Ophthalmol Ther
https://doi.org/10.1007/s40123-022-00481-3

ORIGINAL RESEARCH

Long-Term Graft Survival and Decline in Endothelial Cell Density Following Penetrating Keratoplasty with Organ-Cultured Corneas

Ali E. Ghareeb · Maria S. Figueiredo · Sayali P. Pradhan · Elinor Curnow · W. John Armitage · Francisco C. Figueiredo

Received: November 1, 2021 / Accepted: February 3, 2022

ABSTRACT

Introduction: Endothelial cell density (ECD) changes long after penetrating keratoplasty (PKP) of organ-cultured corneas have been little studied. We aim to calculate the point when ECD decline stabilises following PKP with organ culture stored corneas.

Methods: This is an observational study of first-ever PKPs and first-ever re-grafts, performed over 17 years under a single surgeon. ECDs were acquired at 3 and 6 months, 1 year post-graft and annually thereafter by specular microscopy. Time-dependent ECD data was fitted to a log-biexponential model.

Results: We studied 465 first-ever grafts and 128 re-grafts. Mean recipient age was 59 years (range 0–96 years; SD 22). Median follow-up was 5.7 (range 0.2–17.1) years. Probability of ED at 5 years in first grafts and re-grafts was 4.4% (2.6–7.1%) and 14.8% (8.3–23.2%). In first grafts, ECD loss reached 0.6% per annum at 7.9 (6.2–9.6) years post-operatively. The half-lives of ECD loss during the immediate post-operative period for first grafts, re-grafts, dystrophies, ectasias, and previous ocular surgery are 20.1 (14.9–30.9), 12.8 (6.9–79.4), 19.5 (13.1–37.7), 26.2 (16.2–68), and 11.6 (6.7–41.3) months, respectively. The half-life during this rapid phase of ECD loss has an inverse correlation with graft survival at 10 years ($r = -0.89$, $p = 0.02$).

Conclusions: Rate of endothelial decompensation is higher in first grafts than re-grafts. ECD decline stabilises 7.9 years post-operatively in first grafts but then becomes lower than the physiological loss expected. Further work is needed to verify whether organ-cultured grafts reach physiological levels of ECD loss faster than hypothermically stored grafts.

Keywords: Corneal Endothelium; Corneal Transplant; Endothelial Cell Density; Graft Survival; Organ Culture Techniques; Penetrating Keratoplasty

A. E. Ghareeb · F. C. Figueiredo (✉)
Biosciences Institute, Faculty of Medical Sciences, Newcastle University, Central Parkway, Newcastle upon Tyne, UK
e-mail: francisco.figueiredo@newcastle.ac.uk

A. E. Ghareeb · M. S. Figueiredo · S. P. Pradhan · F. C. Figueiredo
Department of Ophthalmology, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne, UK

E. Curnow · W. J. Armitage
NHS Blood and Transplant, Bristol, UK

W. J. Armitage
Translational Health Sciences, University of Bristol, Bristol, UK

Published online: 18 March 2022

Key Summary Points

Why carry out this study?

Two methods are employed in the storage of donor corneas: organ culture and hypothermic storage.

Endothelial cell loss in the donor cornea accelerates after penetrating keratoplasty.

This study was performed to find out when the rate of endothelial cell loss after penetrating keratoplasty with organ-culture stored corneas reaches physiological levels.

What was learned from the study?

Mathematical modelling suggests that in first-time keratoplasties, the post-operative rate of endothelial cell loss reaches physiological levels after 7.9 years, but then continues to drop to below physiological levels.

The initial rate of endothelial cell loss is strongly correlated with risk of graft failure at 10 years post-operatively.

Head-to-head trials are needed to determine the long-term differences in endothelial cell loss between organ-cultured and hypothermically stored corneas.

INTRODUCTION

When pathology irreversibly impairs the optical properties of the cornea, affecting vision and quality of life, a corneal transplant may be indicated. Endothelial decompensation (ED) accounts for the vast majority of graft failures after the first 5 years post-operatively [1]. It occurs when graft endothelium is unable to maintain corneal deturgescence, the probability of which is thought to increase as the endothelial cell density (ECD) drops [2–4]. Endothelial cell loss occurs post-operatively at an accelerated rate in transplanted corneas, and therefore factors which influence endothelial cell loss and function in corneal grafts are important [3, 5]. Although pre-operative graft ECD does not correlate with graft survival, 6-month post-operative ECD does, indicating that peri-operative ECD loss is important in long-term graft survival [3, 6–8].

Globally, two protocols are employed in the storage of donor corneas: organ culture at 28–37 °C and hypothermic storage at 2–8 °C [9]. While organ culture is used almost exclusively in Western Europe, in the USA 98% of retrieved corneas are stored in hypothermic solution [10, 11]. Whereas hypothermic storage is technically simpler and has a lower cost per unit, corneoscleral rims may be stored for 4–5 weeks in organ culture compared with up to only 14 days in hypothermic storage [10–12]. Theoretically, cell processes can continue in organ culture, and there is evidence of endothelial migration towards wounds in organ culture conditions [13]. There is, however, no conclusive evidence showing superior graft survival with organ culture or hypothermic storage [11, 14, 15]. Some retrospective studies suggest superior graft survival with organ culture, while others report no difference [15]. One prospective trial reported no difference in visual outcomes or survival; however, follow-up was only 2 years [15]. No studies have evaluated the differences in ECD loss or graft survival between organ culture and hypothermic storage using long-term data [10].

In this study, we evaluated the post-operative ECDs and graft survival in patients who received corneas exclusively stored in organ culture with the aim of identifying the point at which ECD loss normalises.

METHODS

The Newcastle Corneal Transplant Registry (NCTR) is a prospectively collected electronic database of donor and recipient characteristics, operative details, and follow-up data. It comprises all consecutive corneal transplants performed by a single surgeon in a single tertiary

referral eye unit at the Royal Victoria Infirmary in the North East of England between February 1997 and June 2014. This is a retrospective analysis of the NCTR data. In order to avoid correlated outcomes between operated eyes from the same patient, and because previous analysis has shown that a graft in the contralateral eye can improve the survival of the first graft [16], we included only the patients' first-ever graft in the analysis of first grafts. For the analysis of re-grafts, we included only the first-ever re-graft. The tenets of the Declaration of Helsinki were followed, and permission was obtained from the Clinical Governance Department at the Royal Victoria Infirmary. All patients signed a consent form agreeing to data collection, storage, and analysis for research purposes. Ethics committee approval was not required for this study owing to its observational nature; however, all patients were aware of the collection of their data for this study and signed a consent form at the time of enrolment. The study protocol was approved by the clinical governance committee.

Demographics and Clinical Outcomes

Data were collected on patients' demographics (age and gender) and clinical information including pre-operative risk factors, operated eye, best corrected visual acuity, indications for PKP, lens status (phakic/pseudophakic/aphakic), graft status and complications.

