phase half-time of 3.4 years which was related to a proportion of 1494 cells/mm$^2$.

## Discussion

The number of patients still available for follow-up and the number of follow-up time-points per patient were both small, which made the fitting of bi-exponential regression models difficult. Also, some over-interpretation of the data may occur. Nevertheless, our results suggest that the intra-operative endothelial cell loss is higher with the donor folding technique (Group B). In the late phase, grafts in Group B showed less cell loss. This may be related to the use of larger diameter grafts, as more endothelial cells were transplanted and less redistribution to the recipient is expected. In Group B the mean age was lower. Also, bullous keratopathy was less often the indication for transplantation. Both factors may diminish endothelial cell redistribution as well. Decline in ECD appears to be higher and faster than in historical PK groups, in all except one of these PLK patients.[231 310 764 1223] However, because our patients constituted the first PLK cases performed worldwide, the ECD-loss may be skewed due to a learning curve, the use of prototype instruments, and selection of patients with pre-existing, possibly endothelial cell density influencing ocular diseases.

## References

See page 287.

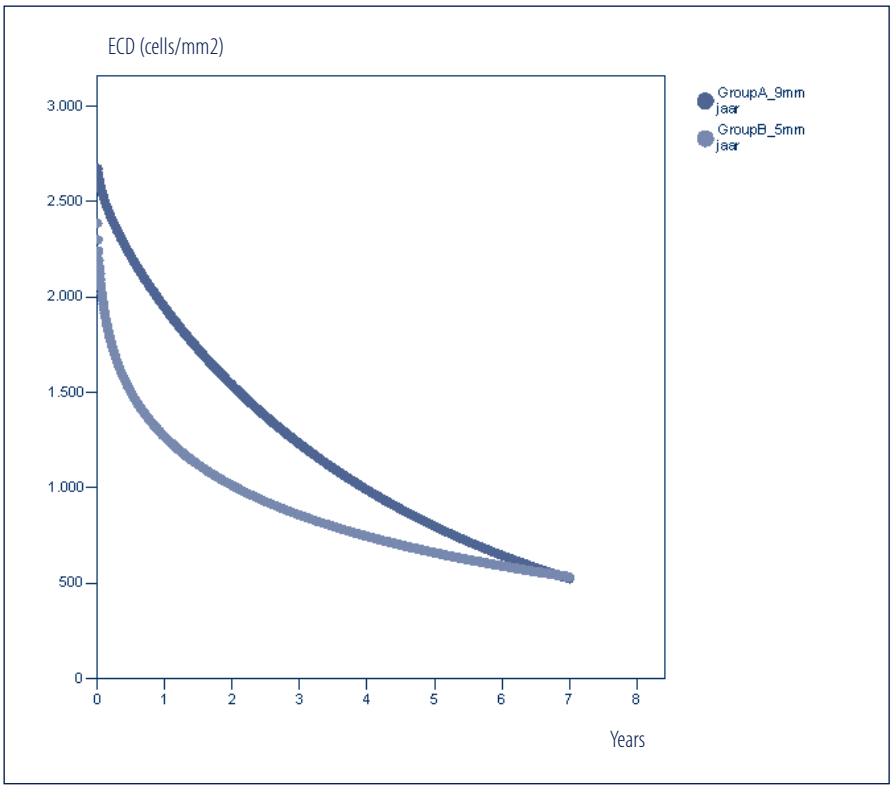
Table 13.1 Endothelial cell density (cells / mm2) after posterior lamellar keratoplasty

| Patient | Age | Sex | Indication | Donor ECD (contralateral eye) | Prospective ECD-loss[11] | | | | Cross-sectional data | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 6 m. | 12 m. | 24 m. | 36 m. | Last FU | At m FU | |
| Group A | | | | | | | | | | | |
| 1 | 79 | F | PPBK | 2700 | 2120 | 2077 | 973 | 582 | 368 | 80 m | Anterior chamber lens in situ |
| 2 | 63 | M | PPBK | 3000 | 2820 | n.a. | 1566 | 842 | decompensated | 72 m | Preoperative: UGH syndrome |
| 3 | 86 | M | FED + PPBK | 2900 | 2255 | 2094 | 2033 | 2064 | 1576 | 72 m | |
| 4 | 74 | F | FED + PPBK | 3100 | 2969 | 2102 | 1407 | 1088 | 820 | 72 m | |
| 5 | 74 | F | FED | 2900 | 2632 | 2508 | 1905 | 1141 | 592 | 84 m | |
| 6 | 72 | F | FED + PPBK | 3100 | 2332 | 2247 | 1932 | 1423 | 607 | 72 m | |
| 7 | 85 | M | ABK | 2700 | n.a. | n.a. | n.a. | n.a. | n.a. | | Lost to follow up 4 m post-op. |
| 8 | 90 | F | FED + PPBK | 2800 | 1896 | 1738 | 1355 | 814 | n.a. | | 12 m post-op. severe HSV keratitis |
| 9 | 77 | M | FED | 2750 | 2412 | 2287 | 1928 | 1574 | 756 | 60 m | |
| 10 | 61 | F | FED + PPBK | 2600 | 2325 | 2177 | n.a. | n.a. | n.a. | n.a. | 13 m post-op. PK for interface scar |
| 11 | 81 | M | FED | 2800 | 1681 | 1338 | 741 | 605 | 645 | 54 m | Postoperative uveitis |
| **Mean** | **76.5** | | | **2850** | **2366** | **2062** | **1538** | **1126** | **766** | | |
| **SD** | 8.7 | | | 158 | 357 | 320 | 434 | 461 | 385 | | |

Table 13.1 Continued

| | | | | Donor | 6 m. | 12 m. | 24 m. | 36 m. | Last FU | At m FU | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Group B | | | | | | | | | | | |
| 12 | 77 | F | FED | 2600 | 1787 | 1465 | 1122 | 1077 | 805 | 54 m | |
| 13 | 64 | M | FED | 2500 | 1916 | 1119 | 1084 | 750 | 595 | 54 m | |
| 14 | 66 | F | FED + PPBK | 2800 | 1168 | 1053 | 821 | 751 | 743 | 54 m | |
| 15 | 65 | M | FED | 2900 | 1268 | 1227 | 1133 | 899 | 647 | 54 m | Mild allograft rejection, glaucoma |
| **Mean** | 68 | | | **2700** | **1535** | **1216** | **1040** | **869** | **698** | | |
| **SD** | 5.2 | | | 183 | 322 | 156 | 128 | 134 | 94 | | |
| **All** | **74.3** | | | **2818** | **2126** | **1859** | **1385** | **1047** | **741** | | |
| SD | 8.8 | | | 181 | 548 | 477 | 451 | 425 | 305 | | |

Fig. 13.1 Predicted endothelial cell density (in cells/mm2) against time (in years) according to the mono-exponential regression models.



part

V

# Summary, references, and abbreviations

chapter

# 14

# Summary and perspective

# I. Summary in perspective.

In *Chapter 1* a short overview is presented on how knowledge of and research on the corneal endothelium and endothelial specular microscopy evolved. It also briefly introduces the background and purpose of the studies in parts II, III, and IV of this thesis.

*Chapter 2* is a review of the literature on the corneal endothelium. Key points include the paramount importance of the endothelium, the innermost corneal layer, for hydration homeostasis. According to the Pump-Leak theory, the endothelium comprises a barrier that is permeable to water and smaller molecules in the anterior chamber (Leak), but not to larger molecules. Simultaneously, the endothelial cells actively pump water back into the anterior chamber (Pump). This hydration homeostasis is necessary to maintain corneal transparency. When in corneal edema the corneal water content becomes higher than normal, the cornea loses transparency. In humans, corneal endothelial cells possess very little mitotic capacity. This affects wound healing and it results in age-related loss of endothelial cells. This decay can be described using the endothelial cell density (ECD), the most important quantitative morphometric parameter of the endothelium. In vivo, endothelial morphology and ECD are often examined with specular microscopy.

Studies on cataract surgery and penetrating keratoplasty (PK) have raised concerns that there is prolonged endothelial cell loss at a higher-than-physiological rate. After uncomplicated cataract surgery with modern techniques, peri-operative endothelial cell loss is relatively low and it may approach physiological rates within a few years. With older cataract extraction and lens implantation techniques, the loss was substantially higher and its rate stayed elevated for a long time. The amount and rate of endothelial cell loss may therefore be considered to be a safety standard for techniques and innovations in cataract surgery.

Increased endothelial cell loss has been observed for at least 10 and probably more years after PK. An important cause of endothelial cell loss is endothelial allograft rejection. But even in the absence of overt immunological rejection, endothelial cell loss occurs at a higher-than- physiological rate. Eventually this may lead to graft decompensation due to late endothelial failure (LEF). Too few endothelial cells are left to maintain the hydration homeostasis and hence to prevent graft opacification. Why the cell loss rate remains high after PK, and for how long, is still in debate.

As endothelial cell density is an important parameter, it is important that its measurements are reliable. Several aspects of reliability can be discerned. In

part II of this thesis we present studies on four of those aspects. We evaluated endothelial morphometric measurements with a Topcon SP2000P autofocus non-contact specular microscope, combined with Topcon IMAGEnet2000 endothelial cell analysis software.

In *chapter 3* we describe how the magnification of the SP2000P can be calibrated. We found incorrect calibrations in 4 different specular microscopes (unpublished data). Once again this proves the necessity of checking the calibration in any biomedical measurement instrument. This is even more important when different types of instruments are used interchangeably.[287,1224] Furthermore, formulas were described for the calculation of correct ECD values, in case measurements had been obtained with an incorrectly calibrated instrument or software.

The validity of several ECD assessment methods was also investigated in this study, using the methods described by Bland and Altman.[910] In our study there was a systematic difference between manual counts, and all ECD assessment methods in the SP2000P & IMAGEnet 2000 combination. However, almost all of these differences were relatively small, except in fully automated ECD assessment. The semi-automated assessment method, in which software-defined cell borders had been manually corrected before ECD calculation, was the most valid method. It had the narrowest limits-of-agreement, which indicated less individual spread in measurements. In most instruments used for endothelial analysis, manual correction of automatedly recognized cell borders increases performance.[1224,1225]

The systematic difference between this assessment method and manual ECD counts implies that a correction factor must be used when semi-automated ECDs are compared to ECDs obtained with manual counting. Similarly, a correction factor should be applied when different assessment methods are used interchangeably.

As Bland and Altman pointed out, calculating correlation coefficients for the comparison of measurement methods is not sufficient, as a systematic difference is not recognized. Nor should linear regression be used when two measurement methods are compared. Both measurement outcomes are independent parameters, and one method is not a function of the other. In literature sometimes the two mistakes can even be found in one study.[313]

A third aspect that affects the reliability of a measurement method is its reproducibility. In the study described in *chapter 4* we found a reasonable to good overall reproducibility of endothelial morphometric parameters including ECD. Reproducibility was foremost affected by a relatively low repeatability,

i.e. a high sampling variability. High sampling variability can be decreased by obtaining more measurements and averaging their results. This way the variance of the measurements decreases with the inverse of the number of measurements.

Inter-observer reproducibility was good as well. ECDs obtained by different observers may be used interchangeably, provided that these observers have been trained well and use identical measurement and analysis techniques. Our results on reproducibility of ECD measurements with the SP2000P & IMAGEnet2000 were similar to those in other studies.[294,1225]

In chapter 4 it was further demonstrated that ECDs from the nasal paracentral (i.e. 3 mm off centre) area may be used to substitute for central ECD, if no clear images can be obtained of the corneal centre. The central ECD of the contralateral eye may substitute as well, provided the medical histories of both eyes were similar. It should be kept in mind that in individual cases a considerable difference between the substitution and substituted values may exist.

The findings in *chapters 3* and *4* show that it is possible to obtain reliable central ECD measurements with the SP2000P & IMAGEnet 2000 combination. Measurements can be reliably compared between individuals, and reliably followed over time within one individual, if the requirements described in these chapters are fulfilled.

In the study described in *chapter 5*, a systematic, fixed difference of 140 cells/$mm^2$ was found between in-vivo SP2000P ECD and in-vitro, light microscopic eye bank ECD in the same corneal buttons. Specular microscopy measured higher ECDs than the in-vitro technique. This is relevant in follow-up studies on keratoplasty, such as those described in chapters 12 and 13 of this thesis, where donor ECDs are compared to specular microscopic ECDs. From now on, in similar studies either 140 cells/$mm^2$ should be added to the donor ECDs or subtracted from the SP2000P ECD. When other specular or confocal microscopes are used, the existence of a systematic difference with donor ECDs obtained with a different method should be investigated using the same methods as in our study.

We did not expect to find a fixed, absolute difference, and it seems in contradiction with the most probable explanations for a possible difference. A relative difference, i.e. a difference that is proportional to the ECD value itself, was thought more probable. Therefore, one might assume that in reality there is a relative difference between in-vivo and in-vitro ECD measurements, but that this nature of the difference is masked by other effects. An explanation for such a masking effect is presented in chapter 5. The linear regression model that is

used in the cornea bank ECD counting method is more inaccurate in corneas with non-average ECDs. The model overestimates the in-vitro ECDs in the low regions, and underestimates them in the highest regions.

