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PhD dissertation

Fuchs' endothelial corneal dystrophy: Pathology and treatment outcome

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Contents

Preface

This PhD dissertation is based on clinical studies and laboratory experiments conducted at the Department of Ophthalmology, Aarhus University Hospital from March 2013 to March 2016.

Preliminary considerations about this project date back to 2009 when I approached Niels Ehlers, the former professor in Ophthalmology. Niels Ehlers, on the verge of retirement from a renowned career in Ophthalmology, arranged for at meeting in his private residence with professor Jesper Hjortdal who came by after work hours. At the time, my experience in ophthalmology was limited, but even so Jesper was kind enough to help me get started.

As work progressed at the Department of Ophthalmology in Aarhus, I met my co-supervisor Anders Ivarsen. Even though Anders was still in his residency at the time, he was already an expert on corneal disease, an experienced surgeon, and an established researcher.

Jesper and Anders form the basis of the Cornea Team and their relentless efforts are an inspiration to the entire staff in Aarhus. I could not have wished for a better team of supervisors, and I would like to take this opportunity to thank them both.

Also thanks to Eva Raagaard Nielsen for valuable help in practical matters, Line Pedersen for excellent assistance in image grading, Olga Kudryavtseva and Kim Nielsen for helpful advice with immunofluorescence, fellow Ph.D. students for a constructive research environment, nurses and secretaries at the Cornea clinic and Cataract clinic, Opticians, Andrey Zhyvov and prof. Rudolph Guthoff in Rostock, Neil Lagali and prof. Per Fagerholm in Linköping, and prof. Mats Lundström.

Finally, my love to my wife Astrid and our three children: Andrea, Matilda and Asger. Thank you for your support and patience with long work hours.

This project has been generously supported by: Øjenforeningen Værn om Synet, Synoptik-Fonden, Jochum Jensen og hustru Mette Marie Jensens f. Poulsens mindelegat, Helga og Peter Kornings Fond, Købmand Marie Kirstine Jensens Fond, Novartis Healthcare a/s.

Esben Nielsen
April 2016

List of papers

This PhD thesis is based on the following papers

Paper I

Nielsen E, Ivarsen A, Erlandsen M, Hjortdal J. Evaluation of endothelial pump function in fuchs endothelial dystrophy before and after endothelial keratoplasty. *Cornea*. 2016;35(6):878-883. doi: 10.1097/ICO.0000000000000821 [doi].

Paper II

Nielsen E, Ivarsen A, Kristensen S, Hjortdal J. Fuchs' endothelial corneal dystrophy: A controlled prospective study on visual recovery after endothelial keratoplasty. *Acta Ophthalmol*. 2016. doi: 10.1111/aos.13126 [doi].

Paper III

Nielsen E, Nielsen K, Ivarsen A, Greenhill NS, Davis PF, Hjortdal J. Fuchs endothelial corneal dystrophy: A systematic immunofluorescence study of collagen type VIII suggests heterogeneous pathophysiology. *Cornea*. 2016;35(6):872-877. doi: 10.1097/ICO.0000000000000848 [doi].

List of abbreviations

ABZ	anterior banded zone
BCVA	best corrected visual acuity
CCT	central corneal thickness
CCTepi	central thickness of the corneal epithelium
CCTgraft	central thickness of the corneal graft
CCTstroma	central thickness of the corneal stroma
CCTtotal	total central corneal thickness
COL8	collagen, type VIII
COL8A1	collagen, type VIII, α1-helix
COL8A2	collagen, type VIII, α2-helix
CS	contrast sensitivity
DM	Descemet's membrane
DMEK	Descemet's membrane endothelial keratoplasty
DSAEK	Descemet's stripping automated endothelial keratoplasty
EC	endothelial cells
ETDRS	early treatment diabetic retinopathy study
FECD	Fuchs' endothelial corneal dystrophy
HOA's	higher-order aberrations
IVCM	in vivo confocal microscopy
LogCS	logarithm to the contrast sensitivity
LogMAR	logarithm to the minimal angle of resolution
PBK	pseudophakic bullous keratopathy
PCL	posterior collagenous layer
PK	penetrating keratoplasty
PNBZ	posterior non-banded zone
SNP	sub basal nerve plexus
TGFBip	Transforming growth factor beta induced proteine

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Acta Ophthalmol. 2016: 94, thesis 104: 6–27

doi: 10.1111/aos.13311

Introduction

The cornea: evolutionary brilliance

Vision is one of the most important senses. In an evolutionary context, the first primitive eyes date back to the Cambrian explosion of species 540 million years ago and eyes have since then evolved in almost all known animal phyla (Land & Nilsson 2002). Evolutionary biologists even argue that the ability to sense light led to an arms race among the species, which caused the rapid evolution of life in the Cambrian period (Parker 2003). While the eye takes on many forms in the animal kingdom, the camera eye that is found in humans and other species provides superior optics by focusing images on the retina. This requires refraction of light, and at the very front of the eye, a remarkable structure provides approximately two-thirds of the refractive power of eyes: the cornea. The cornea is a transparent, dome-shaped structure roughly 11 mm in diameter and 0.5 mm thick. It is comprised of five distinct anatomical layers: epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium.

Small as it may be, this 'window of the eye' is critical for vision, as it needs to be almost completely transparent in order for light to be transmitted further back onto the photoreceptors in the retina. The anatomical position of the cornea subjects it to the perils of the environment, but a dense network of nerves enables sensation of the tiniest foreign bodies. This network contains more nerves per mm^2 than any other tissue in the human body, suggesting the evolutionary benefit of protecting it from harm. Besides the detection of potentially infectious foreign bodies, the cornea must also be able to withstand more brute trauma. This requires strength and durability, and in biological tissues, these features are typically gained by incorporating collagen fibres. However, collagenous tissue is inherently opaque and does not meet the demand for corneal transparency. Nature's brilliant solution to this problem was to engineer a tissue containing a highly ordered, lamellar structure of collagen fibres while leaving out blood vessels. This unique lamellar arrangement of fibres provides not only the necessary resilience to trauma, but also ensures transparency as the lamellae are 'pulled' so close, that light scatter from the individual collagen fibres is ruled out by a phenomenon known as destructive interference. As a result, the cornea scatters only 1–15% of light (wavelength dependable) in the visible spectrum (Levin et al. 2011).

The endothelium

Bringing the collagen fibres into close proximity is made possible in part by keeping the cornea in a relatively dehydrated state through an elaborate hydration control mechanism. This mechanism was described by David Maurice, who termed it the 'pump-leak model'(Maurice 1951). This states that the endothelial cells (ECs) on the inside of the cornea actively pump out ions from the stroma, creating an osmotic gradient, which draws out water. Essential for this pumping mechanism is an average of 4.4×10^{12} Na^+/K^+-ATPase pumps per cell, whose collective energy consumption requires the second highest metabolic rate of all the cells in the eye, only surpassed by the photoreceptors of the retina (Levin et al. 2011). Dehydrating the cornea in this fashion brings negatively charged glycosaminoglycans on adjacent collagen fibrils closer, thus generating anionic repelling forces. This force is termed the swelling pressure as it gives the cornea an innate tendency to swell by imbibing fluid. There are leaky tight junctions between ECs, and this enables a flow of nutrients

back into the avascular cornea (Bourne 2003).

Consequently, the endothelium is key in maintaining both corneal transparency and corneal health. The endothelium (Fig. 1) is a highly specified monolayer of cells. For unknown reasons, the endothelial cells (ECs) in humans are arrested in the G1 phase of the cell cycle and cannot replicate *in vivo* (Joyce & Harris 2010). This means that when ECs are damaged or die, the remaining adjacent cells are forced to migrate and expand in order to maintain the continuity of the endothelium (Levin et al. 2011). Physiological decline is about 0.6% cells/year, but the ECs can compensate for this by increasing the number of Na^+/K^+-ATPase pumps per cell and the activity of the Na^+/K^+-ATPase pumps (Krachmer et al. 2011). This allows hydration to be maintained by as few as 500 cells/mm^2 (Krachmer et al. 2011; Levin et al. 2011). The average EC density of an adult is around 3000 cells/mm^2, and in theory, there are enough ECs in storage for 244 years of life (Levin et al. 2011).

Descemet's membrane

The endothelium is situated on the Descemet's membrane (Jean Descemet, 1732–1810), a basal membrane that is formed in fetal life. The fetal DM contains collagen VIII (COL8) fibrils in a regularly arranged network, giving it a banded appearance when viewed in an electron microscope. This part of the DM is termed the anterior banded zone, the ABZ. Throughout life, the ECs continuously add non-banded material onto the ABZ, eventually forming a posterior non-banded zone, the PNBZ (Krachmer et al. 2011). Consequently, the DM gradually thickens: at birth, the average DM is 3 μm thick, and at 70 years of age, the average DM is 13 μm thick (Bourne 2003).

Fuchs' endothelial corneal dystrophy

In 1910, Austrian Professor Ernst Fuchs in ophthalmology described an eye disease that caused cloudy corneas in a group of elderly patients (Fuchs 1910). For decades, the disorder has been known as Fuchs' endothelial corneal dystrophy (FECD), and it is widely accepted as being a genetic disorder (Elhalis et al. 2010; Hamill et al. 2013; Zhang & Patel 2015). A rare, early-onset variant has been discovered in recent years, and it is caused by distinct mutations within the gene that encodes the α_2-subunit for the COL8 protein (COL8A2; Biswas et al. 2001; Gottsch et al. 2005a). The common, late-onset variant (from here on just labelled FECD) appears to be autosomal dominant as well, but with incomplete penetrance, and 50% of cases appear spontaneously without family clustering (Hamill et al. 2013). FECD is characterized by pathological changes in the DM and accelerated loss of ECs. Inside the DM, a posterior layer of collagenous material is added onto the PNBZ, often referred to as the posterior collagenous layer (PCL). A hallmark of the disease is the formation of small wart-like excrescences in the PCL called 'guttae' (lat.: 'drops'), as they resemble dewdrops on the cornea when viewed by slit-lamp microscopy (Fig. 2).

The loss of ECs eventually impairs corneal hydration control, and patients with advanced FECD often experience diurnal variation of symptoms with blurred vision in the morning that abates during the day. It is caused by a corneal oedema that forms during the night due to eye closure that eliminates evaporation from the corneal surface and decreases oxygen tension and tear film tonicity (O'Neal & Polse 1985; du Toit et al. 2003). When patients wake up and open their eyes, the oedema is slowly removed by a combination of evaporation and fluid pumping by the ECs.

If left untreated, FECD leads to lasting corneal oedema and a substantial decline in visual acuity (Adamis et al. 1993; Borboli & Colby 2002; Krachmer et al. 2011). Clinical diagnosis of FECD is based on the presence of guttae, corneal oedema, lack of inflammation and slow progression (Adamis et al. 1993; Borboli & Colby 2002; Elhalis et al. 2010). Debut may vary, but is often around the sixth decade (Zhang & Patel 2015). The only effective treatment option for FECD is corneal transplantation (Hamill et al. 2013), and in a recent global survey, corneal transplantation was reported to be the most frequently performed type of transplantation worldwide, with FECD being the top indication in industrialized countries (Gain et al. 2016). There is particularly high prevalence of FECD among Caucasians (Adamis et al. 1993; Borboli & Colby 2002; Nakano et al. 2015), and FECD accounts for some 70% of all corneal transplantations in Denmark (Source: the Danish Cornea Bank).

The pathology of Fuchs' endothelial dystrophy

Even though FECD was discovered more than 100 years ago (Fuchs 1910), the cause of the disease remains largely unknown. Substantial progress has been made in recent years, especially by genetic studies. As mentioned above, the early-onset variant of FECD was linked to mutations in the COL8A2 gene, but this was not detected in the common late-onset variant (Aldave et al. 2006). Several mutations in different genes have been associated with the development of FECD (Baratz et al. 2010; Elhalis et al. 2010; Riazuddin et al. 2010a,b, 2012; Wieben et al. 2012; Nakano et al. 2015), and because of this, it has been speculated that different diseases lead to the same phenotype (Hamill et al. 2013).

On a pathophysiological level, early electron microscopic studies of FECD demonstrated the presence of large amounts of disorganized COL8 fibres inside the PCL layer (Levy et al. 1996), and this finding has been confirmed by an immunofluorescence study on DM samples from patients with FECD (Gottsch et al. 2005b). Of further note, COL8 affects plaque stability in cardiovascular disease (Plenz et al. 2003), and a possible link between FECD and cardiovascular disease was reported in 1984 by Olsen who found that patients with FECD had a significantly higher prevalence of cardiovascular disease when compared with an age-matched control group (Olsen 1984).

Further breakthroughs include the detection of apoptosis in endothelial cells (Borderie et al. 2000; Li et al. 2001; Szentmary et al. 2005), ongoing oxidative stress (Buddi et al. 2002; Jurkunas et al. 2008, 2010; Bitar et al. 2012), colocalization of clusterin and transforming growth factor beta-induced protein (TGFBip) in guttae (Jurkunas et al. 2009) and activation of the unfolded protein response (UPR; Engler et al. 2010; Jun et al. 2012; Meng et al. 2013). The UPR is a

cellular clean-up pathway, which can induce apoptosis when triggered by oxidative stress or the presence of misfolded proteins (Wang & Kaufman 2012). The trigger for UPR activation in FECD is unknown, but may be the presence of accumulated clusterin, TGFBip or COL8 proteins.

Considering the implication of COL8 in both FECD and early-onset FECD, COL8 is an appealing starting point for investigating FECD pathology.