Specular Microscopy

ECDs were acquired at 3 months, 6 months, 1 year post-operatively and annually thereafter using the Noncon Robo series specular microscope (Konan Medical, INC; Tokyo, Japan) in semi-automatic mode.

To achieve an accurate measurement of ECD, at least 50 adjacent cells were manually selected on the specular photomicrograph section. The machine performed an automatic analysis of the selected area. This calculated the average number of cells per square millimetre, the endothelial cell population size and provided the minimum, maximum, and average cell size of the selected area. All specular microscopy was performed by trained senior optometrists in a clinic setting according to the same protocol.

Pre-operative donor cornea ECDs were obtained from National Health Service Blood and Transplant eye bank records. The ECD was determined at the end of organ culture storage just before transplantation. The criteria for cornea suitability and the protocol for ECD determination were described by Armitage and Easty [9]. In brief, corneas were stained with trypan blue followed by hypotonic sucrose to render the endothelial cell borders visible under transmitted light microscopy. All endothelial examinations were carried out by experienced eye bank staff.

Surgical Technique and Post-operative Management

All cases were performed with the same PKP surgical technique and post-operative management as previously reported [17]. Fifty-six percent ($n = 333$) of patients received a 7.5 mm graft, 24% ($n = 144$) received an 8 mm graft, and 12% ($n = 69$) received a 7 mm graft. The remaining 8% received various graft sizes between 6.5 and 12 mm. We routinely implanted a graft that was 0.25 mm larger than the host. Exceptions were large grafts (9.00 mm or more), for which we transplanted a graft 0.5–0.75 mm larger than the host. If the host was aphakic we transplanted a cornea 0.5 mm larger than the host.

Statistical Analysis and Modelling

In brief, we explored three different approaches for describing changes in post-operative ECD and found that a log-biexponential model gave the closest fit to our data. We used this log-biexponential model to calculate ECD half-lives and using the derivative of this model we were able to calculate the point at which ECD loss became similar to the physiological rate of 0.6% per annum. This is the average annual loss of total corneal endothelium in an adult [18].

Mathematical models may help understand the mechanisms causing ECD loss post-

operatively and predict graft failure [4]. Price et al. modelled the linear decrease in ECD following Descemet-stripping endothelial keratoplasty (DSEK) [19]. Armitage et al. were the first to describe the biexponential model of ECD loss after PKP [5]. This model describes the initial fast and subsequent slow decline in ECD post-operatively with two exponential terms:

$$d = P \times e^{-at} + Q \times e^{-bt} \quad (1)$$

where d = endothelial cell density (ECD) at time t; $P + Q$ = total ECD at time = 0; a and b are exponential rate constants.

A log transformation of this equation has been shown to have better fit to ECD data after PKP [4]:

$$\log d = \log\left[P \times e^{-at} + Q \times e^{-bt}\right] \quad (2)$$

A log-polynomial model proposed by Riddlesworth et al. accounted for correlation of repeat measures on the same patient over time and was found to have an improved fit to post-PKP ECD data than the biexponential or log-biexponential models, and therefore greater predictive power [4]. However, many biological processes are known to be fundamentally exponential, and furthermore, the interpretation of a third-order polynomial model is less intuitive. As an example of how the fast and slow components of the biexponential model may be linked to underlying biological mechanisms, Böhringer et al. demonstrated that the slow component may be influenced by migration of host endothelium in the remaining cornea bed into the graft, while the fast component may represent loss of donor endothelium in the graft [20].

Previous studies by Bourne et al. have used the biexponential model to calculate half-times for the fast and slow components of ECD loss following penetrating keratoplasty (PKP) for donor corneas which were predominantly stored in hypothermic conditions [5, 21].

In order to account for multiple causes of graft failure as competing risks, we modelled graft survival using cumulative incidence curves [22] with three possible events: graft failure from ED, graft failure from all other causes, and a third outcome for patients who died with grafts which were surviving at last follow-up. Comparison between cumulative incidence curves was made using Gray's test [23]. A graft which had cleared in the initial post-operative period was deemed to have failed if it underwent irreversible changes rendering useful vision impossible.

Biexponential, log-biexponential and log-polynomial models have previously been fitted to ECD data following PKP [4]. We conducted a sensitivity analysis to determine which model would fit our data best.

To fit the biexponential model to our ECD data, we used the non-linear least squares method with the Newton–Gauss algorithm. Only follow-up points with at least five patients were included in the biexponential models. The half-lives of the fast and slow components of ECD (in years) were calculated as $(0.693/a) \times 12$ and $(0.693/b) \times 12$, where a and b are the respective decay constants.

Unlike the biexponential and log-biexponential models, the log-polynomial $\log(\mathrm{ECD}_t) = \beta_0 + \beta_1 \times t + \beta_2 \times t^2 + \beta_3 \times t^3 + \text{error}$ accounts for correlated outcomes from repeated measures on the same subject over time. The parameters $\{\beta_i\}_{i=0}^{3}$ were assumed to be random effects varying between individuals according to a normal distribution [4]. The log-polynomial model was fitted to the ECD data using linear least squares regression.

We limited follow-up to 12 years post-PKP for all ECD analysis, as beyond this point the number of patients lost to follow-up becomes high.

Competing risks regression models [24] were used to study the association of pre-operative ECD and original pathology with graft failure from ED in first grafts. In the model of original pathology, the model included dystrophies, previous ocular surgery (POS), ectasias, infections and injuries as covariates.

Between-group comparison of mean ECD after year 7 post-PKP between ectasias and dystrophies was performed using a two-samples t test after confirming normality using the Shapiro–Wilk test.

Pearson's product-moment correlation coefficient was used to characterise the correlation between the rapid ECD half-life (calculated from parameter *a*) and cumulative risk of graft failure from ED.

All analysis was performed using the statistical programming language R [25].

The partial derivative of Eq. (2) with respect to *t* gives the rate of ECD loss at time *t*:

$$\frac{d}{dt} = \frac{ae^{at} \times P + be^{bt} \times Q}{e^{at} \times P + e^{bt} \times Q} \quad (3)$$

Nelson et al. determined the rate of ECD loss in subjects with healthy eyes and no previous surgery using confocal microscopy at two intervals 10 years apart [18]. Hence, we next calculated the point at which ECD loss equals that previously measured by Nelson et al. (approximately 0.6% per annum or 16.3 cells/mm^2/year) [18]. Solving for when Eq. (3) = 0.6% and using the parameter values from Table 5 allows us to calculate the point at which ECD loss reaches approximately physiological levels.

RESULTS

We studied first-ever PKPs and first-ever re-grafts in our consecutive patients. In total, 465 first-ever PKPs were recorded from 465 patients. Of the 465 recipients, 65 also received a PKP in the other eye, although these were not included in our study. Additionally, 128 patients had a first-ever re-graft, bringing the total number of PKPs in this study to 593. Mean recipient age at the time of operation for all patients was 59 years (range 0–96 years; SD 22) and 51.4% (n = 239) were male. Table 1 summarises the indication for first grafts.