*Chapters 5* and *6* discuss that there are several optical phenomena that could explain a proportional difference, i.e. a difference that increases with the ECD. Another possible explanation for a proportional difference may be found in chapter 3. In this study a similar systematic difference was described between a semi-automated and a manually obtained ECD. This difference may be inherent when fixed-frame and variable-frame counting methods are compared. Especially in small samples, a systematic underestimation of cell area resulting in overestimation of ECD may be introduced by variable-frame counting.[279,280] However, all these explanations remain speculative, since no proportional difference between in-vivo and in-vitro ECD was observed. There might be interaction between the effects of several simultaneously acting factors.

Indeed, the study on optical distortions in non-contact specular microscopy described in *chapter 6* demonstrates that simultaneously several errors are introduced, whose effects oppose each other. We found that the total of optical distortions did not cause substantial errors in corneas with close to average thickness and curvature or in keratoconic eyes. However, this was only true when the error caused by the effect of an oblique angle of observation, the perspectivistic distortion, was eliminated. This appears to be the case in the SP2000P (Pierre Gelissen, Topcon Europe, personal communication, 2004). In instruments in which the oblique observation angle effect is not compensated for, the error increases with the co-sinus of the angle and may therefore be substantial.



The studies in Part II of this thesis are not only valuable for users of the SP2000P and IMAGEnet2000, but also relevant to investigators using other instruments, assessment methods, and techniques for endothelial morphometry. Users of other specular or confocal microscopes may find our methodological considerations, investigative and statistical methods, and interpretations useful. The correction formulas presented in chapter 3 for incorrect magnification are more generally applicable. The physical formulas in chapter 6 are not specific to our specular microscope; they apply in other non-contact and contact types as well.

In part III of this thesis we focused on the effects of trypan blue on the corneal endothelium. The vital stains trypan blue (TB) and indocyanine green (ICG) have long been used in ophthalmology in several applications. From 1998 onwards, both dyes have also been used for intra-operative staining, to enhance visualization of ocular membranous structures. Better viualization facilitates delicate surgical manoeuvres. The term dye-enhanced ocular surgery was coined to describe several surgical techniques in which these and other

dyes are valuable adjunctive tools. In *chapter 7* early applications of both dyes are reviewed, and the development of the intra-operative application of each dye is described. From an increasing number of published studies it may be concluded that the two dyes have a concentration-related and exposure time-related toxicity for ocular cells. In 1998 and 1999, the knowledge about toxic effects of TB on the corneal endothelium was based mostly on experience in cornea banks and a few rare studies. The studies described in *chapters 8* and *9* were initiated to investigate the toxicity of TB in depth, and to establish the safety of TB lens capsule staining in cataract surgery.

The clinical study in *chapter 8* demonstrates the safety of TB (0.06 %) lens capsule staining. One year after bilateral surgery, the endothelial cell loss in the eyes that had undergone phacoemulsification with intra-operative TB application was not higher than in the fellow, control eyes.

The results of the experimental study described in *chapter 9* demonstrate that there is a concentration-related and exposure-time-related toxicity of TB to endothelial and other corneal cells. It was shown that under experimental circumstances especially the vehicle of TB was essential for the degree of toxicity that was caused. We demonstrated that concentrations of and exposure times to TB such as currently applied in the Cornea Bank Amsterdam and during cataract surgery, remain below the toxicity threshold. However, caution is warranted whenever higher concentrations of TB are used or when the endothelium is exposed for a long time.

In animal experiments TB has been shown to have teratogenic effects. The dose used in ophthalmic surgery in humans is about 10,000 times lower than the systemic teratogenic dose in animals. Furthermore, most of the dye is removed from the eye immediately after the application, before it can even get into the systemic circulation. Nevertheless, it may also be advisable not to use TB in pregnant women or in infants. Operating theatre and eye bank staff should take protective measures (e.g. gloves) to prevent repeated exposure to small doses of TB.

The possible toxicity of ICG to retina and retinal pigment epithelium has also been the subject of much discussion. However, dye-enhanced surgery is probably here to stay. Therefore, the search continues for alternative dyes that are equally or even more effective, but less toxic.

In part IV of this thesis, in *chapter 10*, the development of new types of lamellar corneal transtplantation (lamellar keratoplasty, LK) is described in the context of the history of the overall development of corneal transplantation. In LK only diseased layers of the cornea are replaced, whereas in penetrating keratoplasty (PK) a full-thickness part of the cornea is transplanted. Lamellar corneal surgery

has undeniably seen a revival of interest in the last 8 years.[1226] The rationales for anterior and posterior lamellar keratoplasty (PLK) as reviewed in chapter 10 illustrate that both types of surgery have important theoretical advantages on PK. These include more rapid visual rehabilitation, less astigmatism, and less wound-related and suture-related complications. Consequently, anterior and posterior lamellar keratoplasty might become the treatment of choice for many cases for which PK used to be the mainstay treatment. For this to happen it is imperative that the theoretical advantages prove to be clinical assets. Furthermore, transplant survival should be similar or better than after PK. Transplant survival after PK depends on the occurrence of allograft rejection, and long-term non-rejection endothelial cell loss (late endothelial failure, LEF).

In anterior lamellar keratoplasty the recipient endothelium remains in place. This eliminates the possibility of allograft rejection causing a graft failure. Furthermore, non-rejection LEF may be prevented, as anterior LK essentially is extra-ocular surgery.[231,310] The results of the study on ECD-loss after deep anterior lamellar keratoplasty (DALK, Melles' Technique), described in *chapter 11*, indicate that after an initial drop in ECD, the cell loss soon approached a physiological, age-related rate. These findings are comparable to other studies on endothelial cell loss after (anterior) LK.

Our paper had been submitted just before Armitage's important paper on bi-exponential endothelial cell decay models was published.[231] Therefore, Armitage's method was not applied in this study. Nevertheless, our results suggest that with longer follow-up and more measurement points such a decay pattern could be discerned. As in DALK surgery the endothelium is not directly manipulated, several indirect mechanisms for the initial drop in cell loss can be postulated. Mechanical attrition due to deep lamellar dissection just superior/anterior to Descemet's membrane and the endothelium may cause shedding of endothelial cells. Noticed or unnoticed microperforations during deep lamellar dissection will directly damage the endothelium. Possibly, the presence of an air bubble in the anterior chamber during the procedure contributes to cell loss as well.[535,536,899]

In contrast, after posterior lamellar transplantation (PLK), immunological allograft rejection may and does occur, and both peri-operative and late non-immunologic endothelial cell loss are substantial. As has been discussed in chapter 2 paragraph XVI, all three factors affect graft survival. Therefore, the rate and pattern of endothelial cell loss after PLK needed clarification. In the prospective study described in *chapter 12* we found that after 3 years the ECD-loss was somewhat higher compared to that after PK reported in the literature. Concerning these results, some observations need to be brought to attention. First, the donor ECDs were those of the contralateral donor eye. The posterior

lamellar discs were prepared in the operating room from whole globes. Thus, in the cornea bank no ECDs could be obtained from this donor eye itself (see chapter 2 paragraph VIII). As has been shown in the study described in chapter 4, paired eyes usually do not have significantly different ECDs. However, in individual pairs differences in ECD between contralateral eyes may still have been substantial. Secondly, donor ECDs in the contralateral donor eyes were obtained with the usual cornea bank in-vitro light microscopic ECD counting methods, whereas follow-up in-vivo ECDs were obtained with non-contact specular microscopy. If the results of chapter 5 are taken into account, the cell loss after PLK is in fact even larger, on average 140 cells/mm$^2$. Thirdly, in the study in chapter 12 we were not able to fit a bi-exponential decay model, probably because our follow-up was too short at that point (Armitage, personal communication, 2004).

For this last reason we gathered additional, cross-sectional data on ECD in this cohort of patients. With these data successful fitting of bi-exponential models could be performed, as *chapter 13* proves. In the models in this study, the 'fast phase' of endothelial cell loss applies to the first few hours after surgery. Again, because of our relatively short follow-up, this emphasis on the first post-operative hours in our models is probably a result of "over-fitting" of a regression model (Armitage, personal communication, 2006). Still, the results are interesting, because they indicate that in these first few post-operative hours a significantly larger proportion of donor ECD was lost in eyes in which the donor was folded before introduction into the recipient eye. Furthermore it is remarkable that after the use of a folded donor disc the late loss appeared to be slower. As an explanation, in this group a larger diameter donor disc was used, which provides in absolute numbers more donor endothelial cells, with a higher density towards the donor edge. Moreover, this larger donor disc was transplanted into a recipient cornea in which a smaller surface with a poor endothelium needing replenishment remained. For these reasons, substantially less redistribution of endothelial cells to the recipient cornea is to be expected. In the group in which folded donor discs were used, two additional factors may have diminished late redistribution of endothelial cells on to the recipient cornea. The mean recipient age was lower in this group, and the indication for PLK was less often aphakic or pseudophakic bullous keratopathy, and more often Fuchs' endothelial dystrophy. Both these factors may also be associated with diminished redistribution to the recipient cornea.[780]

In our study, endothelial cell loss appeared to be both higher peri-operatively and faster thereafter compared to historical PK groups. Using extrapolated predictions from the regression models an ECD level of 500 cells/mm$^2$ was expected to be reached between 7 and 8 years postoperatively. This level of ECD is often regarded as a threshold below which graft decompensation is

imminent. However, these results may be skewed by small group size, relatively short follow-up, and the use of prototype instruments. As the groups in our studies were the first patients ever operated with PLK, a learning curve may have had substantial influence.

Eyes in the series operated by Terry with comparable techniques (which are called Deep Lamellar Endothelial Keratoplasty or DLEK, instead of PLK) also show an ongoing higher-than-physiologic endothelial cell loss. The level of cell loss however is lower than in our studies, and also lower than in historical PK groups.[1161, 1162] Price, who has operated a large series of patients with a newer PLK-type operation called DSEK (see below), has until now not provided data on endothelial cell loss.[1151,1152]

It is clear that there is an urgent need for more, well-planned, randomized clinical trials comparing the different treatment modalities in corneal transplantation. This does not only apply to the anterior and posterior lamellar keratoplasty techniques discussed above. Rather it should extend to all treatment modalities for corneal disease, including those new emerging techniques that are discussed below in paragraph II of this chapter (Perspectives on future developments). Only with such trials important outcome measures can be compared between any of these new techniques. These outcome measures should in any case include clinical results such as visual acuity, astigmatism, and spherical equivalent, and if possible also contrast sensitivity and/or stray light measurements. Other extremely important outcome measures are graft survival, endothelial cell loss, the occurrence of operative and post-operative complications, and patient satisfaction.



Until recently only one such a comparative trial existed for anterior LK,[1103] but recently the Dutch Lamellar Corneal Transplant Study started, which compares several techniques for both anterior and posterior lamellar keratoplasty to PK.

The results of such trials will be very useful to better define or restrict indications for the different treatment options. For instance, it may be clarified which subgroups of keratoconus eyes will do well with initial anterior LK (either ALTK or deep ALK, see below), which may be treated primarily with intrastromal rings (see below), and for which patients PK or a combined PK-LK variation (see below) should be the first treatment option.
Obviously, especially for posterior lamellar techniques aiming at endothelial replacement, long-term follow-up results on endothelial cell loss are essential. However, even if such trials were to demonstrate that PLK techniques suffered from higher and more rapid ECD-loss and shorter graft survival, there still may be good indications for PLK. Advantages of PLK such as faster visual recovery, fewer follow-up visits to the clinic, and less suture- (removal-) related

complications may be preferable to graft longevity. In elderly or less mobile patients, PLK could still be the technique of choice for treating endothelial disease. In patients with severe dry eyes, where PK is virtually contra-indicated, PLK may even be the only available treatment for endothelial disease. Similar considerations may ensure anterior lamellar techniques a "niche" in the therapeutic armamentarium, even if some of their outcomes fall behind.

Ultimately, preferences of patients and perhaps also preferences and abilities of surgeons will influence the decision for one treatment or another.

## II. Perspective on future developments.

In endothelial imaging, in-vivo confocal microscopy is rapidly gaining ground. Advantages of confocal microscopy over specular microscopy include its capability of imaging the endothelium in edematous corneas. Additionally, confocal microscopy can depict cells in all corneal layers, and is able to quantify light reflectivity in different corneal layers – for instance as a measure for haze.[303] Moreover, many of the optical distortions that affect specular microscopy are not relevant in confocal microscopy (see chapter 6). However, the other considerations regarding reliability, as discussed in part A of this summary, do apply to confocal microscopy as well. It usually is a contact-type examination, which is tolerated less well by patients and has inherent risks for microbial contamination. Therefore non-contact specular microscopy may remain a valuable tool for follow-up examinations on the endothelium, e.g. in patients after ocular surgery.

In dye-enhanced surgery, the search for suitable dyes continues. Dyes that both provide adequate staining and completely lack toxic effects are yet to be found. The dye Coomassie blue a.k.a. brilliant blue G may be a promising addition.[1227]

In the last few years, many new treatment modalities and modifications of existing techniques have emerged for the treatment of corneal diseases.