However, there are inconsistencies regarding COL8 levels in FECD, as neither a proteomic study nor a transgenic COL8A2 gene knock-in mouse model of FECD found elevated COL8 levels in FECD tissue (Jun et al. 2012; Meng et al. 2013; Poulsen et al. 2014). Further, a study of cells that carried a COL8A2 gene mutation known to cause early-onset FECD reported that the absolute levels of COL8 did not differ from normal tissue (Kelliher et al. 2011).

One explanation for these discrepancies might be that different pathophysiological processes are taking place, as the genetic heterogeneity of late-onset FECD implies. Before work towards a future medical treatment for FECD can be begun, this has to be resolved.

Treatment outcome: visual recovery and patient-reported outcomes

For decades, corneal transplantation was performed as penetrating keratoplasty (PK). In 1998, endothelial keratoplasty was introduced (Melles et al. 1998) and subsequently developed into the Descemet's stripping automated endothelial keratoplasty (DSAEK) procedure (Melles et al. 2002; Price & Price 2005; Gorovoy 2006). In DSAEK, the diseased DM is removed and replaced by a thin posterior lamella consisting of endothelium, DM and a layer of stromal tissue. Compared with PK, DSAEK provides faster healing, earlier visual recovery, more predictable refractive outcomes and improved structural–mechanical strength of the globe (Lee et al. 2009; Mau 2009). The procedure has even become rather expedient, as the use of precut donor tissue has eliminated time-consuming donor preparation in the operating theatre (Price et al. 2008; Terry et al. 2009). In uncomplicated cases, DSAEK surgery takes about 25 min to carry out. For these reasons, DSAEK has become the most widely performed type of keratoplasty for endothelial disorders (Ang et al. 2015), and more patients with FECD than ever are receiving corneal transplantation (Coster et al. 2014; Greenrod et al. 2014).

Even though the advantages of DSAEK are important, DSAEK does not provide superior visual outcomes when compared with PK (Lee et al. 2009; Nanavaty & Shortt 2011; Nanavaty et al. 2014). Vision is seldom fully restored after DSAEK – even in otherwise healthy eyes (Lee et al. 2009). The reason for this is unknown, but pathology in either anterior or posterior corneal layers is most likely the reason. In the anterior part of the recipient cornea, preoperative long-standing oedema leads to formation of subepithelial fibrosis, resulting in optical imperfections such as scatter (haze) and higher-order aberrations (HOAs; Kobayashi et al. 2009; Patel et al. 2009; Wacker et al. 2015). In the posterior part of the cornea, interface haze between the donor lamella and recipient cornea could affect vision, and graft asymmetry could lead to aberrations from the posterior surface. Substantial efforts have been made to identify the limiting factor of vision after DSAEK, but reports have been mostly retrospective, and only very few prospective studies exist (Lee et al. 2009; Nanavaty et al. 2014; Wacker et al. 2016a). Results have been contradictory, linking post-DSAEK vision to anterior HOAs (Yamaguchi et al. 2009; Patel et al. 2012; Rudolph et al. 2012; van Dijk et al. 2014), graft thickness (Dickman et al. 2013) and corneal scatter (Koh et al. 2012; Hindman et al. 2013; Ivarsen & Hjortdal 2014). In a recent review on the matter, it was concluded that the limiting factor of vision after DSAEK remains unknown (Turnbull et al. 2016).

What is more, an improvement in best-corrected visual acuity (BCVA) does not necessarily translate well to patient-reported outcomes. For instance, reports suggest that improved contrast sensitivity (CS) rather than BCVA is linked to the quality of vision that patients perceive (Nielsen & Hjortdal 2012; Cabrerizo et al. 2014). In order to ensure progress for patients, a systematic investigation on patient-reported outcomes is warranted (Patel et al. 2014).

Even though BCVA is limited after DSAEK, there is ongoing improvement for years after surgery (Li et al. 2012). This slow recovery is thought to be due to remodelling of subepithelial pathology in the anterior cornea (Wacker et al. 2016a). Also located below the epithelium, the sub-basal nerve plexus (SNP) density is reduced even in early stages of FECD (Schrems-Hoesl et al. 2013), and as with BCVA, the SNP density also recovers slowly after DSAEK (Bucher et al. 2014). Because corneal nerves are known to play a central role in wound healing (Shaheen et al. 2014), a link between the SNP and anterior

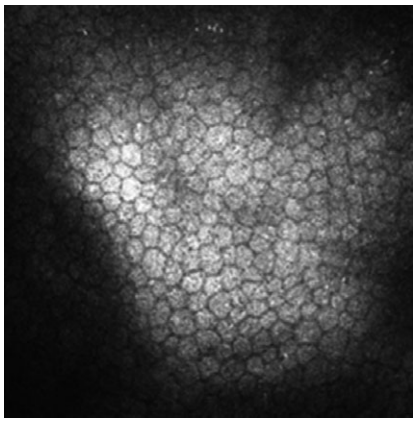

Fig. 1. Normal hexagonal-shaped endothelial cells. The hexagon is the most energy-efficient geometric shape to cover a surface without leaving gaps (Levin et al. 2011; *in vivo* confocal microscopy by EN).

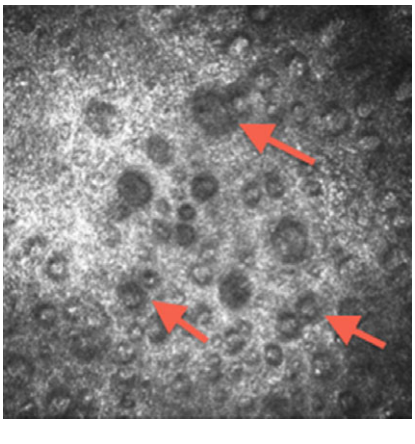

Fig. 2. The endothelium and DM in a patient with Fuchs' endothelial dystrophy. Red arrows indicate guttae. There are no visible endothelial cells (*in vivo* confocal microscopy by EN).

corneal remodelling therefore seems conceivable.

Treatment outcome: graft function

Due to the relative novelty of EK, there are few studies on the long-term graft survival. So far, the evaluation of graft health and its potential for longevity have been limited to measuring the EC density. However, using EC densities as a marker of graft health is not straightforward, and it is unknown whether grafted ECs are fully able to draw on normal compensatory mechanisms such as increasing the number and activity of Na^+/K^+-ATPase pumps (Krachmer et al. 2011).

Hypothesis

On the basis of the above-mentioned considerations, the following hypotheses were formulated:

Pathology

- There is more COL8 present in DM samples from patients with FECD when compared with DM samples from normal controls.

Treatment outcome: visual recovery and patient-reported outcomes

- Visual function after DSAEK is associated with changes in HOAs or densitometry from either the anterior or the posterior corneal surface.
- DSAEK surgery in patients with FECD leads to the improvement in patient-reported outcomes.
- There is an association between changes in SNP density and anterior corneal remodelling.

Graft function

- The functional capacity of the endothelium is comparable to normal corneas after DSAEK surgery.

Methods

Pathology (paper I)

Samples

Three groups were formed: a group of DM samples from patients with FECD (FECD group), a group of samples from patients with pseudophakic bullous keratopathy (PBK) and a group of samples from normal corneas (normal group). Samples were collected in a consecutive manner at the Department of Ophthalmology, Aarhus University Hospital, between March 2013 and September 2015. In the FECD and PBK group, DM samples were collected during DSAEK surgery (see DSAEK and cataract surgery). Normal corneas were collected from patients treated with either enucleation or penetrating keratoplasty (PK) and consisted of an 8-mm trephined corneal full-thickness buttons.

Samples were gently flattened under a light microscope, embedded in OCT compound (Tissue-Tek; Sakura Finetek Co., Tokyo, Japan), snap-frozen in liquid nitrogen, freeze-sectioned at a thickness of 5 μm, placed on super frost slides and stored at -80°C. Permission was obtained by the local ethics committee; written consent was obtained from patients; and investigations adhered to the tenets of the Declaration of Helsinki.

Immunofluorescence procedure

Frozen sections were warmed at room temperature for 10 min. Next, the samples were placed in ice-cold acetone (Sigma-Aldrich, 179973, St. Louis, MO, USA) for 10 min, washed in PBS–Tween (Sigma-Aldrich, P3563) for 3 × 2 min and incubated with rabbit serum block (Sigma-Aldrich, R9133, dilution 1:100) for 30 min at room temperature. After a rinse in PBS–Tween (Sigma-Aldrich, P5368), the slides were incubated with primary antibody (COL8A1, rabbit polyclonal, Sigma-Aldrich, HPA053107, dilution 1:50/COL8A2, rabbit polyclonal, Paul F. Davis/Nicholas Greenhill, dilution 1:50) for 1 hr at room temperature. Afterwards, the samples were rinsed in PBS–Tween and then incubated with FITC–antirabbit fluorophore (Sigma-Aldrich, 0382, dilution 1:100) for 30 min in darkness. The slides were subsequently washed in PBS–Tween for 3 × 2 min and dehydrated in 100% ethanol (Sigma-Aldrich, 02854) for 2 min. Fluoromount (Sigma-Aldrich, F4680) and coverslips were used for mounting. Immediately after mounting, the samples were viewed with an immunofluorescence microscope (HBO 50 Axiolab, Carl Zeiss, Oberkochen, Germany), and a representative section of the DM was selected. Images were captured using a Leica DFC 420c fluorescent camera (Wetzlar, Germany) under controlled ambient light settings. Exposure time, brightness, saturation, gamma and gain were fixed.

Grading of staining intensity

Two masked, independent observers (EN and LP) graded images using a subjective grading scale from 0 to 3 (0: no fluorescence, 1: discrete fluorescence, 2: marked fluorescence and 3: intense fluorescence). The scale was constructed after repeatedly going through the entire pool of images to identify representative images from each of these categories. Two images from each category were chosen as reference images, and these images were used by each of the observers when grading images. Because the anterior banded zone (ABZ) naturally contains COL8, the observers ignored any signal from this region and focused only on the remaining parts of the DM.

LP is an independent observer, not otherwise affiliated with this study. Observers EN and LP graded a total of 112 images and designated a value from the grading system to each image. Observers were blinded from each other's results. In 86 images (77%), values given by observers were identical; in the 26 remaining images, values differed no more than 1. This corresponded to a weighted K of 0.84, indicating a 'very good' interobserver agreement (Altman 1991). The grade from EN was elected in the cases of disagreement.

Treatment outcome: visual recovery and patient-reported outcomes (paper II)

Study design

In a prospective, controlled study, a group of patients with FECD eligible for DSAEK surgery (FECD group) was compared with an age-matched control group (control group). The aim was to identify the determinants of vision after DSAEK surgery, estimate patient-reported outcomes and finally investigate whether SNP density could be involved with anterior corneal remodelling. Patient-reported outcome was estimated by a questionnaire (Catquest-9SF), which was originally developed for cataract surgery. Consequently, the control group consisted of cataract patients with normal corneas eligible for cataract surgery.

In the FECD group, examinations were carried out before surgery and at 3-, 6- and 12-month follow-up. Only

the DSAEK eye was included. In the control group, examinations were carried out before surgery and at 12-month follow-up. Both eyes were included in the control group.

Subjects

Subjects were recruited consecutively among patients referred to the Department of Ophthalmology, Aarhus University Hospital, for treatment between March 2013 and July 2014.

Inclusion criterion in the FECD group was eligibility for DSAEK surgery due to FECD, and inclusion criteria in the control group were eligibility for cataract surgery and a normal cornea by slit-lamp examination. Exclusion criteria were reduced cognitive function, previous DSAEK surgery (FECD group), concurrent eye disease or history of eye disease, which theoretically could affect results. All investigations adhered to the tenets of the Declaration of Helsinki, and all patients gave informed consent. This study was registered at Clinicaltrials.gov, Identifier: NCT01979250.

Investigation outline

Each visit followed the same outline: first, patients filled out the Catquest-9SF Questionnaire. Brief instructions regarding the Catquest-9SF were given in an identical manner to all patients by the same investigator (EN). After instructions, the patient was left alone to fill out the questionnaire and encouraged to take the necessary time. Subsequent examinations were carried out in the same room with controlled light settings in the following order: anterior segment Scheimpflug tomography (Pentacam HR, Oculus Optikgeräte, Wetzlar, Germany), objective refraction and intraocular pressure measurement (Tonoref II, Nidek, Japan), specular microscopy (CEM 530, Nidek) and anterior segment OCT (Spectralis, Heidelberg).

After this, BCVA and contrast threshold were measured, and slit-lamp investigation and finally in vivo confocal microscopy (HRTIII-RCM, Heidelberg Engineering, Germany) were performed in a separate room with controlled lighting.

Best-corrected visual acuity was measured according to the early treatment diabetic retinopathy (ETDRS) protocol. Measurement of contrast sensitivity is described below. Cataract severity was graded on a scale from 0 to 4 by slit-lamp evaluation. One investigator (EN) carried out all investigations, and the same two examination rooms were used throughout the duration of the study.

Catquest-9SF Questionnaire

The Catquest-9SF contains nine questions and has been validated in several languages using Rasch analysis (Gothwal et al. 2009; Lundstrom & Pesudovs 2009; Lin et al. 2014). The Catquest-9SF questions are focused on the difficulty that patients experience in daily life due to poor vision. Scores from the questionnaire range from −3.94 logits, which indicates the least experienced difficulty, to 3.52 logits, which signifies the maximum amount of experienced difficulty. The improvement in patient-reported outcome was reported as the effect size, which is the change in Catquest-9SF scores from pre-op to 12 months divided by the standard deviation of the pre-op measurements: Δ (Catquest-$9SF_{Pre\text{-}op}$ − Catquest-$9SF_{Post\text{-}op}$)/$SD_{pre\text{-}op}$ (Lundstrom & Pesudovs 2009). Thus, an effect size of 0.3 represents a change of approximately one-third of the baseline SD. This measure is recommended as an indicator of responsiveness, and the following standard in effect size evaluation has been suggested: 0.2 or less is considered small, 0.5 is considered moderate, and 0.8 or above is considered large (Husted et al. 2000).