Graft Survival

In total, 121 graft failures and 15 deaths were recorded during a median follow-up of 5.75 years (range 0.2–17.1; interquartile range 2.8–9.3; Table 2). Of the graft failures, 76 (62.8%) were due to ED, 20 (16.5%) to endothelial rejection and 7 (5.8%) to infection. In 10 patients, grafts failed as a result of a variety of other causes such as disease recurrence and trauma. In eight the cause was unknown. Forty-nine (53.8%) of the 76 failures due to ED occurred 5 years post-PKP or later.

Table 1 Summary of the frequency of original pathologies necessitating first penetrating keratoplasty

Indication for graft	Count	Percentage
Dystrophies (Fuchs' dystrophy, other dystrophies)	138	29.7
Previous ocular surgery (pseudophakic corneal oedema, aphakic corneal oedema)	100	21.5
Ectasias (keratoconus, other ectasias)	95	20.4
Infection (viral, bacterial, protozoan)	72	15.5
Injury (chemical, mechanical)	19	4.1
Unknown aetiology (phakic bullous keratopathy, unknown)	17	3.7
Ulcerative keratitis (rheumatoid arthritis, other)	9	1.9
Opacification (interstitial keratitis, other)	8	1.7
Miscellaneous (exposure keratitis, paediatric aphakia, other)	6	1.3
Anomaly (Peters' anomaly)	1	0.2
Grand total	465	100.0

Table 2 Numbers of patients in follow-up at the time points studied

	3 Months	6 Months	12 months	18 Months	2 Years	3 Years	4 Years	5 Years	6 Years	7 Years	8 Years	9 Years	10 Years	11 Years	12 Years	13 Years	14 Years	15 Years	Total
Lost to follow-up	0	5	1	2	2	3	4	6	2	6	2	0	1	3	0	0	0	0	37
In follow-up	458	443	437	385	391	358	317	284	235	192	152	129	113	79	60	49	35	20	
Unknown	1	5	8	14	7	11	17	6	11	9	6	5	8	4	2	1	1	2	118
Total	459	453	446	401	400	372	338	296	248	207	160	134	122	86	62	50	36	22	

The estimated probability of graft failure from ED in first grafts and re-grafts is given in Table 3 and displayed in Fig. 1a. The probability curves for failure of first grafts and re-grafts were significantly different for ED only ($p < 0.0001$, $\chi = 11.2$, Gray's test) and all-cause failure ($p = 0.01$, $\chi = 6.2$, Gray's test).

We compared the cumulative risk of failure due to ED for first grafts between the most common original pathologies and found the curves are significantly different ($p < 0.0001$, $\chi = 28.9$, Gray's test; Fig. 1b). Furthermore, the curves for graft failure due to causes other than ED were significantly different ($p < 0.01$, $\chi = 20.1$, Gray's test; Table 3).

In a regression analysis by original pathology, having an ectasia is associated with lower chance of graft failure due to late ED [$p < 0.01$, HR = 0.17 (0.05–0.6)]. Previous ocular surgery (POS) is associated with a higher chance of graft failure due to ED [$p < 0.0001$, HR = 3.4 (2.0–5.8)]. Dystrophies [$p = 0.27$, HR = 0.7 (0.4–1.3)], infections [$p = 0.31$, HR = 0.6 (0.2–1.6)], and injuries [$p = 0.24$, HR = 1.98 (0.6–6.2)] were not associated with significantly higher or lower risk of ED.

Specular Microscopy

In total, 472 first grafts and re-grafts (79.6%) from 442 patients contributed 2069 ECD measurements during follow-up and 533 pre-operative measurements were provided by the eye bank. Table 4 gives statistical summaries of ECD for each follow-up point.

The biexponential, log-biexponential and log-polynomial models of ECD loss were fitted to the first graft and re-graft ECD data (Fig. 2a and b respectively). Unlike previous analysis, we did not find that the biexponential model tended to overestimate ECD [4]. Furthermore, we found that all three models predicted a gradual rise in ECD after approximately year 7 post-PKP. Although the log-polynomial model accounts for correlated outcomes from repeated measures on the same subject, we found that the log-biexponential model provided an almost identical fit to the data. Furthermore, the log-biexponential model is more intuitive and easier to

Table 3 Summaries of cumulative incidence of graft failure following penetrating keratoplasty from ED and other causes

Subgroup	Cause of failure	Probability of failure				Lower CI				Upper CI			
		3 years (%)	5 years (%)	10 years (%)	12 years (%)	3 years (%)	5 years (%)	10 years (%)	12 years (%)	3 years (%)	5 years (%)	10 years (%)	12 years (%)
Re-grafts	ED	8.4	14.8	31.1	44.0	3.9	8.3	19.6	28.8	15.1	23.2	43.3	58.3
First grafts	ED	1.3	4.4	16.0	19.8	0.5	2.6	11.3	14.1	2.9	7.1	21.4	26.3
Re-grafts	Other	4.5	11.0	17.3	26.3	1.7	5.5	9.1	14.6	9.6	18.7	27.6	39.7
First grafts	Other	3.5	5.0	9.3	11.2	2.0	3.1	6.3	7.4	5.6	7.5	13.0	15.9
Dystrophies	ED	0.0	0.9	13.3	16.9	0.0	0.1	5.5	7.2	0.0	4.5	24.7	29.9
Ectasias	ED	0.0	0.0	4.0	4.0	0.0	0.0	0.7	0.7	0.0	0.0	12.4	12.4
Infection	ED	0.0	2.2	15.4	15.4	0.0	0.2	5.2	5.2	0.0	10.2	30.6	30.6
Injury	ED	0.0	19.6	29.4	29.4	0.3	4.4	7.7	7.7	24.3	42.8	55.7	55.7
Other	ED	0.0	14.5	22.7	22.7	0.2	4.4	6.7	6.7	14.2	30.3	44.4	44.4
Previous ocular surgery	ED	1.2	8.5	27.3	40.7	1.0	3.4	15.0	23.3	9.3	16.6	41.1	57.5
Dystrophies	Other	1.6	1.6	5.5	9.8	0.3	0.3	1.9	2.9	5.1	5.1	12.0	22.0
Ectasias	Other	0.0	1.6	1.6	1.6	0.0	0.1	0.1	0.1	0.0	7.5	7.5	7.5
Infection	Other	7.8	16.3	22.6	22.6	5.0	7.8	11.4	11.4	21.2	27.5	36.1	36.1
Injury	Other	5.9	11.8	31.4	31.4	1.8	1.8	7.7	7.7	31.9	31.9	59.2	59.2
Other	Other	11.0	11.0	11.0	11.0	3.4	3.4	3.4	3.4	23.8	23.8	23.8	23.8
Previous ocular surgery	Other	3.5	9.1	14.6	19.0	1.5	3.9	7.3	8.8	10.7	16.9	24.2	32.2