In this thesis a shift of attention in the treatment of endothelial disease to the selective replacement of the endothelium has been described. The studies described earlier investigated the first clinically applied techniques of PLK. Currently, a newer technique is rapidly surpassing the older types of PLK/DLEK that were described before in popularity, judging by the size of the series in published reports. This new variation is most often called "Descemet stripping and endothelial keratoplasty" (DSEK), and is also known as PLK with Descemethorhexis. In DSEK, the removal of Descemet's membrane with endothelium from the recipient's eye is performed from behind, from

the anterior chamber. Descemet's membrane is removed from within a circumscript area by a combined scraping and tearing off. The reason for the popularity of this specific technique is that it is faster and technically easier than the older PLK/DLEK techniques that used intrastromal dissection. Therefore, it may be more reproducible in the hands of more surgeons. Moreover, in DSEK a smoother wound surface may be expected in the recipient eye than with the older PLK/DLEK types. This, and the fact that the wound interface is at the deepest level possible, i.e. directly anterior to Descemet's membrane, may provide better visual results because of better optical quality of the interface. Even the surgeons who pioneered PLK/DLEK, Melles, Terry, and Price, have generally switched to DSEK except in selected cases.[1149,1151,1152,1228] Recent large DSEK series were promising with regards to visual results and complication rates. However, randomized comparative prospective clinical studies are needed to establish whether any of the posterior techniques has better clinical results, especially when compared to PK.[1151,1152]

In this thesis also a revival of attention for anterior lamellar keratoplasty techniques in the treatment of anterior and ectatic corneal disease has been described. So far we have focused mainly on a deep dissection technique for anterior lamellar keratoplasty, but we did already mention the use of the automated microkeratome in LK. This is an instrument familiar to many refractive surgeons because of its application in the LASIK procedure. The microkeratome may be useful in both anterior and posterior lamellar keratoplasty.[1229] This automated oscillating knife can be used to make lamellar dissections at several chosen depths, both in recipient's and in donor corneas. Usually a very smooth wound surface is obtained which may provide an optical superior interface. Also, microkeratome cutting is faster than manual lamellar dissection. Several authors have applied it in anterior lamellar keratoplasty series.[1092,1215,1216] This technique for anterior LK is also known as Automated Lamellar Therapeutic Keratoplasty. It has been shown to provide promising refractive and visual results, although it is not yet clear whether they compare favourably to other lamellar techniques or to PK. Baring Descemet's membrane, as with the 'big bubble technique', may provide better post-operative visual acuity than ALTK.[1216, 1230,1231] Furthermore, ALTK is not applicable in very steep or thin corneas, or in those with an irregular surface.

In posterior keratoplasty, the microkeratome may especially facilitate and speed up the preparation of the donor posterior disc. The combination of DSEK and the use of the automated microkeratome has been dubbed DSAEK.[1232]

The last few years a new type of photodisruptive laser, the femtosecond ND: Yag laser, has emerged as an alternative to the automated microkeratome. A great number of ultra-short pulses is used to create a dissection plane within a

cornea. Similar to the microkeratome, with this laser very fast and very precise lamellar dissections can be performed. An advantage over the microkeratome is that dissections and incisions can be performed not only horizontally, but also vertically, in a stepped fashion, or even obliquely. This instrument is not only suitable for LASIK and anterior and posterior LK, but also for PK, and PK with stepped or lamellar wound configurations, and for the preparation of tunnels for intracorneal ring segments (see below).[1098,1233-1235] In refractive surgery femtosecond lasers are rapidly gaining ground as they are used for LASIK flap dissection.[1236-1240]

As an alternative to either posterior or anterior LK, new techniques of PK with lamellar wound configuration aspects have also been proposed in the last few years. These combine some of the advantages of both LK and PK. The concepts of these techniques are not new, as most of them have been developed in the middle of the last century.[1061,1241] A combination of the availability of better instruments and microscopes, and an interest in resolving some of the disadvantages of conventional PK has led to their revival. Among the currently more often used techniques is the reversed mushroom PK, a.k.a. "nut-and-bolt PK, a.k.a. "top-hat" PK. Here, the vertical PK wound has a stepwise configuration with the posterior diameter of the graft being larger than the anterior diameter. This technique is foremost used in patients with endothelial disease, as it provides transplantation of a large number of good donor endothelial cells into the recipient. The stepped incision provides a mechanically more stable wound configuration than after PK. Less and less deep sutures are needed, which furthermore may be removed earlier. This supposedly diminishes suture-(removal-)related complications such as wound dehiscence, infection, and high astigmatism. Moreover, in contrast to lamellar keratoplasty, there is no wound interface in the visual axis, which may improve visual acuity.[1108,1242] Similarly, an anterior mushroom PK with a larger anterior diameter and a smaller posterior diameter may be a valuable alternative treatment for keratoconus. This technique preserves more of the recipient's healthy endothelium, and may provide low post-operative astigmatism, while it does not produce a wound interface in the visual axis.[1061] Combinations of lamellar dissections with the microkeratome and deep penetrating buttons for the treatment of full thickness opacities have also been dubbed mushroom keratoplasty. They are akin to endothelial keratoplasty using a microkeratome that is an alternative technique for the selective replacement of endothelium.[1243] The femtosecond laser may give several of these techniques an additional boost as the wound configurations can be made more easily.[1242]

In some possible indications for posterior lamellar keratoplasty, caution is warranted to replace only the endothelium. Secondary stromal changes occur in corneas with persisting edema resulting from endothelial decompensation,

such as in longstanding bullous keratopathy. In long-term edematous corneas, stromal hydropic degeneration (necrosis) of keratocytes occurs. In a recent study confocal microscopy was able to quantify that this keratocyte necrosis itself caused significant stromal scatter, additional to the effects of the edema. Therefore, in these corneas the decrease in corneal transparency did not only result from a widened interfibrillary distance caused by edema, as stated in the classic theory. Unfortunately, this keratocyte degeneration is not reversible. Furthermore, decrease of proteoglycans has also been found in such corneas. Thus, in long-standing bullous keratopathy mere endothelial transplantation may not always suffice to obtain corneal transparency. To obtain significant visual improvement in these cases, PK or a variant of PK with a more complex wound configuration may still be necessary.[874,1244]

A recently emerging, non-keratoplasty surgical treatment in contact-lens intolerant patients for corneal ectatic disease, such as keratoconus or pellucid marginal degeneration, is the placement of intrastromal corneal ring segments. This treatment may provide an alternative or postponement for either PK or LK. Intrastromal rings were introduced as a refractive surgical technique to treat low to moderate myopia (see chapter 2, paragraph XIV), but are becoming less and less used for this purpose because of lack of predictability. Their advantages in ectatic disease include a mechanical "straightening" of ectatic corneas, and reversibility of refractive results. After this treatment refractive errors can persist, but these are often more accessible to correction with spectacles or soft instead of rigid contact lenses. Prerequisite for the placement of ring segments is that the corneas are not too steep or thin, and do not have central corneal opacities. Therefore, in selected cases of mild to moderate keratoconus and other corneal ectasias, ring segments (INTACS or Ferrara rings) may be a useful addition to the therapeutic arsenale.[1192,1245-1256] The use of the femtosecond laser may facilitate the making of tunnels that are needed for the insertion of intrastromal corneal ring segments.

The most recently introduced treatment option in corneal ectatic disease is stromal collagen cross-linking. This process involves topical riboflavin application followed by exposure to UV-A radiation. Cross-links between corneal fibrils are formed by riboflavin radical molecules that are induced by the UV-A irradiation. This ensures biomechanical stiffening of the corneal stroma, and the treatment is predominantly aimed at halting progression of ectasia. As yet, only case series have been reported, which indicate good short- to midterm safety and suggest efficacy.[1257-1259] However, whether this treatment is truly effective remains to be proven, by means of long-term, randomized, comparative, prospective studies. Finally, a few words need to be said about the induction of endothelial cell proliferation, as this may be the future approach for the treatment of all endothelial disease. Cultured endothelia may provide the solution to donor

cornea shortage, and either modification of antigen expression, or culturing or inducing proliferation of a patient's own endothelial cells could provide for less immunogenic transplants. The concept of culturing human endothelial cells for the purpose of transplantation is not new.[1114-1117] Later research investigated suitable carriers for the endothelial cells.[1119,1126,1128,1260] Recently, corneal constructs have been manufactured from cultured endothelial cells combined with preserved human corneal stroma.[1126] Even complete corneal constructs with all cellular layers derived from immortalized and cultured human cells (epithelial, stromal, and endothelial cells) have been reported.[1124] Transfection, for instance with SV40 vectors, may be necessary to achieve sufficiently large ECDs in cultured human endothelial cell monolayers that are prepared for transplantation.[132,1124] Some of these monolayers and corneal constructs have been shown to possess adequate endothelial pump and other physiological functions.[132,1124] Current problems include the continuation of cell divisions in transfected cells even when this is not wanted. On the other hand, non-transfected cultured endothelial cell monolayers provide inadequate ECDs and inadequate pump functions.[132,1126] Currently, none of these endeavours have reached the stage of clinical application yet.

As an alternative to transplanting cultured endothelial cells, a future solution to endothelial disease may be the the in-vivo induction into proliferation of the patients' own endothelial stem cells. Although the endothelial stem cells' suspected locus at the posterior limbus seems to be confirmed more and more, the exact identification of the stem cells themselves remains elusive. The stem cells need to be identified first, before the in-vivo induction of endothelial proliferation can be brought to the next level.[132,874,1261,1262] Until these problems are solved, tranplantation of complete donor endothelia, by means of PK, PLK, or technical variations thereof, remains necessary.



## References

See page 287.

# chapter 15

# Samenvatting en Perspectief

# I. Samenvatting in perspectief.

*Hoofdstuk 1* biedt een kort overzicht over hoe de kennis over en onderzoek naar het hoornvliesendotheel zich heeft ontwikkeld. Ook worden de achtergrond en het doel van de onderzoeken in de delen II tot en met IV van dit proefschrift ingeleid.

*Hoofdstuk 2* is een bespreking van de literatuur over het hoornvlies- (cornea-) endotheel. Enkele essentiële punten hieruit worden hier nu samengevat. Het endotheel vormt de binnenste laag van het hoornvlies en is essentieel in het handhaven van een stabiele waterhuishouding in het hoornvlies. Volgens de "Pump-Leak theorie" vormt het endotheel een barrière die wel water en kleine moleculen vanuit de voorste oogkamer het hoornvlies in laat diffunderen, maar niet permeabel is voor grotere moleculen (Leak). Tegelijkertijd pompen de endotheelcellen actief water uit het hoornvlies terug naar de voorste oogkamer (Pump). Deze hydratie-homeostasis is noodzakelijk om het hoornvlies transparant te houden. Als het watergehalte van het hoornvlies te hoog wordt (cornea-oedeem), verliest het zijn helderheid. Menselijke cornea-endotheelcellen hebben een zeer beperkt vermogen tot celdeling. Dit is niet alleen van belang bij de wondgenezing van het endotheel, maar het is ook verantwoordelijk voor het optreden van leeftijdsgebonden endotheelcelverlies. Dit verval in cellen kan worden beschreven met behulp van de endotheelceldichtheid (ECD), de belangrijkste maat in de morfometrie (kwantitatieve vormbeschrijving) van het endotheel. Kwalitatieve en kwantitatieve aspecten van het de vorm van het endotheel kunnen m.b.v. "spiegelmicroscopie" (specular microscopy) worden onderzocht bij levende patiënten (in vivo).

Onderzoeken na staaroperaties (cataractchirurgie) met kunstlens implantatie, en na hoornvliestransplantaties (penetrerende keratoplastiek, PK) deden het vermoeden rijzen dat er na de operatie langdurig verhoogd endotheelcelverlies is. Bij ongecompliceerde cararact-operaties met moderne technieken is het endotheelcelverlies tijdens en vlak na de operatie beperkt, en bovendien lijkt het tempo van verval binnen enkele jaren al weer te normaliseren. Na staaroperaties met kunstlensimplantatie volgens oudere methoden was dit verlies echter veel hoger en bleef het verval ook langer verhoogd. Momenteel wordt daarom de mate van verlies aan endotheelcellen als veiligheids- en kwaliteitsmaatstaf gebruikt bij cataractchirurgie, zeker als het gaat om technische vernieuwingen.

Tot ten minste tien jaar na PK en waarschijnlijk nog langer is er sprake van een verhoogd verlies aan endotheelcellen. Een belangrijke oorzaak voor dit verlies is immunologische afstoting van endotheelcellen. Maar zelfs als er nooit een duidelijke afstotingsreaktie is waargenomen, is het verval in ECD sterker dan

normaal. Op de lange termijn kan dit uiteindelijk leiden tot decompensatie van het transplantaat, waarbij het aanvankelijk heldere transplantaat vertroebelt. De vertroebeling onstaat door zwelling (oedeem) doordat er te weinig endotheelcellen over zijn om de hydratie-homeostase te kunnen handhaven. Dit proces wordt "laat endotheelfalen" (LEF) genoemd. Het is op dit moment nog steeds niet duidelijk wat de oorzaak van dit verhoogde endotheelcelverlies na PK is en hoe lang het verlies hoger dan normaal blijft.