Translation of the Catquest-9SF Questionnaire

The Catquest-9SF Questionnaire was developed in Sweden for evaluating the outcomes in cataract surgery. Prior to this study, the Catquest-9SF was translated from Swedish to Danish with the assistance from the Danish Language Council and selected linguistic experts (see acknowledgements, paper II). Minor adjustments had to be made in questions 6 and 7. These questions contained the words 'mark' and 'slöjda', which do not readily translate into the same meaning in Danish. In this context, 'mark' in Swedish holds the meaning 'terrain/ground', while the corresponding word 'mark' in Danish holds the meaning 'acre/field'. We chose to use the Danish word 'terræn', literally meaning 'terrain'. The Swedish word 'slöjda' means 'to work with smaller pieces of wood' and there is no equivalent word in Danish. We chose to add a word to the sentence, settling on 'finere træarbejde', meaning 'fine woodworking'. Font size, type and other graphical features remained unchanged apart from adding a 'Region Midt' logo (logo for the Central Region of Jutland) at the upper right corner.

Corneal optics: higher-order aberrations and densitometry

Pentacam® HR (software version 1.20r41) was used in automatic release mode. Scans were repeated until quality score was satisfactory: scans with 'pathological' QS scores were omitted and scans with 'conspicuous' QS scores were examined closer, and if there were warning/error messages regarding a measurement, this was omitted. Anterior and posterior higher-order aberrations (HOAs) were calculated as the total root mean square (RMS) of the aberrations from the third to the eighth Zernike order in a 5-mm-diameter central zone. Anterior densitometry was measured from the anterior 120 μm of the cornea, and posterior densitometry from the posterior 60 μm of the cornea. The Pentacam® software rates the densitometry in arbitrary units from 0 to 100 (100 = complete opaqueness) based on average pixel intensity value (scatter) from Scheimpflug images.

Corneal thickness

Anterior segment OCT was used to monitor the central corneal thickness (CCT_{total}) of the entire cornea and of the following sublayers: epithelium (CCT_{epi}), recipient stroma (CCT_{stroma}) and graft (CCT_{graft}). Of note, the focus light of the anterior segment OCT was turned away from the patient's line of sight to avoid affecting retinal photoreceptors prior to measuring BCVA and contrast vision. Patients fixated on the back of the light casing instead. Measurements were taken on the right side of the light reflex in the Scheimpflug image at 800% magnification. Care was taken to not get close to the light reflex as increased reflections could obscure the true border of the epithelium. The location of the light reflex on the live IR image was controlled along with head positioning and apparatus alignment.

Contrast sensitivity

Contrast sensitivity was measured using the Freiburg acuity and contrast test, FrACT. This system uses an 8-alternative forced choice test using a Landolt C optotype of logMAR 1.1 (Snellen decimal equivalent: 0.08). No patient had trouble seeing this size of optotype at 100% contrast. The Landolt C ring is rotated into eight different positions and the subjects were asked to locate the gap. Based on the patient's answer (correct/incorrect), the program estimates the contrast threshold measured in Weber contrast units with an adaptive staircase procedure, based on maximum-likelihood calculations ('Best PEST'; Hertenstein et al. 2016). This value represents the reciprocal of the contrast sensitivity (CS). The logarithm of the CS (logCS) was used for analysis (Anton et al. 2014).

Ambient light was 200 LUX (LM-81LX, Lutron Electronic) measured at the patient's eyes. Test screen luminance was 300 cd/m^2 (Polyphor light, Block Optics Ltd., Dortmund, Germany). To simulate mild glare, a white light-emitting bulb was placed 25 cm above the test screen. Patients were encouraged to look at the screen until the optotypes had time to fade in and instructed to try as hard as possible to visualize the gap in the Landolt C ring. This was performed to allow for photoreceptor adaptation in the retina.

Nerve density measurement

In vivo confocal microscopy (IVCM) of the cornea was used to measure the density of nerve fibres in the SNP. The procedure was performed as previously described (Nielsen et al. 2013), except the red laser reflex was centred on the corneal apex. Patients focused on a white light source with the other eye. Three representative images of the SNP were chosen. Oblique sections were not included. Criteria were a localization of the nerves in the basal epithelial cell layer or in Bowman's layer. Nerve density was ascertained by the semiautomatic program NeuronJ (http://www.imagescience.org/meijering/software/neuronj/). The average density from the three images was used for data analysis. In cases where three representative images from the basal epithelial cell layer or Bowman's layer did not show any nerves, a nerve density score of 0 was entered into the database.

DSAEK and cataract surgery

DSAEK surgery was performed as previously described, albeit using precut donor tissue (Clemmensen et al. 2015). Briefly, the Descemet's membrane was stripped using Sinsky hook and forceps. The donor lamella was inserted through a 4-mm scleral incision using a Busin glide and forceps (Moria, France). After correct positioning in the anterior chamber, a large air bubble was insufflated to maintain contact between the donor lamella and recipient stroma. Patients were left supine for 2 hr after which the air bubble was reduced to approximately 50%. Patients were controlled daily during the four initial postoperative days, and again after 2 weeks, 1 month and at 3, 6 and 12 months. Postoperative medication was combined tobramycin and dexamethasone (Tobradex, Alcon, Texas, USA) eye drops 6 times/day, which was reduced to 4 times/day after the first postoperative week and slowly tapered over the next 6–9 months. In phakic patients, DSAEK surgery was preceded by cataract surgery.

In both the FECD and control group, cataract surgery was performed through a small (2.2- to 2.6-mm) scleral incision, followed by capsulorhexis, phacoemulsification and finally implantation of the intraocular lens in the capsular bag.

Treatment outcome: graft function (paper III)

Study design

In order to evaluate the functional state of the endothelium before and after DSAEK in patients with FECD, a prospective, controlled trial was performed. A group of patients with FECD eligible for DSAEK surgery (FECD group) and a group of cataract patients with normal corneas eligible for cataract surgery were enrolled (control group). Corneal oedema was induced experimentally and the capacity of the endothelium to control hydration was evaluated indirectly by monitoring the changes in CCT. Both groups were subjected to experiments before surgery and again 12 months after surgery.

Recruitment

Letters of invitation were sent out to FECD and cataract patients that were referred to the Department of Ophthalmology, Aarhus University. Patients were recruited consecutively from June 2013 to September 2014.

Inclusion criteria for the FECD group were the presence of FECD by slit-lamp investigation and eligibility for DSAEK surgery. Inclusion criteria for the control group were normal corneas by slit-lamp investigation and eligibility for cataract surgery. Exclusion criteria were the presence of any other type of corneal disease, prior eye surgery or a history of eye disease that could potentially affect the endothelium, that is glaucoma, uveitis or iridocorneal endothelial (ICE) syndrome.

The ethics committee of the Central Region of Denmark approved the study, case no. 1-10-72-36-13. All patients gave informed consent and investigations adhered to the tenets of the Declaration of Helsinki.

Experiments

Experiments were initiated between 8.00 and 10.00 am. All experiments and measurements were performed by the same investigator (EN) and carried out in the same room with ceiling lights turned on and blackout curtains drawn. This was performed in order to reduce the variation in ambient light.

After preliminary slit-lamp investigation, the central corneal EC density was measured using a Nidek CEM 530 (Nidek, Japan) specular microscope in automatic mode.

Central corneal thickness was measured by anterior segment OCT (AS-OCT; Spectralis, Heidelberg Engineering, Germany). The cornea was then anaesthetized using tetracaine eye drops (tetracaine hydrochloride 0.5%, minims, Bausch & Lomb) and a soft contact lens with low oxygen permeability was placed on the cornea (specifications: 500 μm centre thickness, 8.6 mm back curvature, polymacon, 38%, DK 7.9, Contamac, UK). Slit-lamp investigation was repeated to ensure the correct position and fit of the lens. Any air bubbles under the lens were removed by applying gentle pressure. All lenses achieved a good fit. The eyelid was taped shut and full closure of the eye was ensured. After 2 hr, another tetracaine drop was administered and the contact lens was removed. Central corneal thickness was measured immediately after removal and subsequently at 15, 30,

45, 60, 90, 120, 150, 180, 210 and 240 min. Patients were instructed to avoid prolonged eyelid closure after removing the contact lens. Measurements were obtained slightly adjacent to the central light reflex on the right side of the AS-OCT image. Averages from two measurements were used. Patients fixated on the white light source and the location of the light reflex on the live IR image was controlled along with head positioning and apparatus alignment. CCT of the fellow eye of participants was measured at the beginning and at the end of the experiment. This was performed in order to assess the extent of diurnal variation in the fellow eye.

There were no adverse events or safety issues.

Validation of specular microscopy measurements

A validation study of the specular microscope's precision was performed on DSAEK eyes with least one-year follow-up. These patients were chosen among regular outpatients who attended our clinic. Eyes were measured first in automatic mode and second in 'manually corrected mode'. In 'manually corrected mode', unconvincing cell tracings were manually deleted.

Statistical methods

Software and basic assumptions

Data were analysed using EXCEL version 14.4.7 (Microsoft, Redmond, Washinton, US), GRAPHPAD PRISM version 6 (CA, USA), SAS version 9.4 (NC, USA) and STATA 13.1 (Västervik, Sweden). Results are shown as means followed by the 95% confidence interval unless otherwise specified. A p value <0.05 was considered statistically significant.

Power calculations

Due to the unprecedented nature of the pathology study, a power calculation was not possible.

In the study of treatment outcome, assuming a power of 90% and an α-risk 0.05, nine patients were needed in the control group to detect a significant difference between pre-op mean Catquest-9SF and post-op mean Catquest-9SF (expected pre-op mean: -0.27 (SD $\pm$ 2.04); expected post-op mean -3.45 (SD $\pm$ 2.39). Data are available from Lundstrom & Pesudovs (2009), while no available data on patients with DSAEK.

In the graft function study, sample size showed that a sample size of seven patients was needed in each group (assuming a power of 90%, α-risk 0.05, independent *t*-test, expected baseline mean of 552.5 μm, postcontact lens mean CCT 602.3 μm, SD $\pm$ 28.3, data from pilot studies).

Pathology

In the pathology study, distributions of grades for COL8A1 and COL8A2 were compared between the FECD, PBK and normal groups in contingency tables. This was carried out by Kruskal–Wallis tests with ties, due to frequent recurrences of identical ordinal outcomes among groups. If the overall Kruskal–Wallis test showed significant differences between the three groups, pairwise *post hoc* tests were conducted by Wilcoxon rank sum tests with Bonferroni correction as this takes the ordering of grades into account. Interobserver agreement was assessed using weighted kappa, K.

Treatment outcome: visual acuity and patient satisfaction

In order to analyse the determinants of vision after DSAEK, a mixed-model (multilevel) repeated-measures analysis was performed. The model analyses the association between the entered variables between the specified time-points. This means that in the present study, the model includes the changes from pre-op to 3 months, from 3 to 6 months and from 6 to 12 months.

The model was fitted to the data while allowing for non-parallel development in the FECD and control group over time. The model allowed the variation on an individual level as well as eye level, thus accounting for within-subject correlation in control patients where both eyes were included. Further, the model takes into account that variables may display decreasing changes over time (exponentially decaying correlation structure). An examination of the residuals and fitted values along with QQ plots of the random effects did not give reason to doubt this model.

The dependent variables were BCVA (ETDRS letters) and contrast vision (logCS). The following independent variables were entered as covariates into a preliminary model: CCT_{total}, CCT_{epi}, CCT_{stroma} and CCT_{graft}. From this model, it was determined which of the thicknesses contributed most to explain the changes in ETDRS, and this parameter was entered into the final model. In accordance with the hypothesis, the final model then included the following independent variables (entered as covariates): anterior densitometry, posterior densitometry, anterior HOAs, posterior HOAs and the above-mentioned measurement of corneal thickness that contributed most to the preliminary model.

In a *post hoc* test, densitometry and HOAs from the anterior surface were paired as were densitometry and HOAs from the posterior surface. Each pair was entered into the model in order to assess whether the changes in anterior or posterior parameters had a significant effect on ETDRS.

In the FECD group, the association between SNP density and anterior corneal remodelling was investigated using the same repeated-measures mixed model analysis as described above. Anterior HOAs and anterior densitometry change as the optical quality of the anterior surface changes. Change in the optical quality of the anterior surface should theoretically be affected by anterior remodelling. Therefore, the mixed model included SNP density as the dependent variable the following independent variables (entered as covariates): anterior HOAs, anterior densitometry and CCT_{total}.

The model could not be expanded any further due to the low sample size.

Graft function

In the graft function study, data from the AS-OCT readings were expressed as percentage oedema of the maximum oedema, that is as the percentage CCT above baseline CCT at time = -120 min. These percentages were analysed in a repeated-measures mixed model that accounted for within-subject correlation and a decreasing correlation over time (exponential decay). As the preoperative interindividual variation was larger than both the similar postoperative variation in the FECD group and the similar variation in the control group, an additional variance component was added for the preoperative FECD group. Time, group and period were fixed factors in the model, and the de-swelling curves

could be compared as a whole or at specific time-points between groups as well as within groups. Adjustment for EC density and baseline CCT was made as a subanalysis on the postoperative data by entering these as covariates into the mixed model.