Results are shown for the most common pathologies and for all first-time grafts and all re-grafts at 3, 5, 10 and 12 years post-operation. Results are calculated using competing risks analysis

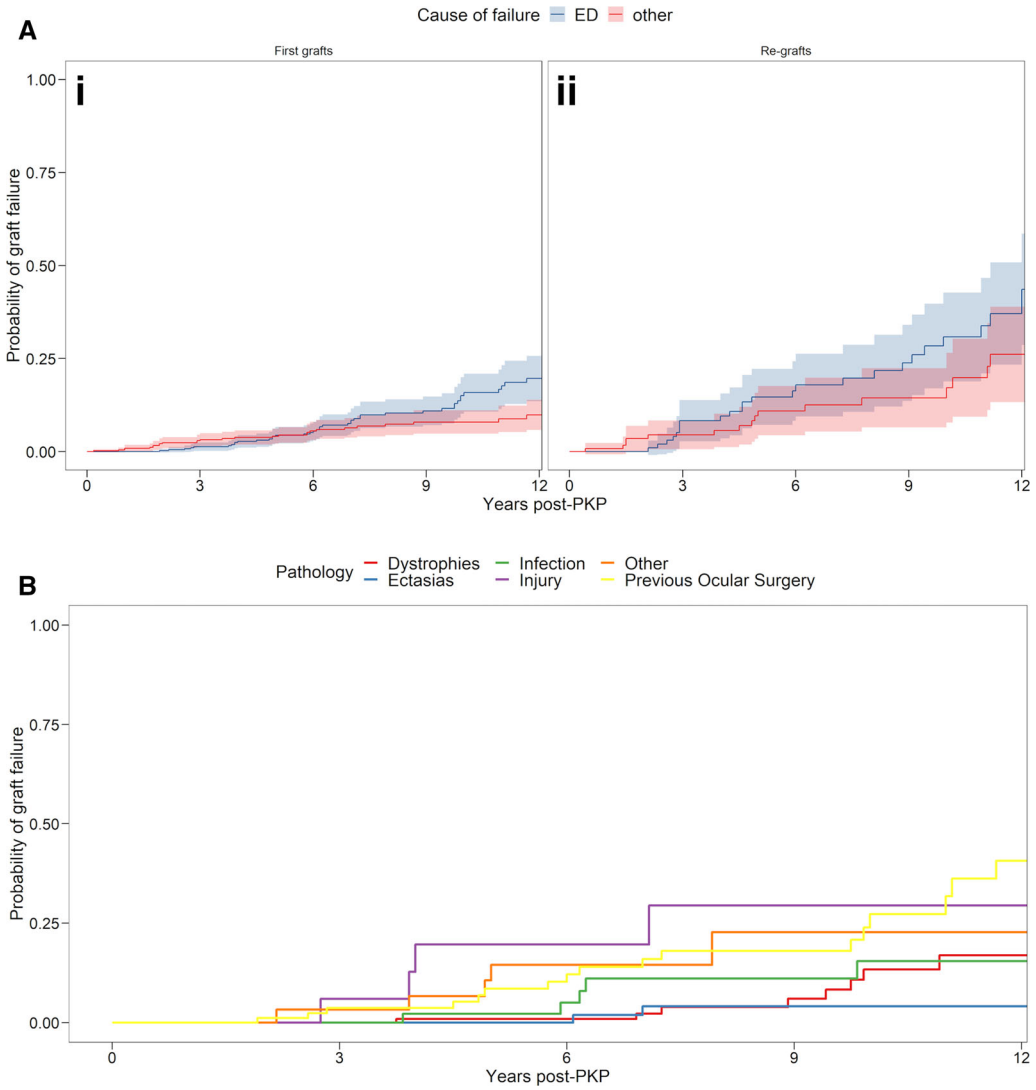

Fig. 1 **a** (i) Cumulative risk of graft failure for first-ever PKPs. (ii) Cumulative risk of graft failure for a patient's first re-graft. In both graphs, cumulative risk of graft failure secondary to ED (blue) and all other causes (red) is shown. Confidence intervals (95%) are shown by the respective shaded regions. **b** Cumulative risk of graft failure secondary to ED for first-ever grafts with subgroups divided by original pathology

interpret as it consists of two components representing fast and slow rates of change in ECD (see "Methods"). We therefore chose to use the log-biexponential model for further analysis.

The calculated parameters of the log-biexponential models along with derived half-lives for the fast and slow components of ECD loss and confidence intervals are given in Table 5. Figure 3 shows the model fit for first grafts, re-grafts and the most common original pathologies.

Our model gives a value of $t = 7.9$ (6.2–9.6) years for first grafts to reach approximately physiological levels of ECD loss (0.6% per annum). For re-grafts, the value is $t = 5.6$ (3.6–12.7) years (see "Methods").

Between years 7 and 10 post-PKP, the mean ECD increases by 74 cells/mm^2, although the confidence intervals overlap (Table 4).

Table 4 Summary statistics for the ECD measurements taken over 15 years of follow-up for first grafts and 11 years for the re-grafts

Time post-PKP (years)	First grafts					Re-grafts				
	n	Mean ECD	SD	Upper CI	Lower CI	*n*	Mean ECD	SD	Upper CI	Lower CI
0	356	2689.4	251.1	2715.5	2663.3	124	2715.7	235.6	2674.3	2757.2
0.25	51	1995.5	492.9	2130.8	1860.2	20	1688.4	685.5	1988.8	1388.0
0.5	122	1806.4	624.3	1917.2	1695.6	26	1673.8	506.6	1868.6	1479.1
1	188	1671.0	580.7	1754.1	1588.0	41	1436.5	554.0	1606.0	1266.9
1.5	136	1442.7	584.3	1540.9	1344.5	27	1111.6	467.8	1288.1	935.2
2	167	1268.0	579.9	1356.0	1180.1	37	1041.2	508.3	1205.0	877.4
3	207	1075.6	594.9	1156.6	994.6	41	933.7	523.2	1093.9	773.6
4	175	926.2	513.4	1002.2	850.1	25	721.8	484.2	911.6	531.9
5	174	839.9	494.2	913.4	766.5	21	733.1	473.4	935.6	530.7
6	125	798.5	476.3	882.0	715.0	16	534.4	133.5	599.9	469.0
7	99	677.6	358.5	748.2	607.0	17	572.0	188.3	661.5	482.5
8	73	676.7	308.8	747.6	605.9	5	1020.2	675.3	1612.1	428.3
9	66	676.3	316.4	752.6	600.0	11	701.9	325.0	894.0	509.9
10	65	751.6	346.0	835.7	667.5	4	702.8	245.0	942.8	462.7
11	36	699.7	362.9	818.2	581.1	6	741.8	286.4	971.0	512.6
12	31	728.7	265.2	822.0	635.3					
13	26	833.4	461.8	1010.9	655.9					
14	18	950.4	514.5	1188.1	712.7					
15	13	741.5	301.2	905.2	577.8					

Follow-up points with fewer than five patients were not used in the modelling

ECD endothelial cell density, *CI* 95% confidence interval, *n* number of patients, *SD* standard deviation, *PKP* penetrating keratoplasty

Nevertheless, ECD is expected to continue to decline at physiological levels, and not plateau. To investigate whether this was due to outlier measurements, we studied the ECD trajectories of individual surviving first grafts which had ECD measurements at both year 7 and year 10 post-PKP. Figure 4 shows that while some first grafts lose ECD between year 7 and year 10 post-PKP, many grafts show increases of around 200 cells/mm^2 or more.