Omdat de ECD zo'n belangrijke maatstaf is, is het belangrijk dat de bepaling betrouwbaar gebeurt. In deel II van dit proefschrift beschrijven we onderzoeken naar vier aspecten van de betrouwbaarheid van een bepaalde techniek van ECD metingen. Alle morfometrische metingen aan het endotheel in deze onderzoeken werden verricht met een Topcon SP2000P non-contact autofocus spiegelmicroscoop (of specular microscope), gekoppeld aan IMAGEnet 2000 endotheel-analyse software.

In *hoofdstuk 3* is aangegeven hoe de vergroting van de SP2000P geijkt (gecalibreerd) dient te worden. De noodzaak van het controleren van de ijking - van welk biomedisch meetinstrument dan ook- is opnieuw aangetoond, toen we bij controles in 4 verschillende opstellingen telkens calibratiefouten aantroffen (niet gepubliceerde gegevens). Deze noodzaak tot controle van de calibratie geldt des te meer als er voor dezelfde soort metingen afwisselend verschillende (typen) instrumenten gebruikt worden.[287,1224] We hebben formules beschreven om de correcte ECDs te berekenen in de voorkomende gevallen waarin incorrecte waarden zijn verkregen met fout gecalibreerde instrumenten. Voorts is in dit onderzoek van verschillende ECD-meetmethoden de validiteit onderzocht met de methode volgens Bland en Altman.[910] We vonden systematische verschillen tussen handmatige tellingen en al de verschillende ECD meetmethodes die in de SP2000P & IMAGEnet 2000 opstelling voorhanden zijn. Deze verschillen waren vrij klein, met uitzondering van bij de volledig geautomatiseerde ECD bepalingen. Bij de semi-automatische ECD meetmethode worden door software getrokken endotheelcelgrenzen eerst handmatig verbeterd voordat de ECD bepaald wordt. Deze semi-automatische ECD-bepaling was de meest valide meetmethode, aangezien hierbij individuele metingen de minste spreiding lieten zien binnen de zogenaamde "limits-of-agreement". Handmatige correctie van automatisch bepaalde celgrenzen blijkt meestal de nauwkeurigheid van ECD-meetinstrumenten te bevorderen.[1224,1225]

Aangezien er een systematisch verschil was tussen deze semi-automatische ECD meetmethode en volledig handmatige tellingen, moet er een correctiefactor gebruikt worden om ECDs die verkregen zijn met beide methodes met elkaar te kunnen vergelijken. Een dergelijke correctiefactor is ook van toepassing als andere resultaten van andere meetmethodes onderling worden vergeleken.

Zoals reeds aangegeven door Bland en Altman volstaat het voor het vergelijken van 2 meetmethoden niet om een correlatie-coëfficiënt te berekenen, omdat hiermee een systematisch verschil niet herkend wordt. Lineaire regressie is voor de vergelijking van meetmethoden ook niet van toepassing, aangezien de uitkomsten van beide methoden onafhankelijke variabelen zijn, en de ene uitkomst niet een functie is van de andere. In sommige onderzoeken kan men echter beide foute methodes tegelijk aantreffen.[313]

Een derde aspect dat van belang is voor de betrouwbaarheid van een meetmethode, is de reproduceerbaarheid. In het onderzoek beschreven in *hoofdstuk 4* werd er een redelijke tot goede reproduceerbaarheid gevonden voor alle morfometrische parameters, inclusief de ECD. De reproduceerbaarheid werd vooral beïnvloed door een relatief geringe herhaalbaarheid (repeatibility), d.w.z. een hoge variabiliteit tussen direct herhaalde steekproeven (sampling variability). Een hoge sampling variability kan worden verminderd door de uitkomsten van meer metingen te middelen. Hierdoor neemt de variantie in de metingen af met de inverse van het aantal metingen.

De reproduceerbaarheid bij wisselende "beoordelaars" (inter-observer reproducibility) was eveneens goed. ECDs die door verschillende beoordelaars zijn verkregen kunnen dus onderling worden vergeleken, mits de beoordelaars dezelfde methodes gebruiken en goed getraind zijn. Onze resultaten t.a.v. de reproduceerbaarheid van ECD metingen met de SP2000P & IMAGEnet 2000 kwamen overeen met die in andere studies. [294,1225]

Verder werd er in hoofdstuk 4 aangetoond dat ECDs in het nasale paracentrale gebied (dat is 3 mm van het centrum aan de nasale zijde) gebruikt kunnen worden in plaats van centrale ECDs, indien er van het centrum geen duidelijke afbeeldingen kunnen worden gemaakt. De centrale ECDs van het andere (contralaterale) oog van een paar kunnen hiervoor ook gebruikt worden, mits er geen verschillen in voorgeschiedenis tussen beide ogen zijn. Desalniettemin moet men hierbij er zich wel van bewust zijn dat er in individuele gevallen toch een aanzienlijk verschil kan bestaan tussen de vervangende en de vervangen waarde.

De resultaten in de *hoofdstukken 3* en *4* tonen aan dat met de SP2000P & IMAGEnet 2000 combinatie betrouwbare centrale ECD metingen verkregen kunnen worden. Als aan de voorwaarden zoals beschreven in deze twee hoofdstukken voldaan wordt, kunnen op betrouwbare wijze ECD-metingen betrouwbaar worden vervolgd bij herhaalde metingen bij dezelfde persoon, en ook betrouwbaar worden vergeleken tussen verschillende personen.

In het onderzoek dat is beschreven in *hoofdstuk 5* werden ECDs vergeleken die eerst in vivo waren gemeten met de SP2000P, en vervolgens in dezelfde

cornea's (na excisie tijdens een transplantatie) opnieuw in vitro bepaald werden met de gebruikelijke lichtmicroscopische meetmethode van de hoornvliesbank. We vonden dat de specular microscopy metingen systematisch en constant 140 cellen/mm$^2$ hoger waren dan die van de corneabank. Dit verschil heeft consequenties voor vervolgonderzoeken na hoornvliestransplantatie, waarbij donor-ECDs vergeleken worden met specular microscopy ECD metingen. Dit is bijvoorbeeld het geval in de onderzoeken beschreven in hoofdstukken 12 en 13 van dit proefschrift. In dergelijke onderzoeken zou van nu af aan ofwel 140 cellen/mm$^2$ bij de donor ECD moeten worden opgeteld, ofwel moeten worden afgetrokken van de SP2000P-ECD. Indien er een andere specular of confocal microscope wordt gebruikt, moet er opnieuw worden nagegaan of er een systematisch verschil bestaat met donor ECDs, op een vergelijkbare manier als in ons onderzoek.

Dat het systematisch verschil tussen de meetmethodes een constant en absoluut karakter had, was een onverwachte bevinding. De meest waarschijnlijke verklaringen voor een eventueel verschil zouden eigenlijk allemaal moeten leiden tot een relatief verschil, d.w.z. een verschil dat evenredig toeneemt met de ECD-waarde zelf. Men zou dus kunnen veronderstellen dat het verschil in werkelijkheid dan ook een relatief karakter had, maar dat dit gemaskeerd wordt door andere factoren. Een van deze maskerende factoren wordt beschreven in hoofdstuk 5. Het lineaire regressiemodel dat in de corneabank gebruikt wordt om de ECD te bepalen heeft de beste "fit" - en levert dus de meest betrouwbare waarden op - bij gemiddelde ECDs. Het model resulteert echter in overschatte ECDs in de lagere regionen en onderschat de ECDs als de waarden veel hoger zijn.



In de "Discussion" paragrafen in de *hoofdstukken 5* en *6* worden diverse optische factoren beschreven die een proportioneel verschil, dat dus evenredig toeneemt met de ECD, kunnen verklaren. Een andere mogelijke verklaring voor een dergelijk proportioneel verschil kan worden gevonden in hoofdstuk 3. In dat onderzoek werd een systematisch verschil van vergelijkbare grootte gevonden tussen een semi-automatische ECD meetmethode en handmatige tellingen op specular microscopy foto's. Het verschil in ECDs zou gekoppeld kunnen zijn aan het inherente methodologische verschil tussen zgn. "fixed-frame" en "variable-frame" tellingen. Bij fixed-frame tellingen wordt het aantal cellen geteld dat binnen een opppervlak van vaste afmetingen ligt, terwijl bij variable-frame bepalingen de oppervlakte gemeten wordt van een bepaald aantal cellen (de ECD is dan de inverse van het gemiddelde oppervlak van die cellen). Bij variable-frame tellingen kan m.n. in kleine steekproeven een onderschatting van het celoppervlak optreden, hetgeen resulteert in overschatting van de ECD.[279,280] Omdat er echter geen proportioneel verschil werd gevonden tussen in-vitro en in vivo ECDs, blijft het onduidelijk of en zo ja welke van al deze mogelijke

verklaringen van toepassing zijn..De interactie tussen diverse verklarende factoren zou van invloed kunnen zijn.

In *hoofdstuk 6* wordt inderdaad aangetoond dat de verscheidene optische vertekeningen die bij non-contact specular microscopy een rol spelen, vaak een tegengesteld effect hebben. We vonden dat alle optische vertekeningen samen geen belangrijke meetfouten veroorzaakten, noch in met ogen met een normale hoornvliesdikte en –kromming noch in ogen met keratoconus. Dit was echter alleen het geval als de meetfout die veroorzaakt werd door perspectivistische vertekening als gevolg van een schuine observatiehoek, werd tenietgedaan. In de SP2000P is dit blijkbaar het geval (Pierre Gelissen, Topcon Europe, persoonlijke mededeling, 2004). Als de perspectivistische vertekening niet wordt gecompenseerd neemt de meetfout namelijk toe met de cosinus van de observatiehoek, en kan zodoende aanzienlijke waarden aannemen.

De bevindingen in de onderzoeken in deel II van dit proefschrift zijn niet alleen van belang voor gebruikers van de SP2000P, maar ook voor onderzoekers die andere apparaten en analysemethoden en –technieken gebruiken voor de morfometrie van het cornea-endotheel. Onze methodologische overwegingen, onderzoeks- en statistische methoden, en interpretaties van gegevens kunnen ook nuttig zijn voor degenen die andere specular of confocal microscopen gebruiken. De correctieformules uit hoofdstuk 3 zijn vrij algemeen toepasbaar in geval van incorrect geijkte of ingestelde vergrotingswaarden. De natuurkundige formules in hoofdstuk 6 zijn eveneens op vele typen specular microscopes van toepassing.

De onderzoeken in deel III van dit proefschrift gaan over de effecten van trypaan blauw op het cornea-endotheel. De vitaalkleurstoffen trypaan blauw (TB) en indocyanine groen (ICG) worden binnen de oogheelkunde al heel lang voor verscheidene doeleinden toegepast. Sinds 1998 worden beide kleurstoffen bovendien tijdens oogoperaties gebruikt voor het aankleuren, en daardoor beter visualiseren, van membraneuze strucuren. Voor deze nieuwere waardevolle toepassingen is in de literatuur inmiddels de term "dye-enhanced ocular surgery" in zwang. In *hoofdstuk 7* wordt besproken hoe deze deze kleurstoffen van oudsher in de oogheelkunde en op andere terreinen werden toegepast. Voorts komt de ontwikkeling van de intra-operatieve toepassingen aan de orde. Verder wordt er een aantal onderzoeken besproken die steeds duidelijker maken dat beide kleurstoffen toxische eigenschappen kunnen hebben voor cellen in het oog. De mate van toxiciteit blijkt afhankelijk van de concentratie van en de duur van de blootstelling aan de kleurstof. In 1998 en 1999 was de meeste kennis over toxische effecten van TB op het cornea-endotheel gebaseerd op ervaringen met TB in hoornvliesbanken en op enkele

onderzoeken. De onderzoeken zoals beschreven in hoofdstuk 8 en 9 werden gestart om de toxiciteit van TB uitgebreider in kaart te brengen, en om de veiligheid van het gebruik van TB tijdens cataractchirurgie aan te tonen.

Het klinische onderzoek in *hoofdstuk 8* toont aan dat het gebruik van TB (0.06 % oplossing) tijdens cataractchirurgie veilig is voor het cornea-endotheel. Eén jaar na staaroperaties aan beide ogen bleek dat het endotheelcelverlies in ogen die geopereerd waren met gebruik van TB niet verhoogd was ten opzichte van de contralaterale ogen die zonder TB waren geopereerd (de controle-ogen).

Het experimentele onderzoek uit *hoofdstuk 9* bevestigt het bestaan van een concentratie- en blootstellingsduurafhankelijke toxiciteit van TB voor cellen in het hoornvlies. Uit de experimenten bleek dat het soort vehikel van de kleurstof van essentieel belang was voor de mate waarin toxische effecten werden veroorzaakt. We konden aantonen dat concentraties van en blootstellingsduur aan TB zoals die momenteel toegepast worden in de Cornea Bank Amsterdam en tijdens cataractoperaties, geen toxisch niveau bereikten. Maar tegelijkertijd werd wel duidelijk dat men zeer voorzichtig moet zijn met hogere concentraties van TB en/of een langere blootstellingsduur.

