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**ENDOTHELIAL EVALUATION FOR CORNEAL TRANSPLANTATION:
FROM PERIOPERATIVE ASSESSMENT TO REAL WORLD OUTCOMES**

Candidato:
Matteo Airaldi

Tutor:
Prof. Vito Romano
Prof. Francesco Semeraro

1 **RIASSUNTO**

2
3 Le cellule endoteliali corneali sono responsabili del mantenimento della
4 deturgescenza corneale, agendo sia come barriere che come pompe attive per
5 preservare la trasparenza corneale mantenendo livelli ottimali di idratazione stromale.

6 La densità delle cellule endoteliali (ECD) è fondamentale, poiché queste cellule non
7 proliferano in vivo; una perdita di cellule endoteliali al di sotto di una soglia fisiologica
8 compromette la deturgescenza e provoca edema corneale.

9 Pertanto, la densità delle cellule endoteliali nei casi di trapianto di cornea è stata
10 descritta come uno dei principali fattori determinanti per la sopravvivenza a lungo
11 termine del lembo corneale. Tuttavia, l'ECD rappresenta solo un indicatore della
12 vitalità dell'endotelio corneale e la sua misurazione e validità hanno mostrato
13 significative carenze. Inoltre, una valutazione finale della qualità per i chirurghi che
14 eseguono il trapianto di cornea non è attualmente disponibile o convalidata.

15 Lo scopo di questa tesi di dottorato è descrivere gli sforzi del nostro gruppo di ricerca,
16 guidato dal candidato, il dottor Airaldi, e dal suo tutor, il prof. Romano, e composto da
17 esperti internazionali e con competenze traslazionali, per ideare, convalidare e
18 implementare un metodo per la valutazione della vitalità endoteliale perioperatoria nel
19 trapianto di cornea. La tesi segue il percorso delle cornee dei donatori, dal recupero
20 alla lavorazione nella banca degli occhi fino al trapianto di cornea, evidenziando il
21 ruolo della valutazione endoteliale in ogni fase e le strategie per ridurre al minimo i
22 danni e migliorare il controllo di qualità.

23 A seguito di un'ampia revisione della letteratura sulle tecniche di conservazione della
24 cornea nelle banche degli occhi, sui metodi di valutazione endoteliale e sulle strategie

per ridurre al minimo il danno endoteliale, il primo studio si concentra sullo sviluppo di un protocollo multicentrico per la valutazione endoteliale perioperatoria dei lembi corneali in sala operatoria e sull'istituzione di una coorte internazionale di pazienti con l'obiettivo di correlare i risultati dei trapianti con le misurazioni della vitalità endoteliale preoperatoria.

Il secondo studio descrive il processo di sviluppo e convalida *ex-vivo* del metodo “Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK)” per analizzare la vitalità endoteliale dei lembi corneali. Questo metodo sfrutta l'uso di un colorante vitale, il Trypan Blue, e un foundation model di segmentazione abbinato ad algoritmi di visione artificiale per misurare automaticamente il grado di mortalità endoteliale nelle immagini al microscopio chirurgico dei lembi corneali.

Il terzo studio descrive l'implementazione del metodo V-CHECK in un sottogruppo di pazienti sottoposti a cheratoplastica endoteliale ultrasottile (UT-DSAEK), collegando la mortalità misurata con V-CHECK a un aumento del rischio di sviluppare un fallimento precoce del lembo corneale. Questo studio descrive anche un elemento peculiare e innovativo, il ritrovamento su numerosi lembi di una macchia a forma di impronta digitale dell'endotelio corneale, che può essere osservata nei lembi di UT-DSAEK ed è un indicatore di un aumento della perdita di cellule endoteliali.

Il quarto studio è un'ampia analisi multicentrica sulla sopravvivenza dei lembi UT-DSAEK che ha coinvolto 379 pazienti sottoposti a cheratoplastica endoteliale presso 8 centri italiani specializzati nella cura della cornea. I trapianti provenienti dalla stessa fonte di quelli che nel nostro studio precedente presentavano una colorazione simile a un'impronta digitale hanno mostrato tassi di sopravvivenza notevolmente inferiori

48 rispetto a quelli provenienti da una banca degli occhi diversa, evidenziando possibili
49 problemi nell'attuale catena di approvvigionamento.

50 Nel complesso, questo lavoro convalida il metodo V-CHECK come strumento
51 affidabile e indipendente di garanzia della qualità. La sua integrazione nei flussi di
52 lavoro chirurgici consente ai chirurghi di identificare lembi corneali ad alto rischio,
53 standardizzando la valutazione preoperatoria e migliorando potenzialmente la
54 sopravvivenza a lungo termine dei trapianti di cornea.