Next, we analysed the first grafts by original pathology. ECD decline for all first-ever grafts and first-ever re-grafts is shown in Fig. 3a and b, for comparison. All pathologies showed a rapid initial decline followed by a plateau and then gradual recovery of ECD (Fig. 3c–f) apart from infections, in which ECD did not enter a plateau or recovery phase but continued to drop gradually throughout follow-up (Fig. 3e). This continued drop in ECD in patients with infections does not seem to be associated with increased

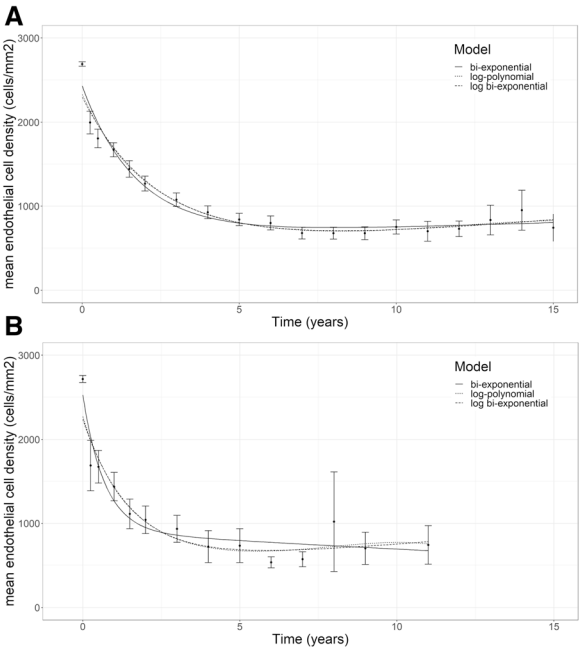

Fig. 2 Change in mean endothelial cell density with time for **a** first-ever grafts and **b** first-ever re-grafts. Biexponential (solid), log-polynomial models (dotted), and log-biexponential (dashed) have been fitted to the data to demonstrate the best-fitting model. Confidence intervals (95%) of the mean endothelial cell densities are shown

graft failure during this period of follow-up (Fig. 1b).

In ectasias and dystrophies, the graft ECDs appear to plateau at different ECDs (Fig. 3c and d). We compared the mean ECD of ectasias and dystrophies from year 7 onwards (by this point both pathologies appear to have plateaued). The mean ECD of ectasias from year 7 onwards was 807 cells/mm^2 versus 625 cells/mm^2 for dystrophies (mean difference = 182 cells/mm^2 (107–258); $p < 0.001$). This seems to correspond with higher numbers of graft failures secondary to ED in dystrophy patients after year 9 (Fig. 1b).

Patients with a history of POS receiving a first graft showed a more rapid early decline in ECD than the overall patient cohort receiving a first graft which corresponds with the relatively greater risk of graft failure due to ED associated with this pathology (HR = 3.4 (2.0–5.8), $p < 0.0001$, Figs. 2 and 3; Table 5).

As expected from the above results, half-life during the rapid phase of ECD loss has a strong inverse correlation with cumulative risk of graft failure for first grafts at 10 years (Pearson's correlation = − 0.89, $p = 0.02$).

Amongst first grafts, regression analysis showed that lower pre-operative ECD was associated with a slightly increased probability of graft failure due to late ED ($p = 0.05$, $n = 356$) in a univariate model, with an associated hazard ratio of 1.15 (95% CI 1.01–1.32) for every decrease in pre-operative ECD of 100 cells/mm^2. The mean eye bank ECD of grafts which failed as a result of ED less than 5 years post-PKP was 2583 cells/mm^2 (SD = 234, $n = 13$) while for grafts which survived it was 2706 cells/mm^2 (SD = 251, $n = 475$).

DISCUSSION

Analysis of graft survival shows similar results to previous large studies in terms of both overall graft survival and survival when only ED is considered an end point. This is the case for American, British and Australian studies regardless of whether predominantly organ culture or hypothermic storage of the donor corneas was used [21, 26–28]. Survival is clearly poorer for re-grafts than first grafts, as expected [28]. There are also clear differences in survival between pathologies, and these correspond with the trends seen in other large studies [21, 28]. Namely, ectasias followed by dystrophies show the best overall graft survival, while POS, infection and injury give a poorer prognosis.

We have fitted our ECD data to the log-biexponential model to obtain half-lives for the fast and slow components of ECD loss. Overall, good fits were obtained, as indicated by the fit lines and model t values. Our data show that for first grafts the half-life during the rapid phase of ECD loss is 20.1 months, which indicates that after less than 2 years post-PKP, half the ECD is lost. For re-grafts, this rate of loss was higher with half of the ECD lost after just over a year. During this rapid phase of ECD loss, no corresponding increase in graft failure is observed.

Table 5 Calculated parameters for a log-biexponential model of ECD loss, where *P, Q, a* and *b* are defined in Eq. (1)