Dierexperimenteel onderzoek heeft laten zien dat TB teratogeen kan zijn. De dosis die in mensen wordt gebruikt tijdens cataractchirurgie is ca. 10.000 keer lager dan de systemische teratogene dosis in proefdieren. Bovendien wordt tijdens de operatie direct na het aankleuren van het kapsel het overgrote deel van de kleurstof verwijderd. Zodoende zal het grootste deel van de toegepaste hoeveelheid TB nooit in de bloedsomloop komen. Desalniettemin dient men te overwegen om TB niet bij cataractoperaties bij zwangere vrouwen of heel jonge kinderen te gebruiken. Verder moet OK- en corneabankpersoneel beschermende maatregelen nemen (b.v. handschoenen) om herhaalde blootstelling aan kleine doses TB te voorkomen.

De mogelijke toxiciteit van ICG voor het netvlies en retinale pigmentepitheel heeft ook tot veel discussie geleid. "Dye-enhanced surgery" lijkt echter bijna niet meer weg te denken uit de huidige chirurgische praktijk. Men blijft daarom zoeken naar alternatieve kleurstoffen met dezelfde of zelfs betere kleurende eigenschappen, maar met minder toxiciteit.

In deel III, in *hoofdstuk 10* is de geschiedenis van de ontwikkeling van hoornvliestrans-plantatie beschreven. In het bijzonder wordt er ingegaan op de ontwikkeling van nieuwe technieken van lamellaire hoornvliestransplantatie (lamellaire keratoplastiek, LK). Bij LK worden alleen de aangedane lagen van het hoornvlies vervangen, in tegenstelling tot de penetrerende keratoplastiek (PK) waarbij het hoorvlies over de volledige dikte wordt getransplanteerd. Er is

een duidelijke opleving in de belangstelling voor LK merkbaar in de laatste 8 jaar.[1226] In hoofdstuk 10 wordt uiteengezet dat er op theoretische gronden een duidelijke rationale is voor zowel de anterieure als de posterieure lamellaire keratoplastiek (PLK). De theoretische voordelen van beide technieken op PK omvatten een sneller herstel van de gezichtsscherpte, een lager postoperatief astigmatisme, en minder wond- of hechtinggerelateerde postoperatieve complicaties. In veel gevallen waarbij tot nu toe PK de algemeen gangbare behandeling was, zou daarom anterieure of posterieure lamellaire hoornvlies transplantatie wel eens de operatietechniek van eerste keus kunnen worden. Het is wel zo dat de genoemde theoretische voordelen dan ook daadwerkelijk in de praktijk van toepassing moeten blijken te zijn. Bovendien zou de overleving van het transplantaat minstens zo goed moeten zijn als na PK. Transplantaatoverleving na PK hangt vooral af van het al dan niet optreden van immunologische afstoting van de donor-endotheelcellen, en van het al eerder genoemde late endotheelfalen (LEF) dat niet door afstoting van endotheelcellen veroorzaakt wordt.

In anterieure lamellaire keratoplastiek worden alleen de voorste lagen van het hoornvlies vervangen en blijft het endotheel van de ontvanger op zijn plaats. Een afstotingsreaktie die leidt tot verlies van het transpantaat is hierdoor nagenoeg uitgesloten. Omdat er bij LK in feite alleen aan de buitenkant van het oog geopereerd wordt, zou ook het niet-afstotingsgerelateerde LEF op de lange termijn voorkomen kunnen worden.[231,310] Ons onderzoek naar endotheelverlies na diepe anterieure lamellaire keratoplastiek (DALK, Melles Techniek) wordt beschreven in *hoofdstuk 11*. De resultaten van dit onderzoek laten zien dat het postoperatieve endotheelcelverlies aanvankelijk duidelijk verhoogd is, maar al vrij snel weer daalt en het fysiologische, leeftijdsafhankelijk niveau nadert.

Ons artikel was net voor publicatie aangeboden toen Armitages belangrijke artikel over bi-exponentiële modellen voor endotheelcelverval werd gepubliceerd.[231] Onze resultaten doen vermoeden dat een dergelijk vervalpatroon ook van toepassing is bij de ogen in ons onderzoek. Waarschijnlijk zou een dergelijk model dus ook wel aan de hand van ons onderzoek geconstureerd kunnen worden, mits er langer vervolgonderzoek met meer meetmomenten per oog zou worden uitgevooerd. Tijdens DALK-operaties vindt er in principe geen directe chirurgische manipulatie van het endotheel plaats. Andere factoren moeten dus het aanvankelijke verhoogde endotheelcelverlies verklaren. Mechanische vervorming en wrijving bij de diepe lamellaire dissectie net boven/voor de membraan van Descemet en het endotheel kan waarschijnlijk endotheelcelverlies veroorzaken. Microperforaties naar de voorste oogkamer die soms tijdens de diepe lamellaire dissectie onbedoeld optreden, beschadigen het endotheel ter plaatse. Een luchtbel die om technische

redenen tijdens de operatie in de voorste oogkamer is kan mogelijk ook tot verlies aan endotheelcellen leiden.[535,536,899]

In tegenstelling tot na anterieure lamellaire transplantatie kan er na posterieure lamellaire keratoplastiek (PLK) wel degelijk immunologische afstoting van endotheelcellen optreden. Verder is na PLK ook het niet-afstotingsgerelateerde endotheelcelverlies, zowel peroperatief als op de langere termijn, aanzienlijk. Elk van deze drie factoren heeft een negatief effect op de transplantaatoverleving – zie hoofdstuk 2 paragraaf XVI. Het is daarom van groot belang de mate en het patroon van endotheelcelverlies na PLK te onderzoeken. Uit het prospectieve onderzoek beschreven in *hoofdstuk 12* bleek dat 3 jaar na de PLK-operatie het endotheelcelverlies licht verhoogd was ten opzichte van het verlies in ECD zoals dat vanuit de literatuur bekend is na PK.

Ten aanzien van deze resultaten moeten er wel enkele kanttekeningen gemaakt worden. Ten eerste waren de donor-endotheelceldichtheden (ECDs) in dit onderzoek die van het andere, contralaterale oog. De achterste lamellaire transplantaten werden op de operatiekamer uit volledige, intacte bulbi geprepareerd. Daarom konden er in de hoornvliesbank geen ECDs van het donoroog zelf bepaald worden (zie hoofdstuk 2 paragraaf VIII). In het onderzoek van hoofdstuk 4 is aangetoond dat er meestal geen significante verschillen bestaan tussen de ECDs van beide ogen van een persoon. In individuele gevallen kunnen er desalniettemin aanzienlijke verschillen tussen beide ogen zijn. Ten tweede zijn de donor-ECDs met behulp van in-vitro licht-microscopische methoden bepaald, zoals gebruikelijk in de hoornvliesbank, terwijl de vervolg ECDs gemeten zijn met behulp van non-contact specular microscopy. Eigenlijk dient men hierbij rekening te houden met de resultaten van hoofdstuk 5, en dat houdt in dat het verlies na PLK nog gemiddeld 140 cellen/mm$^2$ groter is dan gemeten. Ten derde waren we aan de hand van de data in het onderzoek van hoofdstuk 12 nog niet in staat een bi-exponentiëel ECD-verval model te construeren, waarschijnlijk omdat onze follow-up met 3 jaar hiervoor nog te kort was. (Armitage, persoonlijke mededeling, 2004).

Om deze reden hebben we nog aanvullende ECDs gemeten in dit patiëntencohort. Met aanvullende gegevens, die we op cross-sectionele wijze verkregen hebben, konden we zoals *hoofdstuk 13* laat zien wel bi-exponentiële modellen maken. In deze modellen blijkt de snelle fase van het endotheelcelverlies betrekking te hebben op de eerste uren na de operatie. Deze nadruk op de eerste postoperatieve uren is waarschijnlijk mede een gevolg van het "over-fitten" van een regressiemodel. Ook nu wordt dit weer veroorzaakt door een relatief korte follow-up (Armitage, persoonlijke mededeling, 2006). Toch zijn de resultaten wel interessant, omdat ze aangeven dat een grotere hoeveelheid endotheelcellen verloren gaat in de eerste

postoperatieve uren. Dit is zelfs significant meer wanneer de donorlamel gevouwen in het ontvangeroog wordt ingebracht, ten opzichte van het ongevouwen inbrengen. Verder is het opmerkelijk dat met een gevouwen donorlamel het late endotheelcelverlies langzamer bleek te zijn dan met een ongevouwen lamel. Als verklaring hiervoor kan aangevoerd worden dat de diameter van de gevouwen donorlamel groter was. Daarom droeg deze in absolute aantallen meer endotheelcellen en had een hogere ECD aan de rand. Het overgebleven oppervlakte op het ontvangerhoornvlies waar endotheelcellen vervangen moesten worden was daarentegen kleiner. Deze factoren maken het aannemelijk dat er op de langere termijn aanzienlijk minder redistributie van donorendotheelcellen naar het ontvangerhoornvlies plaatsvond. Twee andere factoren kunnen in deze groep waarin gevouwen lamellen werden gebruikt dienen als aanvullende verklaring voor verminderde endotheelcelredistributie. De gemiddelde leeftijd van deze ontvangers was lager, en ze hadden minder vaak een aphake of pseudophake bulleuze keratophatie. Waarschijnlijk is hierdoor de (perifere) ontvanger-ECD relatief hoger.[780]

In onze onderzoeken bleek het endotheelcelverlies hoger te zijn rond de PLK-operatie, en een hoger tempo aan te houden in de fasen daarna, in vergelijking tot gegevens die bekend zijn uit de literatuur over endotheelverlies rond en na PK. Door middel van extrapolatie van onze regressiemodellen konden we voorspellen dat gemiddeld 7 tot 8 jaar na PLK het niveau van 500 cellen/mm$^2$ bereikt zou gaan worden. Dit ECD-niveau wordt meestal aangehouden als de kritische grens voor decompensatie van het transplantaat. Het zou kunnen dat de resultaten in onze onderzoeken vertekend zijn doordat de groepen behandelde ogen klein zijn, de follow-up nog relatief kort is, en doordat er prototype instrumenten zijn gebruikt. Het betrof hier bovendien de eerste groep patiënten die ooit geopereerd is met een PLK techniek, en daarom kan het heel goed zijn dat er ook nog sprake is van een leercurve van de chirurg wat betreft de operatietechniek.

In de groep ogen die door Terry met vergelijkbare technieken geopereerd is (hij noemt deze technieken Deep Lamellar Endothelial Keratoplasty, of DLEK, in plaats van PLK) is er ook sprake van een hoger dan fysiologisch endotheelcelverlies. Bij Terry is het verlies echter lager dan in onze studies en ook lager in vergelijking tot PK onderzoeken uit de literatuur.[1161, 1142] Price heeft een grote groep patiënten geopereerd met een nieuwere PLK-achtige techniek, DSEK genaamd (zie hieronder). Hij heeft tot nu toe geen gegevens m.b.t. endotheelcelverlies gepubliceerd.[1151,1152]

Het is duidelijk dat er een grote en dringende behoefte bestaat aan goed opgezette, gerandomiseerde klinische onderzoeken waarin de verschillende

behandelingsmodaliteiten voor hoornvliesaandoeningen met elkaar vergeleken worden. Dit geldt niet alleen voor de anterieure en posterieure LK technieken zoals hierboven besproken, maar ook voor alle andere recente nieuwe chirurgische behandelingen – zie paragraaf II van dit hoofdstuk (Toekomstperspectieven). Alleen met dit soort onderzoeken kunnen uitkomsten van deze operatietechnieken echt goed vergeleken worden. Het gaat hierbij om klinisch relevante zaken zoals visus, astigmatisme, sferisch equivalent. Zo mogelijk moeten ook metingen van contrastgevoeligheid- en strooilicht-metingen vergeleken worden Verder zijn gegevens zoals transplantaatoverleving, endotheelcelverlies, het optreden van operatieve en postoperatieve complicaties, en patiënt-tevredenheid van het grootste belang.

Tot dusverre bestond er slechts één dergelijk onderzoek en dat vergeleek anterieure LK met PK.[1103] Onlangs is echter de Dutch Lamellar Corneal Transplant Study in ons land van start gegaan, en dit multi-center onderzoek vergelijkt prospectief en gerandomiseerd verschillende technieken voor zowel anterieure als posterieure LK met PK.

Resultaten van dergelijke prospectieve vergelijkende onderzoeken zijn erg nuttig om indicatiegebieden voor de verschillende behandelopties te definiëren en aan te scherpen. Zo kan er bijvoorbeeld duidelijk worden welke subgroepen van ogen met keratoconus het beste behandeld moeten worden met anterieure LK (hetzij ALTK, hetzij DALK, zie beneden), welke ogen aanvankelijk behandeld kunnen worden met intracorneale ringsegmenten (zie beneden), en voor welke patiënten PK of een variant die aspecten van PK en LK combineert (zie beneden) de eerst aangewezen methode is.