Summary of Results

Pathology (paper I)

Thirty-nine samples were collected in the FECD group, 10 samples in the PBK group and seven samples in the normal group. Groups were age- and gender-matched. Patient demographics are in Table 1. Routine negative controls on FECD, PBK and normal tissue samples did not stain.

In the COL8A1 staining experiments, grade 2 or 3 was given to a significantly higher proportion of FECD samples than PBK and normal samples ($p = 0.002$; Fig. 3). *Post hoc* pairwise analysis showed that the FECD group differed significantly from both the PBK ($p = 0.034$) and normal corneas ($p = 0.004$), whereas the distribution of grades was comparable between PBK and normal corneas ($p = 0.37$). In the COL8A2 staining experiments however, the distribution of grades was not significantly different between the groups ($p = 0.39$).

Three distinctive staining patterns were noticed (Fig. 4): a pattern with COL8 staining in linear structures across the entire DM was termed 'refractile strands' as it was similar to the pattern described in 2005 by Gottsch et al. (2005b). Also, a 'diffuse pattern' was noted in which the DM stained homogenously, as well as a 'lumpy' pattern in which the DM stained in confined areas with no significant staining between them.

We further noticed a substantial variation in staining intensity in the FECD group for both antibodies, but more pronounced for the COL8A2 antibody (Fig. 5).

Treatment outcome: visual recovery and patient-reported outcomes (paper II)

Subjects

A total of 41 patients with FECD (41 eyes) and 40 cataract patients (80 eyes) were included for pre-op investigations (see Fig. 6 for an overview of patient flow).

Table 1. Patient characteristics.

Group	*n*	Age Years ± SD	Sex M:F	Ethnicity % Caucasian
FECD	39	73 ± 9.7*	12:26*	≥97%*
PBK	10	69 ± 13	6:4	80%
Normal	7	65 ± 17	2:5	100%
		$p = 0.21^{\dagger}$	$p = 0.21^{\ddagger}$	

FECD = Fuchs' endothelial corneal dystrophy; PBK = pseudophakic bullous keratopathy; M = male; F = female.
* Calculated for 38 samples, because 1 FECD sample label was lost.
† unpaired *t*-test.
‡ chi-squared test.

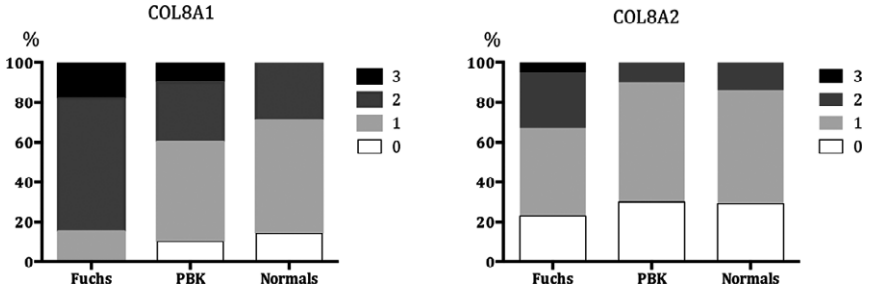

Fig. 3. Relative distribution of staining intensity. Intensity according to grading scale: 0 = no fluorescence; 1 = discrete fluorescence; 2 = marked fluorescence; 3 = intense fluorescence.

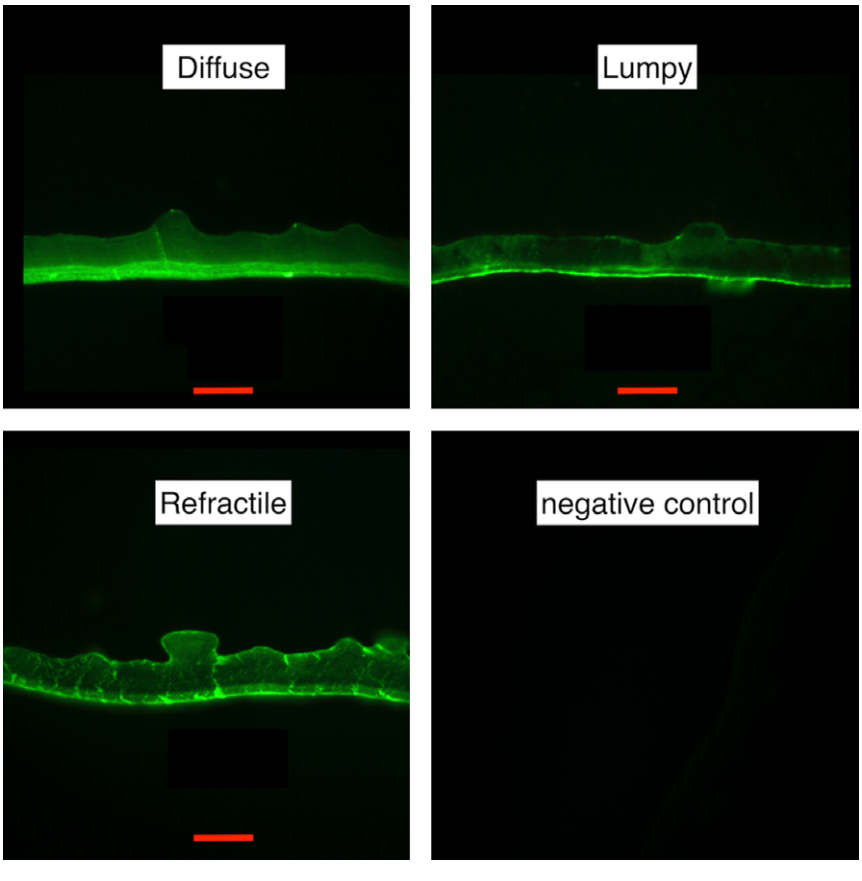

Fig. 4. Immunofluorescence of Descemet's membranes from patients with Fuchs' endothelial corneal dystrophy. Red bars = 20 μm.

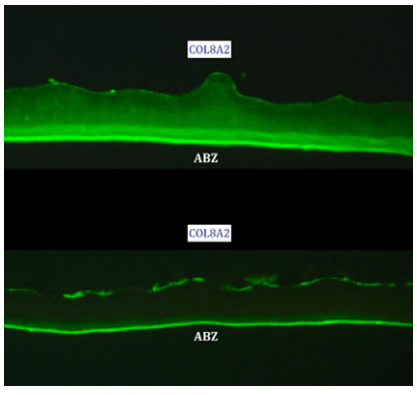

Fig. 5. Example of variation in COL8A2 staining in FECD samples.

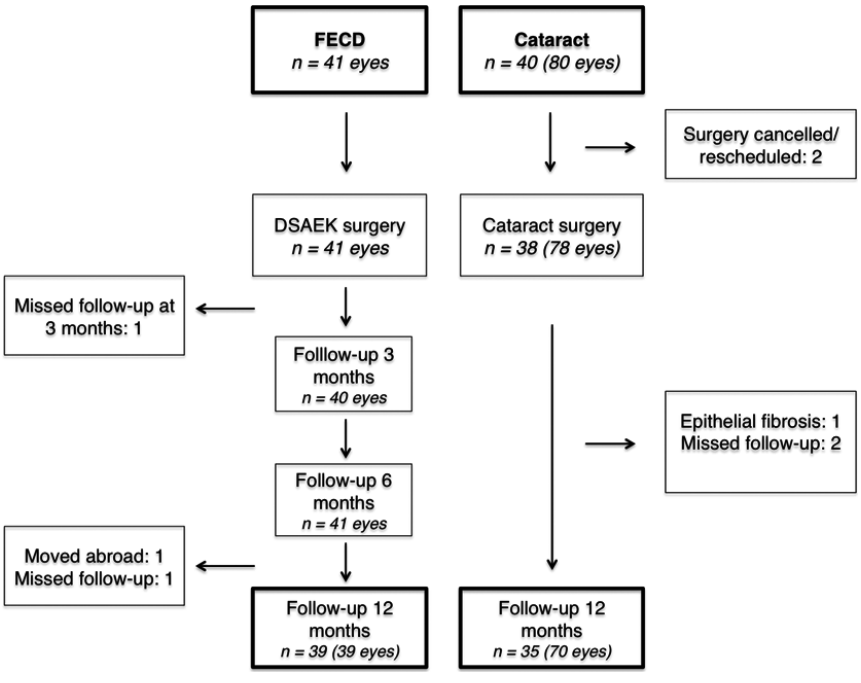

Fig. 6. An overview of patient flow.

In the FECD group, two patients missed follow-up visits at 12 months as one patient moved abroad and one patient was unable to arrange transport. Thus, 39 patients with FECD completed follow-up. In the control group, two patients had surgery cancelled/delayed, one patient had epithelial fibrosis and two patients were unable to attend the follow-up examination. Two eyes developed secondary cataract, received YAG treatment and were re-examined 2 weeks after. Consequently, 35 patients (70 eyes) in the control group completed the follow-up. Patient demographics are provided in Table 2.

Visual acuity, contrast sensitivity and subjective outcome

Briefly, BCVA, logCS and Catquest-9SF scores increased significantly from pre-op to post-op in both groups. BCVA and logCS were significantly better in the control group both pre-op and 12 months post-op. The Catquest-9SF effect size was 1.32 ± 0.84 (mean ± SD) in the control group, 1.84 ± 0.98 SD in patients with FECD who received phako-DSAEK and 1.37 ± 1.3 SD in patients with FECD who received DSAEK only. In the FECD group, 95% had a worse pre-op Catquest-9SF score than the average post-op score. In the control group, 90% had worse pre-op Catquest-9SF score than the average post-op score. Results are presented in Table 3 and Figs 7 and 8.

Determinants of vision after DSAEK

Higher-order aberrations and densitometry data are presented in Figs 9–11.

The preliminary repeated-measures mixed model was performed to investigate whether changes in CCT_{total}, CCT_{epi}, CCT_{stroma} or CCT_{graft} were associated with changes in BCVA. Results are in Table 4. CCT_{total} contributed most, and therefore, the final repeated-measures mixed model was an analysis of the changes in BCVA (ETDRS letters, dependent variable) and the following independent variables: anterior HOAs, posterior HOAs, anterior densitometry, posterior densitometry and total CCT. Results are in Table 5. BCVA had a significantly negative association with anterior HOAs ($p < 0.001$) and total CCT ($p = 0.002$). The regression coefficient for anterior HOAs was −8.4, indicating that an increase of 1.0 μm HOAs would cause decrease of −8.4 ETDRS letters (5 ETDRS letters = 1 Snellen line). The regression coefficient of CCT_{total} was −0.05, corresponding to a decline of five ETDRS letters with a CCT_{total} increase of 100 μm. In a *post*

Table 2. Patient characteristics.

				Cataract grade*				
	n	Age	Gender (F:M)	0	1	2	3	Pseudophakic
FECD	41	68.5 ± 8.1	26:15	9/26 (34%)	15/26 (58%)	2/26 (8%)	0/26	15/41 (37%)
Controls	40	74.3 ± 7.0	21:19	1/80 (1%)	24/80 (30%)	54/80 (68%)	1/80 (1%)	0
		$p = 0.001^{\dagger}$	$p = 0.32^{\ddagger}$	$p < 0.001^{\S}$				

FECD = Fuchs' endothelial corneal dystrophy; F = female; M = male.
Age values are means ± standard deviations.
* There were no patients with cataract grade 4 in either group.
† unpaired *t*-test.
‡ chi-squared test.
§ Wilcoxon rank sum test.

Table 3. Visual quality and patient-reported outcome.

	Group	Pre-op	3 months	6 months	12 months	Pre-op – 12 months*
Catquest-9SF	FECD	0.48 ± 1.47	−1.47 ± 1.31	−1.65 ± 1.48	−1.92 ± 1.25	<0.001
	Control	−1.28 ± 1.46			−3.17 ± 0.83	<0.001
BCVA	FECD	58.6 ± 12.2 (20/63)	66.9 ± 7.7 (20/50)	69.0 ± 8.0 (20/40)	73.7 ± 7.7 (20/32))	<0.001
	Control	71.1 ± 6.3 (20/40)†	–	–	84.9 ± 3.7 (20/20)†	<0.001
LogCS	FECD	0.85 ± 0.23	1.15 ± 0.24	1.23 ± 0.21	1.29 ± 0.21	<0.001
	Control	1.24 ± 0.17†	–	–	1.50 ± 0.14†	<0.001

Pre-OP = before surgery; FECD = Fuchs' endothelial corneal dystrophy; BCVA = best-corrected visual acuity in ETDRS letters followed by the equivalent Snellen acuity in parentheses; LogCS = logarithm to the contrast sensitivity. A higher value corresponds to a better ability to discern contrast; Ant = anterior; Post = posterior; HOAs = higher-order aberrations; Catquest-9SF = the applied questionnaire. A higher value corresponds to more experienced difficulty. Units are logits.
Values are means ± standard deviation.
* Repeated-measures mixed model.
† Statistically significant difference between groups (repeated-measures mixed model).