55

SUMMARY

Corneal endothelial cells are responsible for the maintenance of corneal deturgescence, acting as both barriers and active pumps to preserve corneal clarity through optimal levels of stromal hydration. Endothelial cell density (ECD) is critical, as these cells do not proliferate in vivo; loss below a physiologic threshold impairs deturgescence and results in corneal edema.

Therefore, the density of endothelial cells in cases of corneal transplantation has been described as one of the major determinants of long-term graft survival. However, ECD only represents a proxy of corneal endothelial viability, and its measurement and validity have shown significant shortcomings. Furthermore, a final quality assessment for the surgeons performing the corneal transplantation is currently not readily available or validated.

The aim of this doctoral dissertation is to describe the efforts of our research group, led by the candidate, Dr. Airaldi, and his tutor, Prof. Romano, and composed of international and translational experts, to devise, validate and implement a method for perioperative endothelial viability assessment in corneal transplantation. The dissertation follows the journey of donor corneas from retrieval to eye bank processing to corneal transplantation, highlighting the role of endothelial evaluation at each step and strategies to minimize damage and improve quality control.

Following a comprehensive literature review of techniques of corneal preservation in the eye bank, endothelial evaluation methods and strategies to minimize endothelial damage, the first study focuses on the development of a multicentric protocol for a perioperative endothelial evaluation of corneal grafts in the operating room, and the

institution of an international cohort of patients with the objective of correlating transplantation outcomes with measurements of preoperative endothelial viability.

The second study describes the *ex-vivo* development and validation process of the Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK) method to analyse endothelial viability of corneal grafts. This method leverages the use of a vital dye, Trypan Blue, and a foundation segmentation model coupled with computer vision algorithms to automatically measure the degree of endothelial mortality in surgical microscope images of corneal grafts.

The third study describes the implementation of the V-CHECK method in a subset of patients undergoing Ultrathin Descemet Stripping Automated Endothelial Keratoplasty (UT-DSAEK), linking the V-CHECK measured mortality to an increase in the risk of developing early corneal graft failure. This study also describes a peculiar and novel finding, a fingerprint-like staining of corneal endothelium, that can be seen in UT-DSAEK grafts and is a marker of increased endothelial cell loss.

The fourth study is a large multicenter survival analysis of UT-DSAEK grafts involving 379 patients who underwent endothelial keratoplasty at 8 Italian cornea services. Grafts with the same provenance as those displaying fingerprint-like staining in our previous study showed markedly lower survival rates compared to those coming from a different Eye Bank, highlighting possible issues in the current supply chain.

Collectively, this work validates the V-CHECK method as a reliable, independent quality assurance tool. Its integration into surgical workflows empowers surgeons to identify high-risk grafts, standardizing pre-operative assessment and potentially improving the long-term survival of corneal transplants.

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Chapter 1

BACKGROUND

Corneal transparency is paramount for the correct focusing of incoming light on the retinal surface and, therefore, for clear vision. Transparency is maintained by corneal endothelial cells (CECs), the monolayer of cells that constitutes the innermost part of the cornea. CECs dynamically maintain a correct level of hydration of the corneal stroma through diffusion and ionic pumps mechanisms.¹

Endothelial dysfunction occurs when damage to CECs results in failure to sustain these mechanisms. The corneal edema that ensues is due to fluid accumulation in the corneal stroma. Given that CECs are not capable of regeneration in vivo, the only therapeutic option for severe, visually impairing endothelial dysfunction is surgical transplantation, with the goal of restoring CECs function.²

Corneal Anatomy and Biomechanics

The human cornea is normally around 550 μm thick at its center, where it also assumes its steeper curvature radius, while gradually flattening and thickening towards the periphery. It is comprised of five layers: epithelium, Bowman's layer, stroma, Descemet's membrane, and endothelium.

The epithelium includes epithelial cells and a basement membrane; it provides a barrier for the underlying layers from external agents while also absorbing nutrients and oxygen from the tear film. Bowman's membrane is comprised of a thin, acellular, and transparent layer of collagen. Trauma or injury can disrupt this collagenous film, which may cause scarring that impairs vision, depending on its location and extent. The stroma is the thickest layer of the cornea and is made up primarily of water and collagen. It is vital to the maintenance of the spherical shape of the central cornea. The Descemet's membrane is the collagenous basal membrane produced by CECs. The innermost layer of the cornea is the endothelium. This thin monolayer of hexagonal cells maintains the transparency of the cornea as CECs pump excess fluid from the stroma. CECs do not appear to regenerate in vivo, and their loss leads to endothelial dysfunction.³