Het is duidelijk dat lange-termijn gegevens over endotheelcelverlies van zeer groot belang zijn in de evaluatie van technieken waarbij het endotheel selectief wordt vervangen, zoals in PLK. Gezien bovenstaande bevindingen zouden zulke prospectieve onderzoeken kunnen aantonen dat na PLK-achtige technieken het endotheelcelverlies groter en sneller is in vergelijking met andere technieken, resulterend in een kortere transplantaatoverleving. Toch zouden er nog steeds goede indicaties voor PLK kunnen zijn. Voordelen van PLK, zoals sneller herstel van de gezichtsscherpte, minder polikliniekbezoeken, en minder hechting-gerelateerde complicaties zouden kunnen opwegen tegen langere transplantaatoverleving. Dit zou voor oudere of minder mobiele patiënten PLK nog steeds tot de behandeling van eerste keus kunnen maken voor endotheelaandoeningen. In geval van ernstig droge ogen is PK relatief gecontra-indiceerd en zou PLK wel eens de enige keus voor het behandelen van endotheelaandoeningen kunnen zijn. Vergelijkbare overwegingen kunnen ook anterieure LK technieken verzekeren van een "niche" in het behandelingsrepertoire, zelfs als sommige uitkomsten wat achter zouden blijven.

Uiteindelijk zal vooral de voorkeur van de individuele patiënt, in combinatie met de mogelijke voorkeur, vaardigheden, of voorzieningen van de oogarts met wie de patiënt een behandelingsrelatie heeft bepalen welke behandelmethode er gebruikt wordt.

## II. Toekomstperspectief.

Het cornea-endotheel wordt de laatste tijd in toenemende mate onderzocht en afgebeeld met behulp van in-vivo confocale microscopie. Een van de belangrijkste voordelen ten opzichte van spiegelmicroscopie of specular microscopy is dat confocale microscopie het endotheel ook in beeld kan brengen in troebele, oedemateuze hoornvliezen. Daarnaast kan confocale microscopie ook gebruikt worden om cellen en structuren in alle andere lagen van het hoornvlies in beeld te brengen. Bovendien kan men met deze techniek de reflectiviteit van licht, en daarmee bijvoorbeeld een fenomeen als "haze", in de verschillende hoornvlieslagen kwantificeren.[303] De meeste van de optische vertekeningen die in specular microscopy een rol spelen zijn niet of van onderschikt belang in confocale microscopy (zie hoofdstuk 6). De andere aspecten van betrouwbaarheid zoals die in deel A van dit hoofdstuk zijn samengevat zijn dat wel. Confocale microscopie is meestal een contact-type onderzoek dat enige tijd vergt. Het is daarom minder prettig voor patiënten en draagt bovendien het risico van contaminatie met micro-organismen. Specular microscopy kan daarom wellicht een rol blijven spelen bijvoorbeeld bij vervolgonderzoeken aan het endotheel na oogoperaties.

De zoektocht naar geschikte kleurstoffen voor het adjunctieve gebruik tijdens oogoperaties is nog niet voltooid. Tot dusverre zijn er nog geen kleurstoffen gevonden die wel een goede kleuring kunnen bewerkstelligen maar in het geheel geen toxische effecten veroorzaken. De kleurstof Coomassie blauw, ook bekend als Briljant Blauw G, vormt mogelijk een waardevolle aanwinst in dit opzicht. [1227]

De laatste jaren zijn er opmerkelijke ontwikkelingen geweest in de behandeling van hoornvliesaandoeningen: nieuwe behandelingsmodaliteiten zagen het licht, en bestaande technieken werden aangepast.

In dit proefschift is eerder al beschreven hoe bij de behandeling van aandoeningen van het hoornvliesendotheel de aandacht is verschoven van PK naar de selectieve vervanging van dit endotheel. De in dit proefschrift weergegeven artikelen beschreven de eerste technieken voor PLK die klinisch zijn toegepast. Op dit moment lijken deze "oudere" varianten van de PLK/DLEK technieken al weer enigszins achterhaald en verschuift de aandacht naar een

nieuwere techniek, als men tenminste afgaat op de grootte van de series patiënten in de literatuur die hiermee worden behandeld. De nieuwe variant wordt "Descemet stripping and endothelial keratoplasty" (DSEK) genoemd, en staat ook wel bekend als PLK met Descemetorhexis. Deze techniek is zo snel zo populair geworden, omdat ze sneller en gemakkelijker uit te voeren is dan de oudere PLK/DLEK varianten die nog gebruik maakten van intrastromale dissectie. Hierdoor kan ze door meer chirurgen worden uitgevoerd en leidt ze waarschijnlijk ook tot meer reproduceerbare resultaten. Bovendien mag er aangenomen worden dat er in DSEK een egaler wondbed gecreëerd wordt. Dit, en het feit dat de wond "interface" zich op het diepst mogelijke niveau, namelijk direct boven Descemet bevindt, zouden wel eens een gunstig effect kunnen hebben op de optische kwaliteit en daardoor op de postoperatieve gezichtsscherpte. Ook de pioniers van de oudere varianten van PLK/DLEK (Melles, Terry en Price) zijn in het algemeen overgeschakeld op DSEK.[1148, 1150,1151,1227] Recente publicaties met grote series patiënten die behandeld zijn met DSEK lieten veelbelovende visuele resultaten zien, en bemoedigend lage complicatie-percentages. Er zijn echter vergelijkende, gerandomiseerde prospectieve onderzoeken nodig om vast te stellen of, en zo ja welke van de posterieure hoornvliestransplantaties de betere resultaten heeft, ook in vergelijking met PK.[1150,1151]

De hernieuwde populariteit van anterieure lamellaire keratoplastiek voor de behandeling van cornea-ectasieën en anterieure cornea-afwijkingen is ook al eerder aan de orde gekomen. Tot dusverre hebben we ons met name gericht op een techniek voor diepe anterieure lamellaire keratoplastiek die met behulp van diepe instrastromale dissectie wordt uitgevoerd, maar het gebruik van de automatische microkeratoom bij LK is ook al eerder aangekaart. Dit is een instrument dat tot dusverre zijn belangrijkste toepassing had bij LASIK, en daarom vooral al bekend is bij refractiechirurgen. De automatische microkeratoom kan daarnaast ook in zowel anterieure als posterieure lamellaire keratoplastiek zijn nut hebben.[1228] Met behulp van het oscillerende mes kan men op diverse van tevoren gekozen dieptes lamellaire dissecties verrichten in het hoornvlies van de ontvanger en in de donorcornea. Het dissectievlak van een microkeratoom is meestal erg egaal, wat voordelig is voor de optische kwaliteit van de wond. Daarnaast kan een lamellaire dissectie sneller met de microkeratoom dan handmatig uitgevoerd worden. Verschillende onderzoekers hebben gepubliceerd over series patiënten bij wie ze anterieure LK met behulp van de microkeratoom hebben uitgevoerd. [1092,1215,1216] Deze techniek staat ook wel bekend als Automated Lamellar Therapeutic Keratoplasty (ALTK). Er zijn veelbelovende resultaten mee behaald wat betreft de postoperatieve gezichtsscherpte en refractie. Het is echter nog niet duidelijk of deze gunstig afsteken ten opzichte van de andere technieken voor lamellaire chirurgie of PK. Sommige auteurs rapporteren dat een lamellaire dissectie waarbij de

membraan van Descemet vrijgeprepareerd wordt, zoals met de 'big bubble techniek', een betere postoperatieve gezichtsscherpte oplevert dan ALTK. [1216,1230,1231] De ALTK techniek kan bovendien niet toegepast worden op cornea's die heel dun of steil zijn of een onregelmatig oppervlak hebben.

Het nut van het gebruik van de microkeratoom bij posterieure lamellaire keratoplastiek ligt vooral in het vlugger prepareren van de posterieure donorlamel. Een dergelijke combinatie van DSEK waarbij de automatische microkeratoom gebruikt wordt voor de donorcornea is al wel DSAEK genoemd. [1232]

In de laatste jaren is er een laser op de markt gekomen die een alternatief vormt voor de automatische microkeratoom: de femtosecond ND:Yag laser. Met deze fotodisruptieve laser kan door middel van een groot aantal ultrakorte laserpulsen een snijvlak in het hoornvlies gemaakt worden. Evenals met de automatische mikrokeratoom kan men dus zeer snel en precies een lamellaire dissectie uitvoeren. Een belangrijk voordeel van de femtosecond laser is dat dissecties en incisies in alle richtingen gemaakt kunnen worden; horizontaal, verticaal, trapvormig, en zelfs schuin. Daarom is deze laser geschikt voor LASIK en anterieure en posterieure LK, maar ook voor PK en PK-varianten met getrapte of lamellaire wondconfiguraties (zie hieronder). [1098,1233-1235] De femtosecond laser wordt al steeds meer toegepast in de refractiechirugie, voor het snijden van LASIK-flappen.[1236-1240]

Behalve voor posterieure of anterieure LK kan er de laatste tijd ook gekozen worden voor PK met diverse lamellaire wondconfiguraties. In deze varianten worden sommige voordelen van PK met andere voordelen van LK gecombineerd. De concepten hiervoor zijn niet nieuw maar al in het midden van de twintigste eeuw bedacht. [1061,1241] Ze zijn de laatste jaren echter in populariteit gestegen doordat er steeds meer interesse was ontstaan in het aanpakken van een aantal nadelen van 'conventionele' PK, in combinatie met het beschikbaar komen van betere instrumenten en operatiemicroscopen. Eén van de meest gebruikte technieken is op dit moment de omgekeerde mushroom PK, in de literatuur ook wel benoemd als 'nut-and-bolt' PK, en 'top-hat' PK. De normaal verticale PK-wond heeft hierbij een getrapte configuratie, waarbij de diameter van het achterste deel van het getransplanteerde hoornvlies groter is dan die van het voorste deel. Deze techniek wordt vooral gebruikt bij de behandeling van patiënten met een endotheliaal probleem, omdat hiermee een grote hoeveelheid goede donorendotheelcellen in het ontvangende oog kan worden ingebracht. De getrapte incisie levert een mechanisch stabielere wond op dan bij PK, waardoor er minder en ook minder diepe hechtingen nodig zijn. Die kunnen bovendien vroeger dan gebruikelijk worden verwijderd. In theorie zouden hierdoor er minder postoperatieve complicaties moeten zijn die te maken hebben met hechtingen of het

verwijderen daarvan, zoals wonddehiscentie, infectie, en hoog astigmatisme. Een belangrijk voordeel ten opzichte van LK is dat er geen wond-interface in de visuele as is, waardoor de postoperatieve gezichtscherpte mogelijk beter is.[1108,1242] Op vergelijkbare wijze zou een voorste mushroom PK, met een grotere anterieure en een kleinere achterste transplantaatdiameter voordelen kunnen hebben als chirurgische optie voor de behandeling van bijvoorbeeld keratoconus. Deze techniek zou een groter dan gebruikelijk deel van het normale, gezonde endotheel van het ontvangeroog kunnen sparen, terwijl tegelijkertijd het postoperatieve astigmatisme relatief laag is en er geen wond-interface in de visuele as gevormd wordt.[1241] Een anterieure lamellaire dissectie met de automatische microkeratoom gecombineerd met een dieper penetrerend transplantaat is ook wel 'mushroom' keratoplastiek genoemd. Dit is toegepast voor troebelingen die zich over de gehele dikte van het hoornvlies uitstrekten, en lijkt qua techniek sterk op de endotheliale keratoplastiek met behulp van de microkeratoom, die gepropageerd is voor selectieve endotheeltransplantatie.[1243] De komst van de femtosecond laser kan voor de populariteit van de meeste van deze technieken, waarbij PK met lamellaire dissectie of wondconstructie gecombineerd wordt, een stimulans vormen. [1242]

Het is belangrijk dat men op zijn hoede is met alleen het selectief vervangen van het endotheel in sommige gevallen. Langdurig cornea-oedeem ten gevolge van endotheeldecompensatie leidt tot secundaire veranderingen in het stroma. Dit kan men bijvoorbeeld aantreffen bij al geruime tijd bestaande bulleuze keratopathie. In dergelijke hoornvliezen treed er een hydropische degeneratie, een necrose van keratocyten op. Van deze keratocyten-necrose is recent aangetoond met behulp van confocal microscopy dat het op zichzelf al aanzienlijke verstrooiing van licht veroorzaakte. Dit stond los van de lichtverstrooiing die door het oedeem veroorzaakt werd. Hieruit kan worden opgemaakt dat het verlies aan helderheid in dergelijke hoornvliezen niet alleen voorkomt uit een vergrote afstand tussen de collageenfibrillen, wat volgens de klassieke theorie de oorzaak is van het transparantieverlies bij cornea-oedeem. Helaas is de degeneratie van keratocyten onomkeerbaar. Ook is er in dergelijke hoornvliezen een vermindering van het gehalte van proteoglycanen gevonden. Men dient zich er dus van bewust te zijn dat de selectieve vervanging van het endotheel bij bulleuze keratopathie wel eens niet in alle gevallen afdoende zou kunnen zijn om weer een helder hoornvlies te krijgen. In dergelijke gevallen zou men toch kunnen kiezen voor PK of een variant daarop met een lamellaire wondconfiguratie.[874,1246]