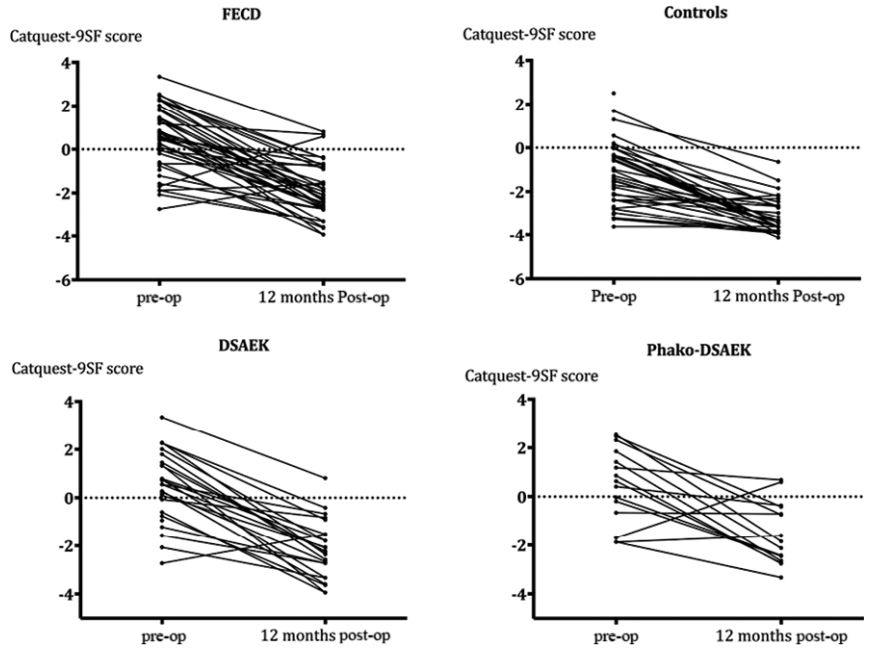

Fig. 7. The development in individual patient-reported outcomes (Catquest-9SF questionnaire). A lower value corresponds to less perceived difficulty in daily life from poor vision.

hoc test, anterior HOAs and densitometry had a significant effect on the model when entered as a pair ($p < 0.001$), but this was not the case when posterior HOAs and posterior densitometry were entered as a pair ($p = 0.52$). Further, CCT_{total} and anterior HOAs ($p < 0.001$) were significantly associated with each other. The regression coefficient of this relationship was 0.002, indicating that an 100-μm increase in CCT_{total} corresponded to a 0.2-μm increase in anterior HOAs.

The mixed model was repeated with logCS as the dependent variable and the same independent variables as in the BCVA. LogCS had a significantly negative (worse contrast vision) association with anterior HOAs ($p = 0.009$) and a significantly positive (better contrast vision) association with posterior densitometry ($p = 0.008$).

Sub-basal nerve density and anterior corneal remodelling

Results are in Table 6 and Fig. 12. In the FECD group, SNP density decreased significantly from pre-op to 3 months post-op ($p = 0.004$). There was no significant change in the FECD group from 3 to 6 months ($p = 0.43$), but the SNP density increased significantly from 6 months to 12 months ($p = 0.002$). There was no significant difference between pre-op SNP density and SNP density at 12 months in the FECD group. In the control group, the SNP density was significantly decreased 12 months after surgery. In the mixed model, there was a significant negative association between SNP density and anterior densitometry ($p = 0.004$), but not with anterior HOAs ($p = 0.63$) or total CCT ($p = 0.89$). Adjusting for age did not affect the results.

Treatment outcome: Graft function (paper III)

Subjects

Seventeen patients were enrolled in the FECD group and 15 patients were enrolled in the control group. One patient with FECD was unable to attend the follow-up measurement at 12 months. Two cataract patients cancelled surgery, and two cataract patients were unable to attend the follow-up experiments; thus 16 patients with FECD and 11 controls completed the follow-up experiments 12 months after surgery. Patient characteristics are in Table 7.

De-swelling curves

Before surgery, the unfitted de-swelling curves in the FECD group were more heterogeneous than curves in the control group, which displayed exponential decay-like properties (Fig. 13), and the fitted de-swelling curves were significantly different in an overall comparison ($p < 0.001$). Fitted de-swelling curves are in Fig. 14. Comparisons between groups at hours 1, 2 and 3 can be seen in Table 8.

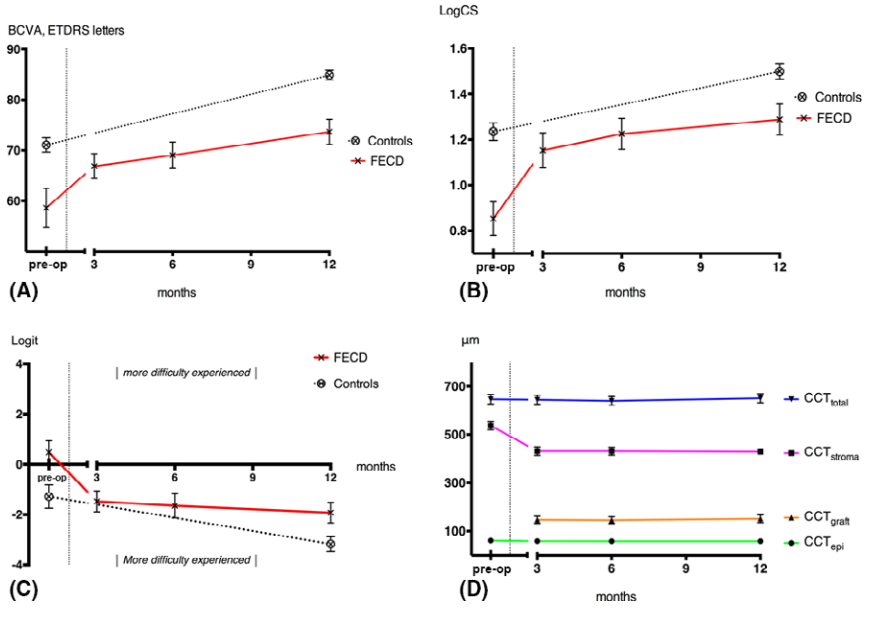

Fig. 8. Primary outcome variables in each group and the development in corneal sublayer thickness. (A) BCVA (ETDRS). (B) contrast vision (logCS). A higher logCS value corresponds to a better ability to discern contrast. (C) Catquest-9SF outcomes. (D) corneal thickness. CCT_{total} = total corneal thickness. CCT_{stroma} = recipient stromal thickness. CCT_{graft} = graft thickness. CCT_{epi} = epithelial thickness. Symbols = means, error bars = 95% confidence intervals.

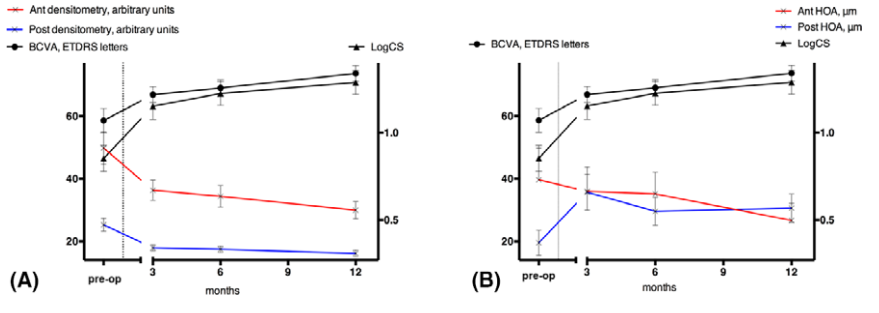

Fig. 9. Data from the FECD group only. Ant = anterior. Post = posterior. BCVA = best-corrected visual acuity. LogCS = logarithm to the contrast sensitivity. HOAs = higher-order aberrations. A higher value corresponds to a better ability to discern contrast. (A) densitometry, logCS and BCVA. (B) HOAs, logCS and BCVA. Symbols = means, error bars = 95% confidence intervals.

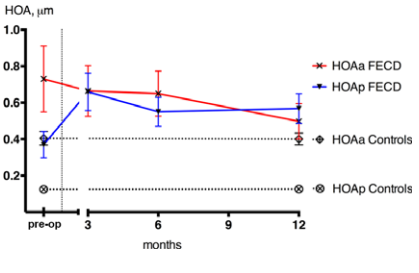

Fig. 10. Densitometry. Ant = anterior. Post = posterior. FECD = Fuchs' endothelial corneal dystrophy. Symbols = means, error bars = 95% confidence intervals.

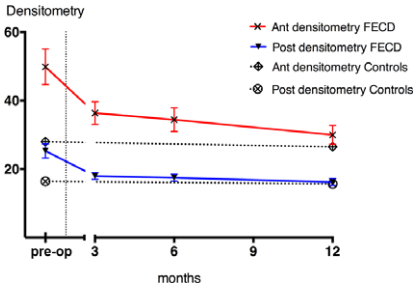

Fig. 11. Higher-order aberrations (HOAs). HOAa = anterior HOAs. HOAp = posterior HOAs. FECD = Fuchs' endothelial corneal dystrophy. Symbols = means, error bars = 95% confidence intervals.

Unfitted de-swelling curves became more homogenous in the FECD group after DSAEK and displayed exponential decay-like properties similar to the control group. In an overall comparison of the fitted de-swelling curves before and after surgery, there was a significant difference in the FECD group ($p = 0.011$), but not in the control group ($p = 0.14$). Twelve months after surgery, the fitted de-swelling curves from the FECD group and the control group surgery were significantly different ($p = 0.04$) in an overall comparison.

Table 4. Preliminary model.

	BCVA Coef.	p-value
CCT_{stroma}	0.01 (0.02; 0.04)	0.33
CCT_{epi}	0.30 (−0.11; 0.17)	0.47
CCT_{graft}	−0.01 (−0.07; 0.05)	0.48
CCT_{total}	−0.04 (−0.10; 0.01)	0.08

Preliminary repeated-measures mixed model. Coef.: regression coefficient.
Values in parentheses are 95% confidence intervals.

EC density and CCT

In the validation of the specular microscope readings, 22 DSAEK eyes with at least 1 year of follow-up were enrolled. The number of cells counted in automatic mode and manually corrected mode were 69 ± 25 and 62 ± 26 cells, respectively. EC density was 1611 (1468–1754) cells/mm^2 in automatic mode and 1604 (1443–1765) cells/mm^2 in manually corrected mode ($p = 0.65$). When entered into the mixed model as covariates, neither EC density ($p = 0.67$) nor baseline CCT ($p = 0.47$) had a significant impact on the model.

Discussion

Pathology (paper I)

In the pathology study, DM samples from FECD corneas had a higher proportion of marked/intense staining for COL8A1 than samples from PBK or normal corneas. This was not the case for the COL8A2 subunit.

In contrast to these findings, Gottsch et al. (2005b) used a similar immunofluorescence technique and reported increased staining for both COL8A1 and COL8A2 in DM samples from late-onset FECD when compared with DMs from normal corneas. The sample size in their study is not clearly specified, but it appears that they are studying three late-onset FECD corneas, one early-onset cornea and one control cornea. Also, a proteomic study from 2014 compared COL8A1 and COL8A2 levels between 10 FECD samples and four PBK samples using

Table 5. Final model.

	BCVA Coef.	p-value	LogCS Coef.	p-value
Ant HOA, μm	−8.4 (−12.5; −4.1)	<0.001	−0.19 (−0.3; −0.07)	0.002
Post HOA, μm	2.4 (−2.7; 7.4)	0.35	−0.09 (−0.22; 0.05)	0.20
Ant densitometry	−0.2 (−0.3; 0.0)	0.11	0.00 (−0.09; 0.00)	0.14
Post densitometry	−0.11 (−0.2; 0.4)	0.43	0.01 (0.00; 0.02)	0.008
Total CCT	−0.05 (−0.07; −0.01)	0.001	0.76 (0.30–1.21)	0.37

BCVA = best-corrected visual acuity; LogCS = logarithm to the contrast sensitivity; Coef. = regression coefficient; Ant = anterior; Post = posterior; HOAs = higher-order aberrations, root mean square from third to eighth Zernike order (μm); CCT = central corneal thickness.
Final repeated-measures mixed model. Values in parentheses are 95% confidence intervals.

proteomics and did not find significant differences in COL8A1 or COL8A2 levels (Poulsen et al. 2014). An often cited study by Levy et al. from 1996 reported large amounts of disorganized COL8 fibres in the PCL region of the DM in three FECD samples using immunocytochemistry (Levy et al. 1996), but the authors did not look at either subunit separately making it unsuitable for direct comparison with this study.

As noted here, the FECD samples displayed clear variation in staining intensity for both subunits. This was especially pronounced for COL8A2, where 23% of the FECD samples appeared completely devoid of staining despite the advanced disease state. As the studies mentioned above were based on small sample sizes, the results become susceptible to this variation. By comparison, this study used a systematic approach with a relatively large sample size and very good interobserver reproducibility. This provided a more robust approach, and for these reasons, it seems reasonable to assume that this variation is real, thus implying that pathophysiology in FECD is heterogeneous. This result is in line with the diverging findings from genetic studies of FECD (Baratz et al. 2010; Riazuddin et al. 2010a; Iliff et al. 2012; Wieben et al. 2012).

Unfortunately, one cannot assume proportionality between the emitted fluorescence and binding of the fluorophore to the primary antibody. This makes a numerical quantification of COL8A1 or COL8A2 subunits inaccessible. To overcome this problem, it was decided to use a subjective scale, and because two independent observers blinded from each other's answers achieved a weighted K of 0.84, the subjective scale was deemed a solid approach. Finally, an expert on immunofluorescence (Alexander V. Ljubimov Ph.D. D.SC., Cedars Sinai Medical Center, US) was consulted, who concurred with the subjective approach.

A potential source of error in this study is the risk of regional variation in FECD pathology. This is supported in part by the clinical appearance of FECD, which sometimes vary. Some areas may present with confluent guttae, while other areas have only few guttae, but in this study, the staining pattern and intensity varied independently on the presence of guttae. More importantly, the relatively large sample size in this study helps to minimize the influence of regional variation.

The increase in COL8A1 staining could in theory be caused by several different cellular mechanisms and does not necessarily translate into the increased production of the α1-chain. Other possible causes include the impaired degradation of the α1-chain and the improper protein folding that could lead to unmasking of COL8A1 epitopes recognized by the primary antibody (Meng et al. 2013). Consequently, this study does not answer why COL8A1 staining is increased.