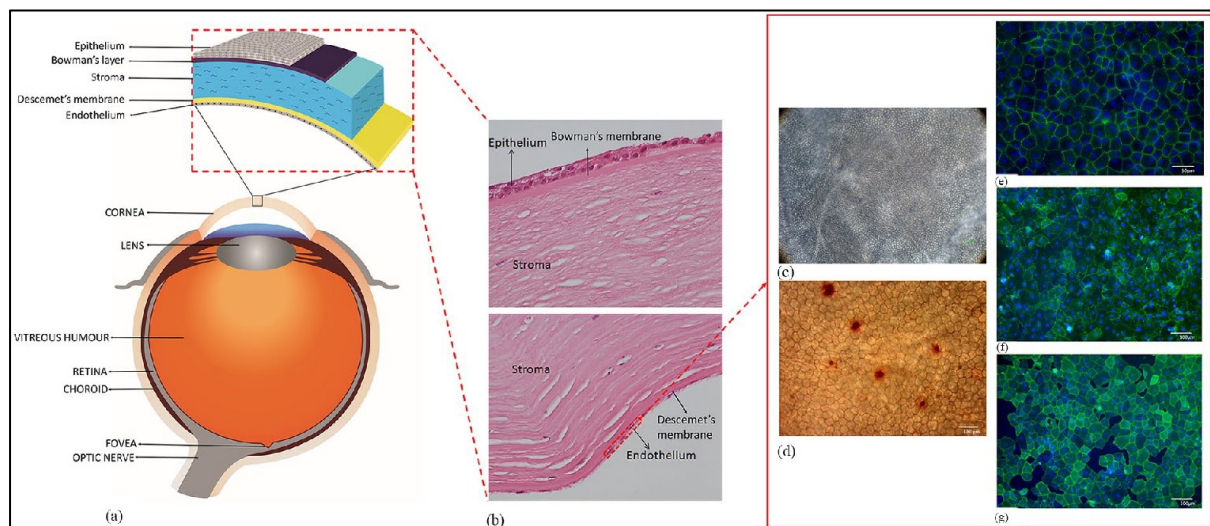


Figure 1. Anatomy of the human eye and cornea: (a) The figure illustrates different parts of the eye and layers of the corneal tissue, (b) histological section of human cornea showing different layers of the tissue, (c) corneal endothelial cell viability using

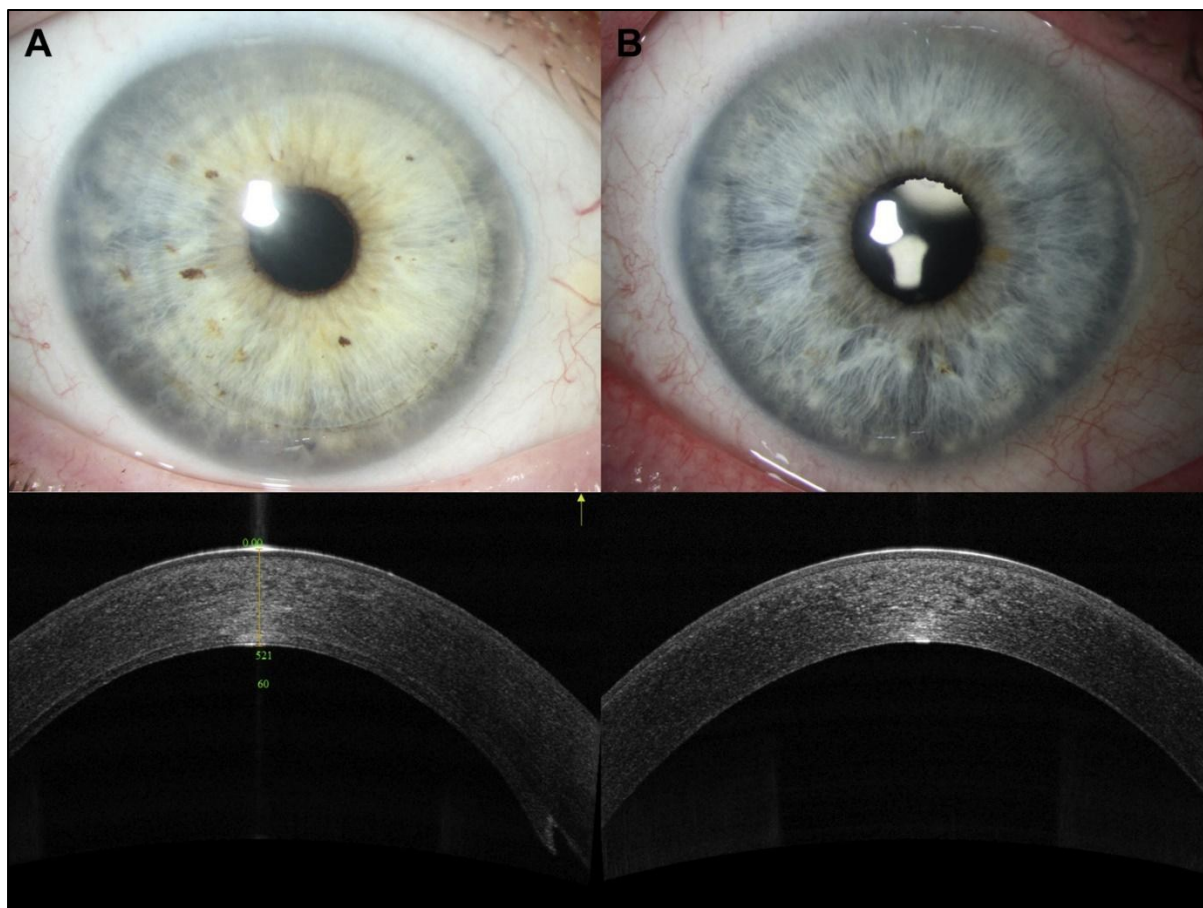
trypan blue staining observed under light microscope and (d) alizarin red staining showing the hexagonality of endothelial cells. Biomarkers such as (e) ZO-1, (f) Tag-1A3, and (g) Tag-2A12 to determine the presence of specific proteins like tight-junction and surface epitopes.