Subgroup	Par	Estimate	Lower CI	Upper CI	SE	*t* value	*p* value	Half-life (months)	Lower CI	Upper CI[a]
First grafts	*a*	0.4	0.3	0.6	0.1	6.1	<0.0001	20.1	14.9	30.9
First grafts	*b*	0.0	− 0.1	0.0	0.0	− 2.2	0.04	− 218.2	− 4911.7	− 111.6
First grafts	*P*	1825.5	1566.6	2084.4	121.5	15.0	<0.0001			
First grafts	*Q*	472.1	251.6	692.5	103.4	4.6	<0.001			
Re-grafts	*a*	0.7	0.1	1.2	0.2	2.7	0.02	12.8	6.9	79.4
Re-grafts	*b*	0.0	− 0.2	0.1	0.1	− 0.8	0.45	− 214.2	114.1	− 55.3
Re-grafts	*P*	1730.1	1087.4	2372.9	288.5	6.0	<0.001			
Re-grafts	*Q*	509.2	10.9	1007.4	223.6	2.3	0.05			
Dystrophies	*a*	0.4	0.2	0.6	0.1	4.5	<0.001	19.5	13.1	37.7
Dystrophies	*b*	0.0	− 0.1	0.0	0.0	− 1.0	0.32	− 207.6	191.2	− 67.3
Dystrophies	*P*	1892.6	1524.2	2261.0	169.1	11.2	<0.0001			
Dystrophies	*Q*	406.0	29.3	782.8	172.9	2.3	0.04			
Ectasias	*a*	0.3	0.1	0.5	0.1	3.4	0.003	26.2	16.2	68.0
Ectasias	*b*	0.0	− 0.1	0.0	0.0	− 1.0	0.34	− 264.5	221.3	− 82.8
Ectasias	*P*	2020.0	1517.7	2522.2	236.9	8.5	<0.0001			
Ectasias	*Q*	516.6	− 8.0	1041.1	247.4	2.1	0.05			
POS	*a*	0.7	0.2	1.2	0.2	3.1	0.01	11.6	6.7	41.3
POS	*b*	0.0	− 0.1	0.1	0.0	− 0.6	0.55	− 307.1	117.0	− 66.4
POS	*P*	1684.3	1140.9	2227.6	243.9	6.9	<0.0001			
POS	*Q*	533.8	101.2	966.3	194.1	2.7	0.02			
Infection	*a*	0.6	0.0	1.1	0.3	2.3	0.04	14.1	7.2	239.8
Infection	*b*	0.0	0.0	0.1	0.0	1.1	0.31	351.4	116.0	− 341.7
Infection	*P*	1344.1	763.9	1924.3	272.2	4.9	<0.001			
Infection	*Q*	851.8	375.4	1328.2	223.5	3.8	0.002			

Dystrophies, POS, ectasias and infection are first grafts only. Values for *P* and *Q* are in cells/mm^2, while *a* and *b* are given in years^{-1}. Half-lives are given in months. Fast half-lives are calculated from *a* and slow half-lives from *b*

Par parameter, *CI* 95% confidence interval, *SE* standard error

[a]Estimated confidence interval for the half-life calculated from parameters *a* and *b*. Where the value is negative, this signifies a doubling time i.e. time taken for the ECD to double

First grafts and re-grafts appear to plateau at the same ECD (approximately 800 cells/mm^2) despite re-grafts having a more rapid ECD loss initially. As a result, re-grafts reached a physiological level of ECD loss earlier than first grafts, which is to be expected from an exponential

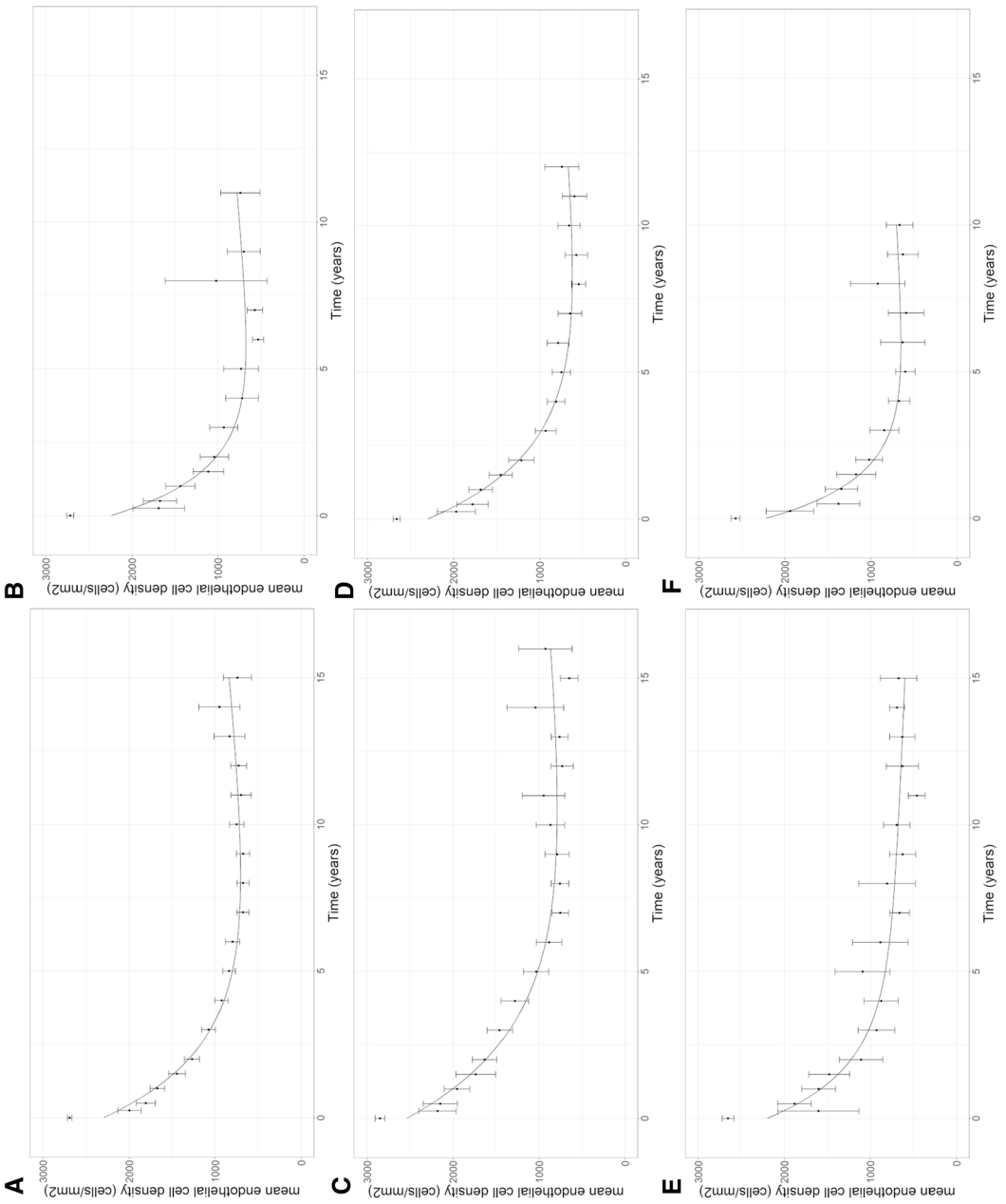

Fig. 3 Change in mean endothelial cell density with time for **a** first-ever grafts, **b** first-ever re-grafts, **c** ectasias, **d** dystrophies, **e** infection and **f** previous ocular surgery. A log-biexponential model is fitted in each graph. Confidence intervals (95%) are shown