Finally, it would have added a significant weight to this finding if links between COL8 staining variations and genetic variations could have been established and if another laboratory could have confirmed our findings by a different technique, such as proteomics.

Treatment outcome: visual recovery and patient-reported outcomes (paper II)

Vision and Catquest-9SF outcome

In this study, the results in terms of BCVA and contrast vision are on a par with the published literature on outcomes after both DSAEK and cataract surgery (Nielsen & Hjortdal 2012; Patel et al. 2012; Anton et al. 2014; Wacker et al. 2016a). Considering patient-reported outcome, a study from Sweden on 10886 cataract patients reported a mean effect size of Catquest-9SF of 1.35 (Lundstrom & Pesudovs 2009), which is similar to 1.32 that was found in the control group here. Pseudophakic FECD patients who received DSAEK surgery had a mean effect size of 1.37. Furthermore, 95% of the patients with DSAEK had worse pre-op Catquest-9SF score than the average post-op score. In support of these findings, Trousdale et al. (2014) reported a significant improvement in patient-reported outcomes using the 25-National Eye Institute Visual Function Questionnaire on 40 FECD patients treated with DSAEK. However, the 25-National Eye Institute Visual Function Questionnaire is not Rasch-validated, which impose serious limitations to its validity (Pesudovs 2006; Lundstrom & Pesudovs 2009).

Table 6. SNP density.

	Group	Pre-op	3	6	12	Pre-12 months
SNP density, μm/frame	FECD	613 ± 683	274 ± 341	391 ± 409	738 ± 606	0.21
	- DSAEK	325 ± 547	70 ± 135	301 ± 368	621 ± 520	0.05
	- Phako-DSAEK	769 ± 708	396 ± 371	454 ± 434	821 ± 660	0.7
	Control	1539 ± 700*	–	–	1298 ± 788*	0.003

FECD = Fuchs' endothelial corneal dystrophy; SNP = sub-basal nerve plexus; EC = endothelial cell; N/A = not available.
SNP density. Values are means ± standard deviations.
p-Values are from the repeated-measures mixed model.
* statistically significant difference between FECD and control group.

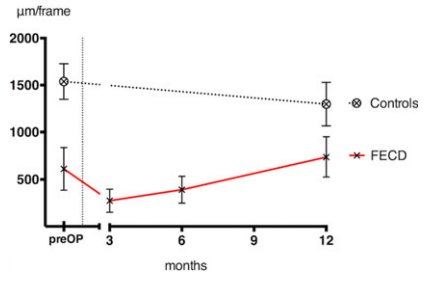

Fig. 12. Sub-basal nerve plexus density in the control group and FECD group. Symbols = means, error bars = 95% confidence intervals.

By contrast, the Catquest-9SF has been developed and validated by Rasch analysis, which is a prerequisite for high-quality questionnaires. However, the Catquest-9SF is not validated for DSAEK surgery, but a current study is investigating this, and so far, data suggest that the questionnaire retains good psychometric properties in patients with DSAEK (Mats Lundström, personal communication). For these reasons, it seems reasonable to conclude that DSAEK surgery leads to considerable alleviation of the difficulties experienced in daily life activities by patients with FECD.

The Catquest-9SF Questionnaire had to be translated from Swedish into Danish as mentioned, and there were no major obstacles in this process. Swedish and Danish are quite similar as they descend from a common ancient Nordic language, 'urnordisk' (prior to AD 500; Woodard 2008). After miniscule adjustments in question 6 and 7, we concluded that the meaning – as well as the intended meaning – of the sentences remained intact after translation. Therefore, there was no reason to suspect that the results were affected by the translation, but this cannot be ruled out, because a validation of the questionnaire in Danish has not been made. It would have been preferable to do a validation of the translated questionnaire by Rasch analysis before the study. We are currently undertaking such an investigation, but data are still too preliminary to warrant analysis.

Apart from the translation, there are other issues that merit attention when interpreting the Catquest-9SF results. Patients in the control group received surgery on both eyes, while only one eye was treated in the FECD group. Further, FECD can be regarded as a more grave diagnosis as opposed to cataract, which is a common disease with far better outcomes. Finally, the patients with FECD had worse mean Catquest-9SF pre-op when compared with controls, and it is possible that the scores are 'more easily' shifted in this part of the Catquest-9SF scale. For instance, the BCVA in the FECD group increased from 20/63 pre-op to 20/32 post-op Snellen units. This has a profound effect on the patient's capabilities. For instance, the patient would be able to drive a car again after surgery. This might have greater impact on subjectivity than improving from 20/40 Snellen units pre-op to 20/20 Snellen units post-op in the control group, where the image quality is 'simply' improved.

These factors may all have introduced confounding and selection bias with unpredictable psychometric effects, and therefore, a comparison of Catquest-9SF effect scores between groups was omitted. This study cannot be used to draw conclusions on whether DSAEK surgery or cataract surgery has the highest impact on Catquest-9SF effect size, but rather obtain a measure of overall effect size from each group as a stand-alone value.

Determinants of vision

Anterior HOAs and CCT_{total} exhibited a negative association with changes in BCVA after DSAEK. However, CCT_{total} did not change significantly in the follow-up period. Further, CCT_{total} was positively associated with anterior HOAs. Therefore, the detected association between CCT_{total} and BCVA was more likely due to poorer outcomes in patients with preoperatively thicker (i.e. more oedematous) corneas with advanced pathology.

Contrast vision was negatively associated with anterior HOAs and positively correlated with posterior densitometry. The latter does not hold meaning *per se*, but could be a result of a larger logCS gain in FECD patients with higher posterior densitometry pre-op (more pronounced FECD pathology). This could be detected as a positive association by the model, but as implied by a regression coefficient very close to 0, the effect was unimportant. Further, *post hoc* tests in the repeated-measures analysis on BCVA showed that the collective effect on posterior optical parameters did not have an effect on BCVA, whereas the anterior parameters did. For these reasons, it seems unlikely that posterior densitometry or CCT_{total} should be important determinants of vision after DSAEK. In contrast, anterior HOAs had a strong and meaningful association with post-DSAEK vision. The regression coefficients further indicated that the impact on BCVA and logCS was considerable.

In accordance with our findings, the existence of elevated anterior HOAs in patients with FECD before surgery has been demonstrated elsewhere and is believed to be a consequence of long-standing preoperative oedema (Amin et al. 2014; van Dijk et al. 2014; Wacker et al. 2015; Zhang & Patel

Table 7. Patient characteristics.

	n		Age	Sex	CCT*		EC density*	
	Pre-op	Post-op	Years	M:F	Pre-op	Post-op	Pre-op	Post-op
FECD	17	16	68.5 ± 6.7	7:10	634 ± 71	624 ± 44	N/A	1926 ± 334
Controls	15	11	70.4 ± 6.8	4:11	572 ± 35	577 ± 31	2261 ± 355	2226 ± 300
Donor	–	–	64.3 ± 13	13:3	N/A	N/A	2623 ± 248	N/A
			$p = 0.19^{\dagger}$		$p < 0.004^{\dagger}$	$p = 0.005^{\dagger}$	$p < 0.001^{\dagger}$	$p = 0.045^{\dagger}$

CCT = central corneal thickness in µm; Pre-OP = preoperative values; Post-OP = postoperative values; M = male; F = female; FECD = Fuchs' endothelial corneal dystrophy; N/A = not available.
Values are means ± standard deviation.
* endothelial cell density in cells/mm^2.
† unpaired *t*-test.

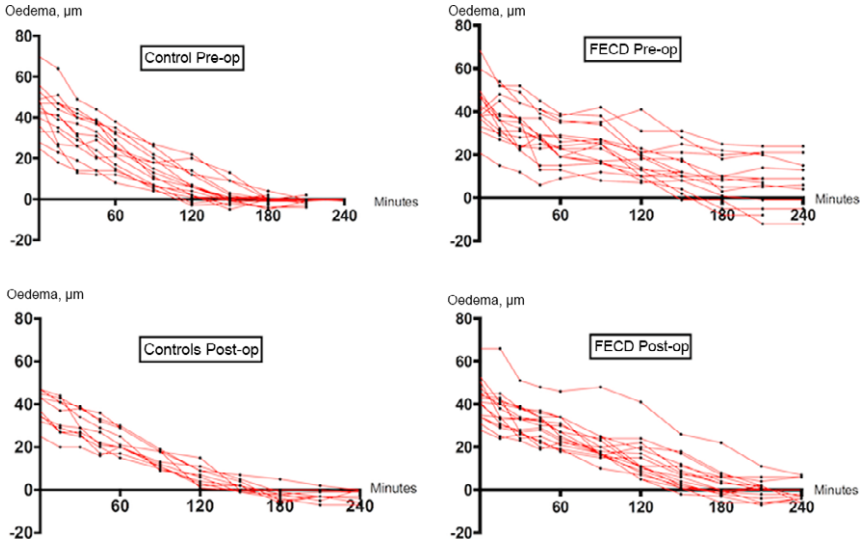

Fig. 13. Raw data presenting the unfitted de-swelling curves.

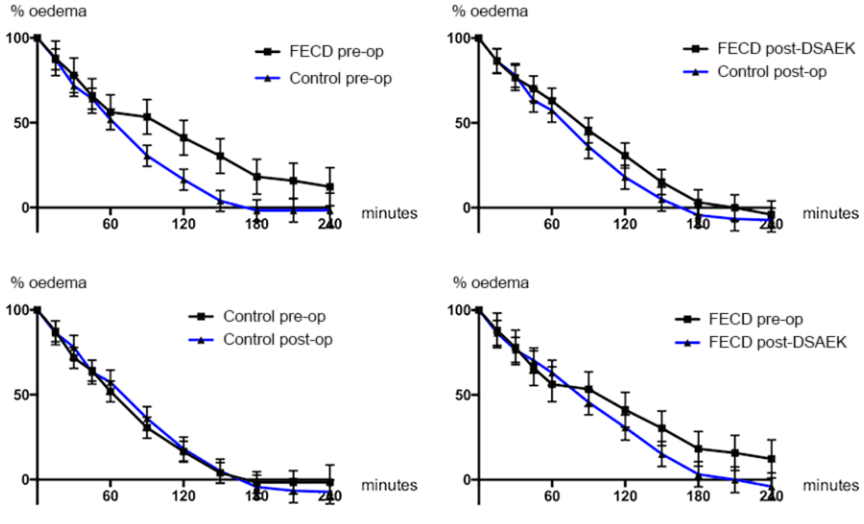

Fig. 14. Fitted de-swelling curves. Dots = means; error bars = 95% confidence intervals.

Table 8. Comparison of % Swelling Present Between Groups at Selected Time Points.

Label	Estimate*	p-Value	95%-CI Lower	Upper
FECD (pre-op versus post-op): 60 min	−6.8	0.256	−18.7	5.1
FECD (pre-op versus post-op): 120 min	10.5	0.085	−1.5	22.4
FECD (pre-op versus post-op): 180 min	15.0	0.015	3.1	26.9
Control (pre-op versus post-op): 60 min	−5.5	0.202	−14.0	3.0
Control (pre-op versus post-op): 120 min	−1.5	0.722	−10.0	7.0
Control (pre-op versus post-op): 180 min	2.7	0.538	−5.9	11.2
FECD versus Control (pre-op): 60 min	4.3	0.446	−7.0	15.6
FECD versus Control (pre-op): 120 min	24.8	<0.001	13.5	36.1
FECD versus Control (pre-op): 180 min	19.9	0.001	8.6	31.3
FECD versus Control (post-op): 60 min	5.6	0.231	−3.6	14.9
FECD versus Control (post-op): 120 min	12.8	0.007	3.5	22.1
FECD versus Control (post-op): 180 min	7.6	0.108	−1.7	16.9

FECD = Fuchs' endothelial corneal dystrophy; Pre-OP = preoperative; Post-OP = postoperative.
* Estimated differences in % swelling.

2015). Furthermore, the level of HOAs typically found in post-DSAEK patients sufficed to induce a significant reduction in vision in a study by McLaren & Patel (2012). In this study, they reported a 6.4 ETDRS letter decrease when normal subjects looked at custom-made aberrated ETDRS charts, which were aberrated to simulate typical HOAs after endothelial keratoplasty. This was in contrast to a modest 2.7 ETDRS letter drop, after forward scatter was introduced (subjects looked at charts through a standardized scattering solution; McLaren & Patel 2012). This makes sense as aberrations primarily affect the small angle domain of the retinal point-spread function, whereas scatter asserts an effect on the large angle domain (Seery et al. 2011). The resultant effects are that aberrations primarily affect BCVA, while forward scatter primarily affects CS. Further, two previous prospective studies (Yamaguchi et al., 2009; Patel et al., 2012) also reported a correlation between anterior HOAs and BCVA after endothelial keratoplasty (Yamaguchi et al. 2009; Patel et al. 2012). However, associations were calculated as Pearson's correlations at specific points in time, thereby limiting power. This yielded inconsistent results in the study by Patel et al., which imposed a limitation to conclusions. In the study by Yamaguchi et al., sample size was limited (n = 13), and two outliers could be seen in a scatter plot depicting logMAR VA versus HOAs. Therefore, this result should be viewed with caution.

In this study, we used a repeated-measures mixed model. This provides an important advantage, as this analysis includes changes in parameters between all time-points. This adds considerable power to our design, and this is the first prospective study that unequivocally points to anterior HOAs as a determinant of BCVA after DSAEK surgery.