Recent is er voor ectatische afwijkingen van het hoornvlies, zoals keratoconus en pellucide marginale degeneratie, nog een nieuwe chirurgische behandelingsoptie bijgekomen die niets te maken heeft met hoornvliestransplantatie. Deze behandeling, de intrastromale ringsegmenten, kan als alternatief voor PK

of LK toegepast worden, maar een transplantatie zou er ook enige tijd mee kunnen worden uitgesteld. De ringsegmenten werden reeds toegepast als een refractiechirurgische techniek ter behandeling van milde tot matige bijziendheid (ie ook hoofdstuk 2, paragraaf XIV). Voor dit doel worden ze echter steeds minder gebruikt, omdat de resultaten niet erg goed te voorspellen waren. Een voordeel van deze ringsegmenten bij de behandeling van cornea-ectasieën is dat ze het uitgestulpte hoornvlies vlakker en strakker trekken. Ook zijn de gevolgen van deze behandeling voor de refractie (de brilsterkte) omkeerbaar. Na een behandeling met ringsegmenten kan er nog steeds wel een afwijkende refractie overblijven, maar vaak blijkt deze gemakkelijker dan voorheen te corrigeren te zijn met een bril of met een zachte contactlens (i.p.v. een harde contactlens). Cornea's die te steil of te dun zijn, of waarin zich centraal een vertroebeling bevindt zijn niet geschikt om met deze methode behandeld te worden. In een geselecteerde groep van ogen met niet al te ver voortgeschreden keratoconus kunnen ring segmenten (INTACS dan wel Ferrara ringen) een bruikbare aanvullende behandelmethode zijn[1192,1245-1254] Met behulp van de femtosecond laser kan in een aantal gevallen op een makkelijkere manier de tunnels waarin de ringsegmenten moeten worden opgevoerd in het hoornvlies worden gemaakt.

De meest recent beschreven nieuwe behandeloptie voor cornea-ectasieën is het cross-linken van het stromale collageen. Bij deze behandeling wordt het oog met bijvoorbeeld keratoconus blootgesteld aan een UV-A stralingsbron, nadat er riboflavine in is gedruppeld. De UV-A straling zorgt ervoor dat er riboflavine-radicalen ontstaan, en deze moleculen zijn in staat chemische cross-links tussen de collageenfibrillen in het hoornvliesstroma te maken. De cross-links zorgen voor een biomechanische verstijving van het stroma. Deze behandeling is met name bedoeld om het progressie van de ectasie, dus het verergeren van de uitbochting, tegen te gaan. Tot nu toe zijn er alleen nog maar 'case series' gepubliceerd. Hieruit blijkt dat de behandeling op de korte en middellange termijn veilig is, en er zijn aanwijzingen dat ze ook effectief is.[1257-1259] Om echter daadwerkelijk de effectiviteit te bewijzen zijn er lang lopende, gerandomiseerde, vergelijkende prospectieven onderzoeken nodig.

Tot slot moet er nog kort worden ingegaan op het induceren van celdelingen in endotheelcellen. Als het gaat om endotheelaandoeningen zou dit wel eens de therapie van de toekomst kunnen worden. Een endotheel dat opgebouwd is uit gekweekte endotheelcellen is een antwoord op een tekort aan donorweefsel, en door modificatie van antigeenexpressie mogelijk ook op transplantaatafstoting. Dit laatste zou natuurlijk ook bereikt kunnen worden via het kweken of induceren van proliferatie van eigen endotheelcellen van de patiënt. Het concept van het kweken van humane endotheelcellen voor transplantatiedoeleinden is in het verleden al beschreven.[1114-1117] Nieuwer

onderzoek richtte zich op het vinden van geschikte dragers waarmee deze cellen getransplanteerd zouden kunnen worden.[1119,1126,1128,1260] Recent onderzoek laat zien dat met gekweekte humane endotheelcellen en gepreserveerd humaan corneaweefsel hoornvliesconstructen kunnen worden vervaardigd.[1126] Er zijn zelfs publicaties over complete hoornvliesconstructen waarvan alle lagen opgebouwd zijn uit geïmmortaliseerde, gekweekte humane cellen (epitheliale, stromale en endotheliale cellen).[1124] Transfectie met bijvoorbeeld SV40 virus vectoren is wellicht noodzakelijk om voor transplantatiedoeleinden een gekweekte endotheelcellaag te krijgen met een voldoende hoge ECD. [132,1124] Van dergelijke getransfecteerde gekweekte endotheelcellagen is in sommige onderzoeken een adequate endotheelpompfunctie aangetoond.[132,1124] Deze getransfecteerde cellagen hebben echter vaak het probleem dat de celdelingen doorgaan ook als dat niet meer gewenst is. Maar cellagen die opgebouwd zijn uit niet-getransfecteerde cellen hebben vaak een te lage ECD of een onvoldoende endotheelpompfunctie. [132,1126] Tot nu toe heeft nog geen enkele van deze experimenten geresulteerd in een klinische toepassing.

In plaats van het transplanteren van gekweekte endotheelcellen zou men endotheelaandoeningen ook kunnen proberen te verhelpen door in vivo de eigen endotheelcellen van de patiënt aan te zetten tot celdeling. Hiervoor is het exact identificeren van de endotheliale stamcellen noodzakelijk en dat is tot dusverre nog niet gelukt, hoewel wel steeds duidelijker wordt dat ze zich in de regio van de posterieure limbus bevinden. Dit probleem moet worden opgelost voordat deze benadering verder ontwikkeld kan worden. [132,874,1261,1262] Tot het zover is, zal het transplanteren van complete donor-endothelia door middel van PK, PLK, of varianten daarop, nodig blijven.



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Zie pagina 287.

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# Abbreviations

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|---|---|
| ABK | Aphakic bullous keratopathy |
| AC | Anterior chamber |
| ACAID | Anterior chamber associated immune deviation |
| AJC | Apical junctional complex (of endothelial cells) |
| a.k.a. | Also known as |
| ALTK | Automated lamellar therapeutic keratoplasty, a.k.a. Lamellar keratoplasty with an automated microkeratome |
| ANOVA | Analysis of variance |
| B(S)CVA | Best (spectacle) corrected visual acuity |
| BSS | Balanced salt solution |
| CCC | Continuous circular/curvilinear capsulorhexis |
| CE (+ I) | Cataract extraction (plus implantation of an IOL) |
| CHED | Congenital hereditary endothelial dystrophy |
| CI | Cytotoxicity index |
| CRT | Corneal refractive therapy (= Orthokeratology, OK) |
| CT | Corneal thickness |
| CV | Coefficient of variation (= standard deviation divided by mean), of endothelial cell size |
| D | Diopter(s) |
| DALK | Deep anterior lamellar keratoplasty |
| DLCTS | Dutch lamellar corneal transplant study |
| DLEK | Deep lamellar endothelial keratoplasty ( = PLK) |
| DM | Descemet's membrane |
| DSAEK | Descemet's stripping with automated endothelial keratoplasty (= DSEK/PLK with Descemetorhexis, with donor preparation using an automated microkeratome) |
| DSEK | Descemet's stripping with endothelial keratoplasty (= PLK with Descemetorhexis) |
| ECCE | Extracapsular cataract extraction |
| ECD | Endothelial cell density |
| ELK | Endothelial lamellar keratoplasty ( = MAPK) |
| EMEM | Eagle's minimal essential medium, for organ culture tissue preservation. |
| ERM | Epiretinal membrane |
| FBS (= FCS) | Fetal bovine/calf serum |
| FED | Fuchs' endothelial dystrophy |
| HLA | Human leukocyte antigen |
| HSV | Herpes simplex virus |
| ICC | Intraclass correlation coefficient |
| ICCE | Intracapsular cataract extraction |
| ICE syndrome | Iridocorneal endothelial syndrome |

| | |
|---|---|
| ICG | Indocyanine green, also: infracyanine green |
| ICL | Implantable contact lens (= posterior chamber phakic IOL) |
| ICRS | Intrastromal corneal ring segments |
| i.e. | Id est (= that is) |
| ILM | Internal limiting membrane (of the retina) |
| INTACS | Type of ICRS |
| IOI | Interuniversitair oogheelkundig instituut (=NORI), now NIN |
| IOL | Intra-ocular lens (artificial) |
| IOP | Intra-ocular pressure |
| IP | Imbibition pressure |
| KC | Keratoconus |
| KP | Keratoprosthesis |
| L | Imaged length of corneal endothelium (in specular microscopy) |
| LASIK | Laser in-situ keratomileusis |
| LASEK | Laser-assisted subepithelial keratomileusis |
| LC50, LD50 | Concentration/dose at which 50 % of maximum toxic effect is reached |
| LEF | Late endothelial failure |
| LK (= LKP) | Lamellar keratoplasty |
| M | Magnification |
| MAPK | Microkeratome assisted posterior keratoplasty |
| MHC | Major histocompatibility complex |
| MK-medium | McCarey-Kaufman medium, for cold donor cornea preservation |
| MMC | Mitomycin-C |
| MMT assay (Mossman's) | Mossman's 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay |
| MS | Morphology score |
| n | Refractive index, or number of patients/eyes in study group |
| $Na^+/K^+$ ATPase | Sodium-potassium ion exchanger = endothelial metabolic pump |
| NIIOC | Netherlands institute for innovative ocular surgery in Rotterdam |
| NIN | Netherlands institute for neurosciences |
| NM | Normalized magnification |
| NORI | Netherlands ophthalmic research institute in Amsterdam, (=IOI in Dutch), now NIN |
| OK | Orthokeratology (= corneal refractive therapy (CRT) = corneal reshaping with contact lenses) |
| OOKP | Osteo-odonto-keratoprosthesis |
| P | Corneal power |
| PBS | Phosphate buffered solution (laboratory) |
| PCL | Posterior collagenous layer |
| PIOL | Phakic intraocular lens |
| PK (= PKP = PKPl) | Penetrating keratoplasty (a.k.a. perforating keratoplasty) |
| PLK | Posterior lamellar keratoplasty |

| | |
|---|---|
| PMMA | Polymethyl-methacrylate |
| PPBK | Pseudophakic bullous keratopathy |
| PPD (= PPMD) | Posterior polymorphous dystrophy |
| PRK | Photo-refractive keratectomy |
| PTK | Photo-therapeutic keratectomy |
| PVR | Proliferative vitreoretinopathy |
| r | Corneal radius |
| RGP | Rigid gas-permeable (= hard) contact lens |
| RK | Radial keratotomy |
| RPE | Retinal pigment epithelium |
| SD | Standard deviation |
| SEM | Scanning electron microscope |
| SP | Swelling pressure |
| TASS | Toxic anterior segment syndrome |
| TB | Trypan blue |
| TECD syndrome | Toxic endothelial cell destruction syndrome |
| UCVA | Uncorrected visual acuity |
| UGH syndrome | Uveitis glaucoma hyphaema syndrome |
| UV-A, -B | Ultraviolet radiation of type A, B |
| VA | Visual acuity |
| 5-FU | 5-Fluorouracil |

# Colour figures

Fig. 2.VII.1 Example of a specular microscopic image, analysed with the "borders" variable frame endothelial assessment method. In this example, automatedly drawn cell borders were corrected manually.