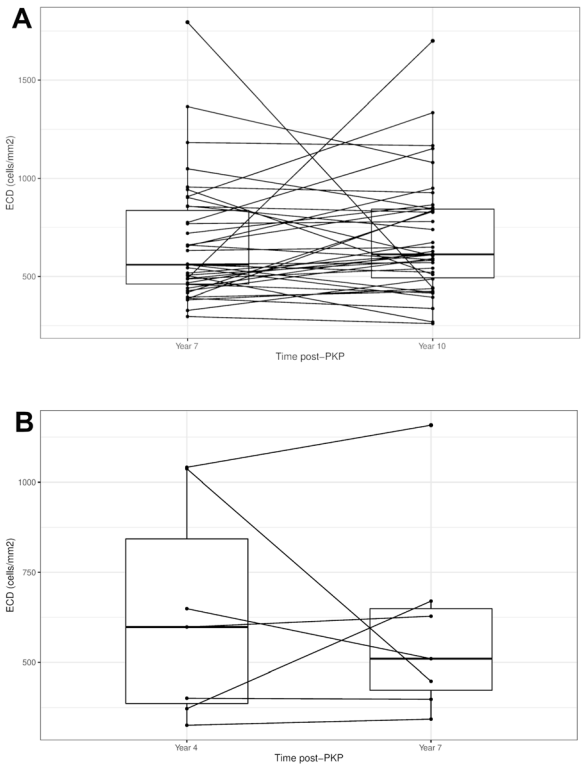

Fig. 4 Change in ECD between two time points for patients who underwent specular microscopy at both time points. This analysis shows that the observed increase in ECD is not due only to outliers. **a** Trajectories of individual patient's ECD counts between year 7 and 10 post-PKP (first-ever grafts). Box plots show the medians and interquartile ranges at years 7 and 10. **b** Trajectories of individual patient's ECD counts between year 4 and 7 post-PKP (first re-grafts)

model where there is a rapid initial decline in ECD. This seems to be the case for both the polynomial and log-biexponential models, as judged by the almost identical curves in Fig. 2b, giving support to the notion that this is not simply an artefact of exponential modelling.

We calculated the time point at which first grafts in our study reach a physiological level of ECD loss, which in this study we defined as an annual loss of 0.6% [18]. We found this to be 7.9 years. Patel et al. [21], who analysed a cohort of patients whose grafts were stored in predominantly hypothermic conditions, found an ECD half-life of 8.2 months and demonstrated that ECD stabilised at approximately physiological levels by year 10 post-PKP, 2 years later than seen in our cohort of strictly organ-cultured grafts. This data suggests our first grafts showed a lower rate of ECD loss initially and less time taken to reach physiological levels of ECD loss than the cohort reported by Patel et al. Furthermore, the more rapid initial rate of ECD loss seen by Patel et al. should have been associated with reaching physiological levels of ECD loss earlier than our data, as suggested by Böhringer et al. [20] and by the ECD data in this paper. However, this is not the case and it appears that our first grafts stabilise earlier. Unfortunately, a direct comparison with their data and our study cannot be made as the patient cohorts may not be comparable and the chosen mathematical models differ.

Our low rate of ECD loss in first grafts is supported by one of the earliest analyses of organ-cultured graft ECD. Redmond et al. found an ECD half-life of 41 months in the initial post-operative years, a much slower rate of ECD loss than was found in hypothermic stored corneas at the time [29].

As found by others who have used the log-biexponential model to study ECD loss [4], the half-life of the slow component was negative, indicating either very slow loss or growth in ECD, or stability of the ECD. This may partly reflect error in the measurement of low ECDs and the fact that relatively fewer patients are seen many years after the original operation. Patel et al. found in their cohort of patients between 10 and 15 years post-PKP that the mean rate of endothelial loss was − 1.0% (SD 5.4) [21]. Increases in graft ECD may be a result of migration of healthy host endothelial cells, as previously hypothesised by Böhringer [20]. Supporting this theory, we found that patients with dystrophy (with presumably reduced endothelial reserves) stabilised at a lower ECD than patients with ectasia. However, we also observed increases in mean ECD several years after PKP in patients with POS, who would presumably have depleted host endothelial cell reserves. This is not clearly explained by increased uncertainty in counting endothelial cells at very low ECD as the confidence intervals do not increase at these time points. Spatial measurements or cell tracking experiments may offer further explanation. The only patient

group in which we did not see a recovery of ECD loss to infra-physiological levels was infections. It is possible that disease recurrence, ongoing low-grade inflammation and a depletion of host endothelium inhibit the host's ability to recover central graft ECD. Furthermore, fitting a log-polynomial model to the first graft data showed a similar slow growth in ECD several years after operation. This was not seen in the previous analysis of hypothermically stored grafts by Riddlesworth et al. [4]

We also fitted the log-biexponential model to patients grouped by original pathology and found that pathologies with poor prognosis such as POS tended to have shorter half-lives during the rapid phase of ECD loss. There was an inverse correlation between the rapid half-life calculated from the model and risk of graft failure from ED at 10 years. This is consistent with a previous analysis, which indicated that the 6-month ECD is a prognostic indicator of long-term graft survival [6].

Price et al. found a linear relationship between ECD loss and time after (DSEK) [19]. This is unusual because it suggests the same number of cells are lost per unit time regardless of the number of endothelial cells remaining on the graft. With the DSEK patients, studied by Price et al., the rate of cell loss does not appear to normalise even after 10 years of follow-up [19]. Looking only at ECD loss in our patients with dystrophy and POS, the dynamics of ECD loss after PKP are clearly different.

As is the case with similar retrospective studies, there is substantial patient loss to follow-up for the collection of ECD data. One concern is that selective dropout of patients with graft failure will cause the long-term ECD to plateau artificially. However, Riddlesworth et al. demonstrated using a Bayesian model of ECD loss that the effect of selective dropout of failed grafts is small when graft survival is good [4]. Furthermore, another retrospective study of ECD after PKP in a US cohort found little change in results after imputing missing ECD follow-up data [6].

Although ECD loss is very rapid during the initial post-operative period, first-time grafts reach approximately physiological levels of ECD loss several years post-PKP, but then continue to reduce the level of ECD loss to infra-physiological levels for years after. Importantly, our results suggest that organ-cultured grafts may normalise to physiological levels of ECD loss more quickly than grafts stored in hypothermic conditions. Further work is needed to compare the rates of ECD loss several years after PKP between hypothermic and organ culture-stored grafts. Ideally, this would be achieved by transplanting first grafts in a cohort of patients randomised to either organ-cultured corneas or corneas stored under hypothermic conditions. Additionally, further work should map the change in ECD in the peripheral and central cornea to determine whether the long-term changes in central ECD are due to migration of cells from the periphery, differential changes in cell area across the cornea, a limited mitotic ability [30] or a combination of mechanisms.

As we have mentioned, our study experienced patient loss to follow-up, which was not surprising given the long duration of our study. This limits the power of subgroup comparisons within our cohort. Additionally, we are unable to make a direct comparison with hypothermically stored donor corneas in this study.