There are, however, studies that do not support this conclusion. A recently published prospective study by Wacker et al. (2016a) found no correlation between BCVA and anterior HOAs at 5-year follow-up. The analysis performed was Pearson's correlation analysis at 5 years. This could be enough time for HOAs to become normal, but there was no control group and this remains speculative. This finding could also be a type 2 error (false negative)

due to low power even though the sample size was 34. Further, anterior HOAs decreased by a modest 10% over a 5-year period in the study by Wacker et al. This is contrast to the 38% decrease over a 12-month period noted here. We cannot explain this discrepancy, but the application of different measuring devices prevents direct comparisons. Wacker et al. used the Atlas system (Zeiss, Dublin, CA) and looked at the RMS of Zernike polynomials from the third to the sixth order, whereas we used the Pentacam HR as described in Corneal optics: Higher-order aberrations and densitometry. In a head-to-head test on healthy subjects, the Pentacam was superior to the Altas in terms of repeatability (de Jong et al. 2013).

Further, a prospective study by Hindman et al. could not detect a significant correlation between post-DSAEK BCVA and HOAs. They used a Spearman correlation analysis. Again, the sample size was 20, and the lack of power could be the issue. The authors used a customized Hartman Shack wave front sensor, which measures whole-eye aberrations. This means that one measurement is obtained from all of the refractive media in the eye: the entire corneal, intraocular lens and vitreous body. Therefore, the contribution of anterior HOAs from the cornea could be somewhat drowned out by 'noise' from HOAs from other parts of the eye, which do necessarily impact BCVA as much as HOAs from the anterior corneal surface.

Pantanelli et al. (2012). used adaptive optics to ascertain the level of BCVA after full aberration correction (lower-order and higher-order aberration) in patients after DSAEK surgery. Snellen acuity increased from 0.6 to 1.0 Snellen decimal units after correction, but as normal subjects can achieve BCVA of 1.6–2.3 Snellen decimal units after full aberration correction, other factors seem to limit vision. However, this study had a small sample size of 5, and the results should be regarded with caution.

From the start, it was deemed important to focus on clinically relevant parameters that can be easily obtained from commercially available devices in daily clinical practice. The intention was to provide a meaningful link to clinical practice. This also imposed certain limitations. The standard measure of posterior densitometry from the Pentacam is limited to the posterior 60 μm of the cornea. This means that scatter from the interface was not included, which could potentially have affected the results. Just one study has reported a significant correlation between interface reflectivity and BCVA (Heinzelmann et al. 2014). However, this study suffered from being retrospective, using a small sample size (n = 14) and including patients with a very large variation in follow-up after surgery (range: 2–11 months). Larger studies did not support this finding (van Dijk et al. 2014; Wacker et al. 2016a), and a recent review concluded that light scatter from the interface probably only has an effect in extreme cases (Turnbull et al. 2016). For these reasons, it is unlikely that a significant effect from interface scatter was missed in this study. A limitation of this study is that the sample size did not support a calculation of a 95% prediction interval of final BCVA based on pre-op anterior HOAs. Future studies of larger samples sizes are warranted.

Of note, the age matching was not optimal, as patients in the control group were slightly older. This was a consequence of differences in patient demographics as FECD causes difficulties earlier in life than cataract. However, this age difference was not a cause for concern as the corneal parameters in the control group were still superior to the FECD group.

What is more, phakic patients in the FECD group could have introduced selection bias by influencing pre-op measurements of BCVA and CS. This could also have influenced between-group comparisons, but 92% of the patients with FECD had no or slight cataract (grade 0 and 1) and such an effect is likely to be negligible.

Graft thickness – does it matter?

The influence of graft thickness on vision after DSAEK merits discussion in its own right, as the subject has been debated in recent years. Several developments in endothelial keratoplasty technique, such as 'ultrathin' DSAEK (Busin et al. 2013) and DMEK, are propelled by a belief that thinner grafts lead to improvements in visual outcome. Yet the evidence of this is lacking – both in terms of the published literature and on a theoretical level. In a recent meta-analysis including all published studies on the matter, it was concluded that there is insufficient evidence to support a clinically relevant impact of graft thickness on BCVA after DSAEK (Wacker et al. 2016b). In this paper, the authors elegantly argue that it is unclear why the addition of a thin posterior lenticule should impose a limitation on BCVA. They make two strong points. (1) Why should the addition of a relatively thin posterior lenticule limit vision given the wide range of normal CCT that does not affect vision? (Doughty & Zaman 2000), (2) The Beer–Lambert law states that even a doubling of the corneal thickness will only lead to a decrease in light transmittance of 4%, which will not have a great effect on BCVA. As mentioned above, an increase in light scatter from a thicker cornea will primarily reduce contrast vision and not BCVA (Seery et al. 2011). The findings presented here support that graft thickness has little impact on visual outcome after DSAEK.

However, these considerations are in discord with the reports of superior visual outcomes after DMEK compared with those after DSAEK (Kruse et al. 2014). This discrepancy could be explained in part if patients with DMEK in general are treated early, thus harbouring less anterior pathology. This was actually the case in a recently published paper comparing BCVA outcomes after the DSAEK and DMEK (Hamzaoglu et al. 2015). The conclusion was that DMEK performed superior to DSAEK as post-op BCVA was higher in the DMEK group. However, Table 2 shows that mean pre-op BCVA was 0.41 logMAR (0.4 Snellen decimal units) in the DSAEK group and 0.27 logMAR (0.5 Snellen decimal units) in the DMEK group ($p < 0.001$).

A recent review from 2015 on the DMEK literature concluded that 'most of the published literature on DMEK results are from similar cohorts by surgeons who pioneered and advocate this (DMEK) procedure'(Ang et al. 2015). On the other hand, paired studies of fellow eye comparisons of DMEK versus DSAEK showed better BCVA in the DMEK eye (Tourtas et al. 2012; Goldich et al. 2015), suggesting that there is an effect of the posterior lenticule after all.

In order to finally conclude on this issue, it may be required to do a randomized clinical trial of DMEK versus DSAEK in order to could avoid confounding and selection bias. This has not yet been performed.

Sub-basal nerve density

We find a significant decrease in SNP density after DSAEK followed by a subsequent recovery within 12 months after surgery. This is in agreement with the findings in a similar prospective study by Bucher et al. (2014) on FECD patients treated with DMEK. Of note though, the SNP densities reported by Bucher are almost double of what we find here, despite pre-op BCVA being comparable. However, Bucher et al. preselected 10 images for analysis from which they selected three images for final analysis, and this different approach may account for some of the discrepancy.

The SNP density in the control group was lower 12 months after surgery. This is in accordance with recent prospective studies by Misra et al. and De Cilla et al., who reported a similar decrease in SNP density after cataract surgery (De Cilla et al. 2014; Misra et al. 2015). De Cilla et al. ($n = 30$) reported that the SNP density recovered at 6 months after surgery, whereas Misra et al. found that SNP density did not recover at 6 months ($n = 23$). Both studies used similar methods, and discrepancies could possibly be due to differences in age, as patients were younger in the study by Cilla et al. (mean: 66 years $\pm$ 11). Yet, it is controversial whether age affects nerve health in the cornea (Shaheen et al. 2014). Due to low sample size, we could not test whether age was associated with changes in SNP density in the control group in this study.

In accordance with our findings, a previous study by Patel and McGhee reported that the SNP changed when investigating it at 6-week intervals, thus suggesting that the SNP is a dynamic structure (Patel & McGhee 2008).

There was a significant association between SNP density and anterior densitometry in the FECD group. These parameters are derived from the same corneal anatomical region, and as corneal nerves are known to play an important role in wound healing (Shaheen et al. 2014), this relationship suggests a link between anterior corneal remodelling and SNP density. This is an interesting finding, as the results from this study point to an association between post-DSAEK visual recovery and changes in the optical quality of the anterior cornea.

In vivo confocal microscopy scans were performed with the brightness setting in automatic mode. This feature is helpful when doing a scan, as the software constantly optimizes images by adjusting the brightness according to the reflectivity of the tissue. However, this also means that analysis of scatter from IVCM scans was unavailable. Another issue with IVCM scans is repeatability. The area analysed by IVCM (400 μm $\times$ 400 μm) represents approximately 0.13% of the corneal surface area, meaning that repeated scans most likely originate from different areas. This introduces random error, which is reflected by the large standard deviations (Table 6). This must be counterbalanced by a significant sample size. Based on the means, standard deviations, and sample size from this study, the power to detect a difference was 0.95; thus indicating a robust approach.

Treatment outcome: graft function (paper III)

For the first time, we were able to indirectly study the functional capacity of grafted endothelium after DSAEK, by inducing corneal oedema and monitoring its subsequent clearance.

Corneal hydration control is the result of a balance between the pumping action of Na^+/K^+-ATPase in the endothelium, corneal swelling pressure (SP) and the permeability of the endothelium (see the pump-leak model, The cornea: evolutionary brilliance), which means that the effects of both SP and endothelial permeability must be considered when interpreting the results.

As mentioned in The cornea: evolutionary brilliance, swelling pressure results from anionic repelling forces on glycosaminoglycan molecules as they are forced into proximity by corneal deturgescence (Fischbarg & Maurice 2004). Consequently, the SP decreases when corneal oedema increases the distance between the anionic charges (Olsen & Sperling 1987). Before surgery, corneal oedema in the FECD group resulted in a higher baseline CCT than in the control group, and after surgery, baseline CCT was still higher in the FECD group, this time due to the added posterior graft. Further, the posterior part of the cornea tends to swell more than the anterior part of the cornea (Sondergaard et al. 2013). Consequently, the SP was presumably less in the FECD group than in the control group both before and after surgery. This should make it easier for the endothelium to remove the oedema due to less opposing force, but on the other hand, the increased volume would also take longer to clear. We cannot be sure of the resultant effect of these forces on our results, but an analysis of the potential influence of baseline CCT as a covariate on de-swelling curves did not detect a significant effect.

Concerning the permeability of the cornea, it has been demonstrated previously that the endothelium retains normal permeability in various stages of FECD (Wilson et al. 1988), implying that leak should be comparable between the FECD and control groups.

In similar experiments conducted by Polse et al. in 1985, it was demonstrated that recovery from corneal swelling was slower and incomplete when evaporation was precluded (O'Neal & Polse 1985). Here, the corneal surfaces were intact in both groups, and evaporation should therefore be similar.

Therefore, it seems reasonable to compare the de-swelling curves between the FECD group and control group.

During the first hour, de-swelling curves were strikingly similar and looked linear (Fig. 14). This phenomenon cannot be explained fully, but may result from corneal evaporation being the dominant force during this time, as suggested in experiments by O'Neal & Polse (1985).

Before surgery, the de-swelling rate slowed down in the FECD group, and at the end of the experiment, 12.3% (1.9–22.7) oedema remained. Such a finding is expected, as the number of Na^+/K^+-ATPase pumps/cell is reduced in advanced stages of FECD (McCartney et al. 1987), but de-swelling capacity in patients with FECD could also be caused by reduced EC density. Unfortunately, as EC density readings were unavailable prior to surgery, this remains unknown. Also in line with previous studies, the variability between subjects was high in the FECD group

(Mandell et al. 1989; Saini & Mittal 1996). Taken together, these observations add weight to the integrity of the experimental model.

Comparing the de-swelling curves in the FECD group before and after surgery, there was a significant improvement after surgery, where baseline CCT was reached as fast as in the control group. This implies that grafted ECs are viable and working at near-normal capacity. Further indicative of an improvement in endothelial function, the unfitted de-swelling curves became more homogenous after DSAEK and displayed exponential decay-like properties similar to the control group (Fig. 13). In the control group, the fitted de-swelling curves did not change significantly.

A significant difference was detected when comparing post-op de-swelling curves between the FECD group and the control group: After 2 hr of de-swelling time, there was 12.8% (3.5–22.1) more oedema in the DSAEK-grafted patients than in the control group. The implication of this finding is unclear, but it could be caused by a kind of 'delayed endothelial response' or slightly more hydrated stroma in DSAEK eyes from diurnal variation. Further, the DSAEK group had increased baseline CCT as mentioned above, which could take longer to clear. These explanations are hypothetical, and the true meaning of this finding is unclear.

In terms of percentage swelling induced, the results from this study were in unison with a study by Saini & Mittal (1996) who did not find a difference in the amount swelling induced when comparing 15 FECD eyes with 15 control eyes. In contrast, a study by Mandell et al. reported less swelling induced in a group of 22 patients with FECD when compared with eight controls. Both of these studies used an experimental model similar to the one applied here. However in these experiments, corneal oedema is a result of an osmotic effect from the accumulation of lactate in the stroma, which is caused by epithelial anaerobic metabolism (Bourne & McLaren 2004), and the informative value of percentage swelling induced as a parameter of endothelial function is limited.

There are several potential limitations to this study. Investigations were initiated in the morning, and diurnal variation in the FECD group could have influenced the results. However, we evaluated the extent of diurnal variation in patients by monitoring CCT of the fellow eye as well. There was no significant change between CCT at the beginning of the experiment and CCT at the end of the experiment, indicating that diurnal variation was unimportant. Yet, as the worst eye is chosen for DSAEK surgery, some degree of diurnal variation cannot be ruled out.

An effect of baseline CCT could not be detected, but because sample size was small in this study, this could be a type 2 error (false negative) from insufficient power. A true effect of baseline CCT could still exist, but the finding implies that such an effect was most likely of limited size.

Further, the specular microscope could not acquire images in the FECD group pre-op due to corneal opacities. This limits the interpretation of the pre-op results as discussed above. Of note, we learned from the prospective study on BCVA outcome after DSAEK that IVCM was able to produce images of the posterior corneal surface in all patients with FECD. However, the endothelial cells were often obscured by the pronounced pathology and ECs could not be identified. It seems that *in vivo* visualization of ECs in advanced stages of FECD is unavailable by today's technology.