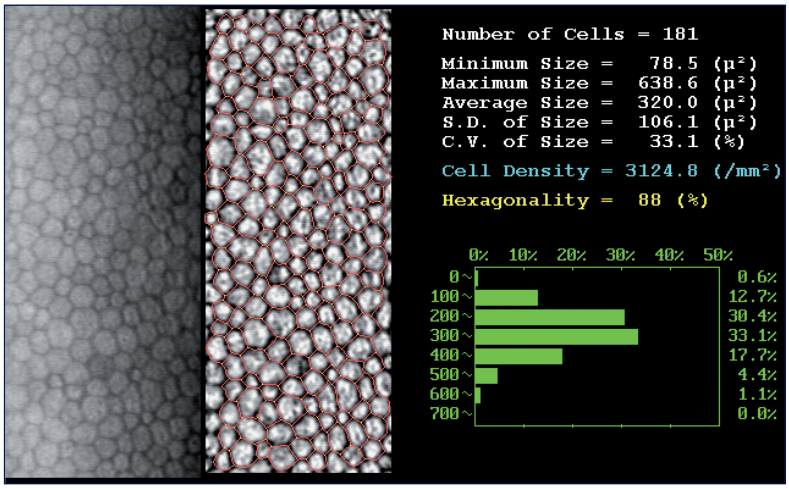
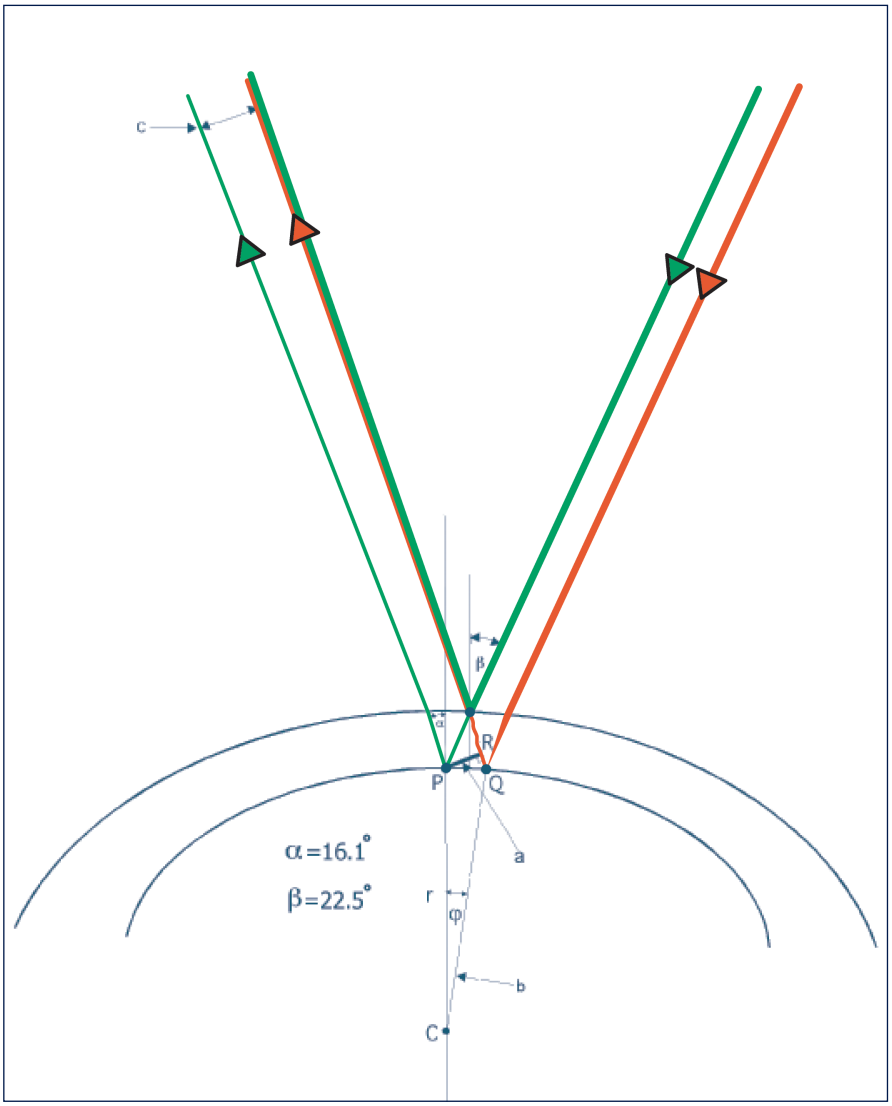


Fig. 6.1 Diagrammatic representation of the optic distortions in non-contact specular microscopy.




Notice the dominant epithelial reflex (heavy green line). The maximal endothelial width that can be observed $L_{max}$ is determined by the corneal thickness CT and the incident angle after anterior corneal deflection α, according to $L_{max} = 2 * CT * \tan \alpha$. In the SP2000P, $\alpha = 16.1°$.

a. An oblique observation-angle introduces underestimation of the observed dimension. β is the incident angle (without anterior corneal deflection). $L_{assumed}(PR) = \cos \beta * L_{max}(PQ)$ (assuming that PQ were not curved). In the SP2000P $\beta = 22.5°$, and hence: $L_{assumed}(PR) = 0.924 * L_{max}(PQ)$.

b. Observation of a cuved surface (PQ) instead of a straight surface introduces slight underestimation of the observed dimension. See text for details.

c. Magnification by the anterior corneal surface introduces overestimation of the observed area. See text for details.

Fig. 7.1 Trypan blue capsule staining to aid visualisation of the anterior lens capsule in mature cataract phacoemulsification.

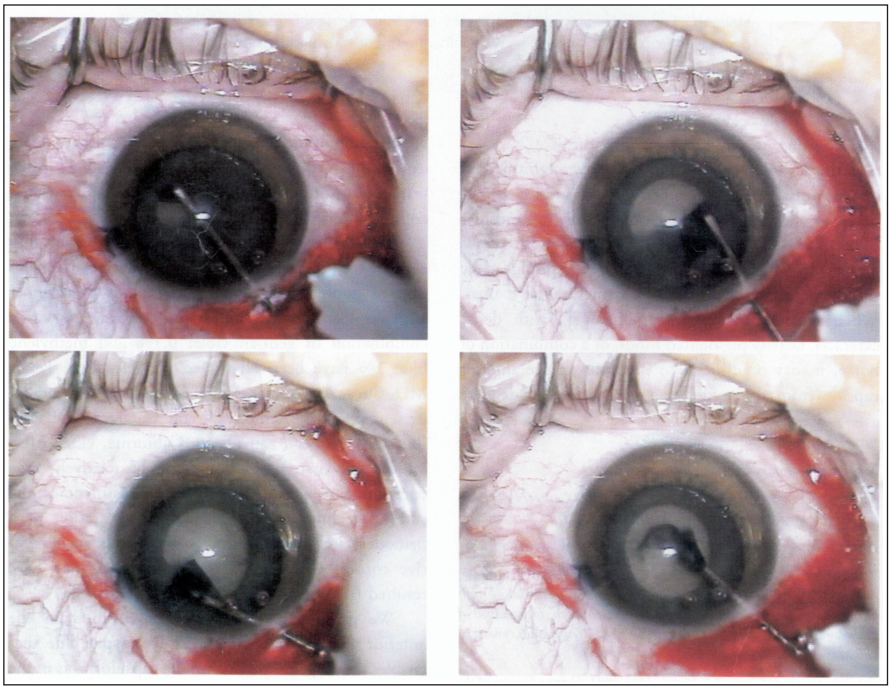
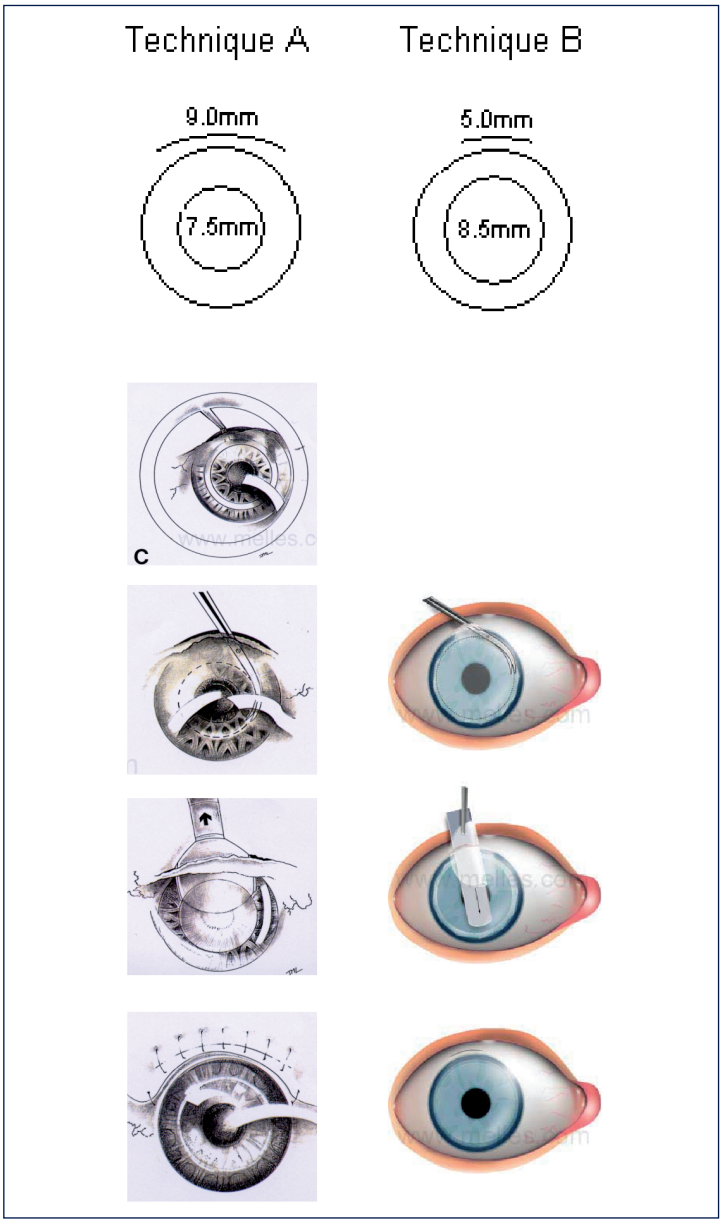


Fig.12.1 Diagrammatic representation of the techniques for posterior lamellar keratoplasty.




In Technique A, a 9.0 mm sclerocorneal pocket incision was made, and a recipient posterior lamellar disc was excised with an instra-stromal trephine and microscissors. The donor posterior lamellar disc was inserted endothelium side down on a spoon-shaped glide covered with visco elastic to protect the endothelium. The scleral incision was sutured, but no sutures were used to fixate the donor tissue. In Technique B, a 5.0 mm sclerocorneal tunnel incision was made, and the recipient posterior corneal tissue was excised with microscissors only. The donor disc was folded prior to insertion with the endothelium on the inside, and the tissue was unfolded inside the anterior chamber and positioned against the posterior recipient cornea. Because the scleral incision was self-sealing, no sutures were used in the procedure.

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Het avontuur van het promotieonderzoek dat nu zijn neerslag in dit proefschrift heeft gekregen, is voor mij in augustus 1998 begonnen. Het werk aan dit proefschrift is nauw verweven met mijn tijd in het Oogziekenhuis Rotterdam, eerst als arts-onderzoeker, later als AGNIO, vervolgens als AGIO/AIOS, en tenslotte nu als cornea-fellow. De promotie is in mijn ogen dan ook een toepasselijke afsluiting van mijn werkzaamheden in het OZR.

Dit proefschrift had nooit tot stand kunnen komen als niet vele mensen mij daarbij hadden gesteund en geholpen. Al deze mensen wil ik daarvoor hartelijk danken. Allereerst alle patiënten en vrijwilligers die de afgelopen jaren, soms herhaalde malen, belangeloos en welwillend hebben meegewerkt aan het promotieonderzoek.

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Bart van Dooren
Rotterdam, 1 november 2006

# Curriculum Vitae

Bart T.H. van Dooren werd op 5 juni 1968 geboren te Geleen. In 1986 haalde hij zijn gymnasium-β diploma (magna cum laude) aan de RKSG Sint Michiel te Geleen en begon hij met de studie geneeskunde aan de Rijksuniversiteit Groningen. Het doctoraalexamen werd cum laude behaald in 1991, en het artsexamen in 1994.

Zijn aanvankelijke carriéreplannen waren gericht op specialisatie in de neurochirurgie, en in dit kader doorliep hij in 1993 en 1994 gedurende een half jaar keuzeco-schappen in de Verenigde Staten. Deze vonden plaats op de afdelingen Neurosurgery van het Presbyterian University Hospital te Pittsburgh, en de Mayo Clinics te Rochester, Minnesota. Na zijn artsexamen vervulde hij eerst in 1994 en 1995 zijn militaire dienstplicht als eerste-luitenant-arts bij de geneeskundige compagnie van de 11. Luchtmobiele Brigade van de Koninklijke Landmacht. Vanaf april 1995 werkte hij daarna gedurende 3 jaar als AGNIO neurochirurgie, in achtereenvolgens het Academisch Ziekenhuis Groningen, het Academisch Ziekenhuis Utrecht, en het Academisch Ziekenhuis St.Radboud in combinatie met het Canisius-Wilhelminaziekenhuis, beide te Nijmegen.

Gedurende deze laatste betrekking werd het hem steeds meer duidelijk dat een specialisatie als neurochirurg niet zijn ideale carrière-invulling zou zijn en daarom besloot hij zich in andere richtingen te oriënteren. Dit omvatte in 1998 het opstarten van een studie bouwkunde aan de Technische Universiteit Eindhoven, en daarnaast het beginnen met het onderhavige promotieonderzoek, in het Oogziekenhuis Rotterdam.

In de loop van 1999 besloot hij zich volledig op een loopbaan in de oogheelkunde te richten. Vanaf 2000 werkte hij 23 maanden als AGNIO in het OZR, waarbij hij deels werkzaam was in de patiëntenzorg en deels verder werkte aan het promotieonderzoek. Vanaf 1 maart 2002 doorliep hij in het OZR voltijds de opleiding tot oogarts, en rondde deze onlangs (per 31 augustus 2006) af. Momenteel voltooit hij in dezelfde kliniek zijn fellowship cornea- en refractiechirurgie. Per 1 januari 2007 zal hij toetreden tot de oogartsenmaatschap van het Amphia Ziekenhuis, te Breda en Oosterhout.

De auteur is getrouwd met Gertrude Smit, en ze hebben samen een zoon: Jonathan. In februari 2007 verwachten zij hun tweede kindje.