CONCLUSIONS

The rate of ED is higher in first grafts than re-grafts, as previously demonstrated. ECD decline stabilises 7.9 years post-operatively in first grafts but then becomes lower than the physiological loss expected. The cause of this sub-physiological rate of ECD loss is not clear. Further work, such as a head-to-head clinical trial, is needed to verify whether organ-cultured grafts reach physiological levels of ECD loss faster than hypothermically stored grafts.

ACKNOWLEDGEMENTS

The authors would like to thank the participants of the study.

Funding. No funding or sponsorship was received for this study. The journal's Rapid Service Fee was funded by the authors.

Authorship. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Author Contributions. All authors contributed to the study conception and design. Material preparation, datacollection and analysis were performed by Ali E. Ghareeb, Maria S. Figueiredo, Sayali P. Pradhan, Elinor Curnow, W. John Armitage and Francisco C. Figueiredo. The first draft of the manuscript was written by Ali Essam Ghareeb and Francisco C. Figueiredo; and all authors contributed to previous versions of the manuscript. All authors read and approved the final manuscript.

Disclosures. Ali E. Ghareeb, Maria S. Figueiredo, Sayali P. Pradhan, Elinor Curnow, W. John Armitage and Francisco C. Figueiredo declare that they have no conflict of interest.

Compliance with Ethics Guidelines. NHS Research Ethics committee approval was not required as this study was observational and therefore does not meet the definition of research held by the UK Policy Framework for Health and Social Care Research. Our study was performed in accordance with the Helsinki Declaration of 1964, and its later amendments. All patients were aware of the collection of their data for this study and signed a consent form at the time of enrolment.

Data Availability. The datasets generated during and/or analysed during the current study are availablefrom the corresponding author on reasonable request.

REFERENCES

1. Ing JJ, Ing HH, Nelson LR, Hodge DO, Bourne WM. Ten-year postoperative results of penetrating keratoplasty. Ophthalmology. 1998;105:1855–65.
2. Claerhout I, Beele H, Kestelyn P. Graft failure: I. Endothelial cell loss. Int Ophthalmol. 2008;28:165–73.
3. Patel SV, Lass JH, Benetz BA, et al. Postoperative endothelial cell density is associated with late endothelial graft failure after descemet stripping automated endothelial keratoplasty. Ophthalmology. 2019;126:1076–83.
4. Riddlesworth TD, Kollman C, Lass JH, et al. A mathematical model to predict endothelial cell density following penetrating keratoplasty with selective dropout from graft failure. Investig Ophthalmol Vis Sci. 2014;55:8409–15.
5. Armitage WJ, Dick AD, Bourne WM. Predicting endothelial cell loss and long-term corneal graft survival. Investig Ophthalmol Vis Sci. 2003;44:3326–31.
6. Lass JH, Sugar A, Benetz BA, et al. Endothelial cell density to predict endothelial graft failure after penetrating keratoplasty. Arch Ophthalmol. 2010;128:63–9.
7. Muraine M, Sanchez C, Watt L, Retout A, Brasseur G. Long-term results of penetrating keratoplasty. A 10-year plus retrospective study. Graefes Arch Clin Exp Ophthalmol. 2003;241:571–6.
8. Wakefield MJ, Armitage WJ, Jones MNA, et al. The impact of donor age and endothelial cell density on

graft survival following penetrating keratoplasty. Br J Ophthalmol. 2016;100:986–9.

9. Armitage WJ, Easty DL. Factors influencing the suitability of organ-cultured corneas for transplantation. Investig Ophthalmol Vis Sci. 1997;38:16–24.

10. Elisabeth P, Hilde B, Ilse C. Eye bank issues: II. Preservation techniques: warm versus cold storage. Int Ophthalmol. 2008;28:155–63.

11. Jirsova K, Dahl P, Armitage WJ. Corneal storage, hypothermia, and organ culture. In: Light and specular microscopy of the cornea. Cham: Springer; 2017.

12. Devasahayam R, Georges P, Hodge C, et al. Implementation of Organ Culture storage of donor corneas: a 3 year study of its impact on the corneal transplant wait list at the Lions New South Wales Eye Bank. Cell Tissue Bank. 2016;17:377–85.

13. Doughman DJ, Van Horn D, Rodman WP, Byrnes P, Lindstrom RL. Human corneal endothelial layer repair during organ culture. Arch Ophthalmol. 1976;94:1791–6.

14. Armitage WJ. Preservation of human cornea. Transfus Med Hemother. 2011;38:143–7.

15. Pels L. Organ culture: the method of choice for preservation of human donor corneas. Br J Ophthalmol. 1997;81:523–5.

16. Steger B, Curnow E, Cheeseman R, et al. Sequential bilateral corneal transplantation and graft survival. Am J Ophthalmol. 2016;170:50–7.

17. Raj A, Salvador-Culla B, Anwar H, Sykakis E, Figueiredo MS, Figueiredo FC. Long-term outcomes on de novo ocular hypertensive response to topical corticosteroids after corneal transplantation. Cornea. 2020;39:45–51.

18. Nelson L, Hodge D, Bourne W. Central corneal endothelial cell changes over a ten-year period. Am J Ophthalmol. 1997;124:273–4.

19. Price MO, Calhoun P, Kollman C, Price FW, Lass JH. Descemet stripping endothelial keratoplasty ten-year endothelial cell loss compared with penetrating keratoplasty. Ophthalmology. 2016;123:1421–7.

20. Böhringer D, Böhringer S, Poxleitner K, et al. Long-term graft survival in penetrating keratoplasty: the biexponential model of chronic endothelial cell loss revisited. Cornea. 2010;29:1113–7.

21. Patel SV, Hodge DO, Bourne WM. Corneal endothelium and postoperative outcomes 15 years after penetrating keratoplasty. Am J Ophthalmol. 2005;139:311–9.

22. Putter H, Fiocco M, Geskus RB. Tutorial in biostatistics: competing risks and multi-state models. Stat Med. 2007;26:2389–430.

23. Gray RJ. A class of K-sample tests for comparing the cumulative incidence of a competing risk. Ann Stat. 1988;16:1141–54.

24. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc. 1999;94:496–509.

25. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2019.

26. Beckingsale P, Mavrikakis I, Al-Yousuf N, Mavrikakis E, Daya SM. Penetrating keratoplasty: outcomes from a corneal unit compared to national data. Br J Ophthalmol. 2006;90:728–31.

27. Gal RL, Dontchev M, Beck RW, et al. The effect of donor age on corneal transplantation outcome results of the cornea donor study. Ophthalmology. 2008;115:620-626.e6.

28. Keryn WA, Keane MC, Coffey NE, JonesVJ. The Australian Corneal Graft Registry 2018 Report. 2018.

29. Redmond RM, Armitage WJ, Whittle J, Moss SJ, Easty DL. Long-term survival of endothelium following transplantation of corneas stored by organ culture. Br J Ophthalmol. 1992;76:479–81.

30. Joyce NC. Proliferative capacity of corneal endothelial cells. Exp Eye Res. 2012;95:16–23.