The issue of evaporation could have been avoided if subjects had been instructed to keep their eyes closed for the duration of the experiment. This approach could be considered for future studies.

Also, it would have added great value if a group of FECD patients treated with PK could have been included in this study. This is highly warranted considering the reports of increased late graft failure after DSAEK when compared with PK (Coster et al. 2014; Greenrod et al. 2014). Including a PK group in this study could have elaborated on whether late graft failures are related to endothelial function.

Conclusions

Pathology (paper I)

In an immunofluorescence study of FECD, the staining of COL8 subunits α1 and α2 (COL8A1 and COL8A2) within Descemet's membrane (DM) samples was analysed. Using a systematic approach, 39 DM samples from patients with FECD were compared with 10 DM samples from patients with PBK and 7 DM samples from normal corneas. Results showed increased staining for COL8A1, but not COL8A2, in FECD samples. Further, there were clear variations in the staining intensity, especially for COL8A2. These findings indicate pathophysiological heterogeneity, which mirrors the results from genetic research.

Treatment outcome: visual recovery and patient-reported outcomes (paper II)

A prospective, controlled study comparing 41 patients with FECD with 40 cataract patients with normal corneas was carried out. Patient-reported outcome was ascertained by Catquest-9SF Questionnaires, and the results showed that DSAEK surgery leads to a remarkable alleviation of the difficulties experienced in daily life activities by patients with FECD. This is an important finding, as the socio-economical benefit of medical treatment in today's society is a high priority.

Results showed a significant association between anterior HOAs and visual recovery after DSAEK. This finding suggests that the sustained anterior corneal pathology could be a primary obstacle for visual recovery. Finally, anterior corneal remodelling after DSAEK may be linked to the changes in nerve density.

Graft function (paper III)

Using an experimental model, we were able to induce a safe yet measurable amount of corneal oedema in human subjects and monitor the subsequent de-swelling as an indirect measure of endothelial function. In a prospective study, a group of patients with FECD was compared with an age-matched control group. Twelve months after DSAEK, the grafted endothelium cleared the induced oedema as fast as controls. This indicates that grafted endothelium is viable and most likely close to physiological capacity 12 months after DSAEK surgery. However, significant differences in de-swelling curves were detected after

surgery, which may be explained by the added stromal volume from the graft.

Perspectives

A medical treatment for FECD is highly warranted, as there is a shortage on donor tissue. In the pathology study, the results suggested heterogeneous FECD pathology, which may have implications for future research in FECD pathology. If FECD is indeed comprised of a group of underlying diseases that ultimately lead to the same phenotype, then it could be thought of as a clinical 'entity' – much like 'uveitis' or 'glaucoma' – rather than a specific disease. This is important as the mechanisms of these different diseases may differ as well, thus requiring unique therapies aimed at different targets. The next step along this line of research would be to confirm these findings with other technologies, and further try to establish links to genetic research.

Ultimately, we still lack an understanding of FECD pathology and medical therapy could be far off into future. Until then, the mainstay of treatment is corneal transplantation, but visual outcomes are still not optimal after DSAEK. This study pointed to a strong association between visual rehabilitation after DSAEK and anterior HOAs. This provides a basis for optimizing outcome after DSAEK through improved patient selection. If DSAEK surgery is performed before a certain level of anterior HOAs is reached, then visual recovery will depend less on slow anterior corneal remodelling. This could speed up recovery times and perhaps also improve final visual outcome.

In this regard, anterior HOAs are presented on screen from a normal Pentacam scan, not requiring any additional postanalysis modifications of any kind. Further, performing such a scan does not require specially trained personnel. Measurement of anterior HOAs is therefore readily accessible for future preoperative assessments of patients with FECD.

We also noted a link between anterior corneal remodelling and corneal nerve density, and this opens up the theoretical possibility for targeting corneal nerve health in order to facilitate corneal recovery. As FECD is the leading cause of corneal transplantation in industrialized countries, the benefits of improved outcome for patients and society are appreciable.

Finally, the results further suggest that graft endothelial function after DSAEK is close to normal 12 months after surgery, which bodes well for graft longevity. The model used here could be applied in future studies of DMEK surgery in order to ensure the long-term durability of these grafts as well.

Danish Summary

Dette projekt havde til formål at undersøge uafklarede forhold vedrørende hornhindesygdommen Fuchs' endoteldystrofi (FECD). Sygdommen fører til ophobning af proteinholdigt materiale på hornhindens inderside, og henfald af endotelceller som normalt sørger for at opretholde hornhindens transparens. Disse forandringer fører til alvorlig synsnedsættelse. Den eneste behandlingsmulighed er hornhindetransplantation, der med FECD som den førende årsag er den hyppigst udførte vævstransplantation blandt verdens industrialiserede lande.

Årsagen til FECD er uafklaret, men genetiske studier har vist at FECD kan skyldes mutationer i flere forskellige gener. Forskning i sygdommens patofysiologi indikerer, at proteinet kollagen type VIII (COL8) spiller en central rolle, men resultaterne har været svingende idet nogle studier har vist at der ophobes COL8, mens andre ikke har kunnet genfinde dette. COL8 består af to dele, COLA1 og COL8A2, der sættes sammen til det færdige protein. Vi foretog et systematisk studie af disse proteiners forekomst på 39 vævsprøver fra patienter med FECD og sammenlignede disse med 10 vævsprøver fra patienter med pseudofak bulløs keratopati (PBK) og 7 vævsprøver fra normale hornhinder. Resultaterne viste, at der var en øget mængde af FECD vævsprøver der indeholdt ophobet COL8A1, mens dette ikke var tilfældet for COL8A2. Derudover kunne det ses, at der var stor variation i proteinmængden blandt FECD prøverne, hvilket understøtter den variation der ses i resultater fra genetiske studier. Samlet set indikerer disse resultater at FECD kan skyldes flere forskellige underliggende sygdomme, hvilket har betydning for fremtidigt arbejde mod en medicinsk behandling af sygdommen.

Hornhindetransplantation har udviklet sig over det seneste årti. Tidligere udskiftede man hele den centrale del af hornhinden, hvorimod man nu anvender en teknik der udelukkende udskifter hornhindens syge inderside med en rask inderside fra en donor. Denne teknik kaldes Descemet's stripping automated endothelial keratoplasty (DSAEK). Teknikken har flere fordele i forhold til den tidligere udførte metode, og det er blevet den hyppigst udførte metode til hornhindetransplantation. Ofte bliver synet dog ikke fuldt rehabiliteret, selv i øjne der i øvrigt er raske. Dette har man ikke kunne finde en entydig forklaring på, og forskningsresultaterne på området har peget på både forreste og bageste forandringer i hornhinden som årsagen. Derudover er der ingen studier af patient tilfredsheden efter DSAEK med Rasch validerede spørgeskemaer. Disse forhold blev undersøgt nærmere i et prospektivt studie. En gruppe af 41 FECD patienter der blev behandlet med DSAEK, blev sammenlignet med en gruppe af 40 grå stær patienter med normale hornhinder (kontroller) der blev behandlet med grå stær operation. FECD gruppen blev undersøgt før operationen og 3, 6 og 12 måneder efter. Kontroller blev undersøgt før operation og 12 måneder efter. Optiske måleparametre fra hornhinden og nervetætheden i hornhinden blev monitoreret undervejs, og patienterne udfyldte desuden et Rasch-valideret spørgeskema. Resultaterne viste, at patienterne oplevede en betydelig lindring af det besvær de oplevede i deres dagligdag som følge af synsnedsættelse. Derudover viste resultaterne, at ændringer i synet efter DSAEK primært har en sammenhæng med ændringer i den optiske kvalitet fra hornhindens forflade, samt at ændringer i lysgennemskinneligheden af hornhindens forreste del har en signifikant sammenhæng med ændringer i nervetætheden. Disse resultater kan bruges til at bedre synet efter DSAEK ved at udvælge patienterne til operation på et optimalt tidspunkt hvor højere-ordens aberrationer fra hornhindens forflade endnu er begrænsede. Dette vil muligvis kunne medføre både hurtigere genvinding af synet efter DSAEK, og et bedre endeligt syn.

Desuden var det en målsætning at undersøge funktionsdygtigheden af den indsatte donorskive. På indersiden af hornhinden findes et lag af endotelceller, der sørger for at holde hornhinden relativt dehydreret ved kontinuerligt at pumpe vand ud. Dette er en forudsætning for hornhindens transparens. Funktionsdygtigheden af donorskivens er ikke tidligere beskrevet, og ved at anvende en eksperimentel model var vi i stand til at skabe en kontrolleret mængde hornhinde ødem (øget vandmængde), og derefter indirekte observere hvorledes endotelcellerne var i stand til at fjerne dette ødem igen ved at monitorere ændringer i hornhindetykkelsen. I et prospektivt forsøg blev 17 FECD patienter sammenlignet med 15 grå stær patienter med normale hornhinder. Patienterne blev udsat for forsøg før operationen og 12 måneder efter. Som en indikation på modellens reproducerbarhed viste resultaterne som forventet en nedsat evne til at fjerne vand blandt FECD patienterne før operationen. Et år efter operationen var donorskivens endotelceller i stand til at fjerne vandet lige så hurtigt som dem i kontrolgruppen, hvilket indikerer at donorskiven er noget nær fuldt funktionsdygtig. Dog observerede man en signifikant forskel 4 timer inde i forsøget, hvor der var signifikant mere ødem i FECD hornhinder sammenlignet med normale hornhinder. Betydningen af dette er uklar, men kan skyldes at hornhinderne i FECD gruppen var tykkere.

English Summary

The purpose of this project was to investigate unresolved matters regarding the corneal disease Fuchs' endothelial corneal dystrophy (FECD). The disorder leads to the accumulation of protein on the inside of the cornea and accelerated loss of endothelial cells. These changes cause a substantial decline in visual acuity. The only treatment option is corneal transplantation, which is the most frequently performed tissue transplantation globally with FECD being the top indication. It is unknown what causes FECD, but genetic studies have demonstrated that mutations in several different genes can generate the phenotype. Research in pathophysiology indicates that the protein collagen type VIII (COL8) plays a central role, but the results have been divergent in that some studies reported an accumulation of COL8 on the inside of the cornea, whereas other studies did not. COL8 is comprised of two subunits, the COL8A1 and COL8A2. We conducted a systematic study on 39 corneal tissue samples from patients with FECD and compared these with 10 corneal tissue samples from patients with pseudophakic bullous keratopathy and seven tissue samples from normal corneas. Results showed a significantly higher proportion of FECD samples with increased amounts of COL8A1 when compared with the pseudophakic bullous keratopathy and normal samples. This was not the case for COL8A2. Furthermore, there was large variation in protein amounts in the FECD samples. This supports the variation found in genetic studies and suggests that FECD may be a result of different diseases that lead to the same phenotype. This has implications for future work towards a non-surgical treatment approach. Corneal transplantation has changed over the last decade. Previously, the entire central cornea was replaced by donor tissue, but the technique has changed into replacing only the diseased inside of the cornea. It is known as Descemet's stripping automated endothelial keratoplasty (DSAEK), and it has become the most frequently used method of corneal transplantation. DSAEK has several advantages when compared with replacing the entire cornea, but visual acuity is seldom fully restored even in otherwise healthy eyes. The reason for this has not been established as the results from studies on the matter have been in discord, linking post-DSAEK vision to different layers of the cornea. Further, no studies using Rasch-validated questionnaires have investigated the patient-reported outcome after DSAEK. These matters were investigated in a prospective, controlled trial. A group of 41 patients with FECD eligible for DSAEK surgery was compared with a group of 40 cataract patients with normal corneas (control group) eligible for cataract surgery. The FECD group was examined before surgery and at 3, 6 and 12 months after. The control group was examined before surgery and at 12 months after. The optical quality of the cornea (higher-order aberrations and densitometry) and the nerve density of the cornea were monitored. Subjects further filled out a Rasch-validated questionnaire. Results showed that changes in vision after DSAEK surgery are significantly associated with changes in higher-order aberrations from the anterior corneal surface. Also, a significant association between changes in densitometry from the anterior corneal surface and changes in nerve density was found. Further, the results demonstrated a remarkable alleviation of the difficulties experienced in daily life activities by patients with FECD from poor vision. These results can be used to improve vision after DSAEK by selecting patients at an optimal time where higher-order aberrations have not yet formed. This will presumably lead to faster visual recovery and improved final outcomes after DSAEK. Another goal of this project was to assess the functionality of the donor tissue (graft) after DSAEK. Endothelial cells reside on the inside of the cornea, and they continuously pump out water in order to maintain corneal transparency. The functional capacity of the graft after DSAEK has not yet been described, and by using an experimental model, we were able to induce a safe amount of corneal oedema. We subsequently observed how the endothelial cells removed this oedema indirectly by monitoring the changes in corneal thickness. In a prospective controlled study, 17 patients with FECD eligible for DSAEK surgery were compared with 15 cataract patients with normal corneas eligible for cataract surgery. Patients were subjected to experiments before and 12 months after surgery. As expected, the FECD corneas exhibited less capacity for removing oedema before surgery, thus giving weight to the reproducibility of the model. One year after surgery, the FECD corneas were able to clear the oedema as fast as the control group, thus indicating that the graft is at a near-normal functional state 12 months after DSAEK surgery. However, a significant difference was observed 4 hr into the experiment, where there was significantly more oedema in the FECD group compared with normal corneas. The significance of this finding is unclear, but it may be due to the differences in baseline corneal thickness.

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