Endothelial Keratoplasty

During the last two decades, endothelial keratoplasty (EK) techniques have largely overtaken penetrating keratoplasty (PK) as the leading procedure for endothelial failure.^{4,5} Unlike PK, which is a full-thickness transplant of a donor cornea, EK procedures are characterized by the transplant of only a lamella of donor tissue containing the CECs monolayer and its basement membrane, the Descemet membrane, with (DSAEK) or without (DMEK) a portion of the donor stroma.

In particular, both procedures involve the selective removal of the patient's Descemet membrane and endothelium. Following either manual stripping of the donor Descemet membrane – endothelium complex (DME, thickness around 10-15 μm) in DMEK, or the automated cut of a thin stroma – DME lenticule (thickness $<100\ \mu\text{m}$) in ultra-thin DSAEK (UT-DSAEK), the graft is injected in the patient's eye and apposed to the recipient stroma via a gas/air bubble.

Evidence from large clinical trials suggests that DMEK, performed by experienced surgeons, might have superior visual acuity outcomes and more rapid recovery compared with UT-DSAEK in patients undergoing cornea transplant with isolated endothelial dysfunction and similar complication rates. The better visual outcomes could be explained by the lower refractive shift and less induced higher order optical aberrations associated with a thicker lenticule in UT-DSAEK compared to DMEK.⁶



290

291 **Figure 2.** Clinical photographs and anterior segment OCT images from patients who
292 underwent ultrathin Descemet stripping automated endothelial keratoplasty (UT-DSAEK) (A)
293 and Descemet membrane endothelial keratoplasty (DMEK) (B).

294

295 Regardless of the technique chosen, EKs avoid full-thickness corneal trephination and
296 intra-operative “open sky” situations, associated with greater peri- and post-operative
297 complications, maintain corneal biomechanics, and provide overall faster and greater
298 visual recovery compared to PK.

299

THE SHORTCOMINGS OF ENDOTHELIAL CELL DENSITY

Endothelial cell density (ECD) is calculated via specular microscopy and defined as the number of cells per mm² of endothelial surface. ECD currently represents the single most important indicator of donor corneal tissue status, and its relevance as a determinant of long-term graft survival has long been proven.⁷ The minimum requirements for delivering a donor cornea might vary from eye bank to eye bank, however ECD is universally adopted to set a threshold for transplantation suitability. This metric is one of the few indicators of endothelial status available to the surgical team performing the keratoplasty, yet ECD does not necessarily reflect total corneal endothelial health.^{8,9} For this reason most eye banks, especially in Europe, also evaluate endothelial cells mortality, specifying a 5% mortality threshold to be deemed suitable for transplantation.¹⁰

There is a need for a greater degree of standardization of outcomes, both functional and survival-wise, in corneal transplant surgery, a demand whose satisfaction is strongly dependent on developing metrics that better reflect global endothelial health of donor tissues.^{11,12} Pan-corneal assessments of endothelial viability in donor tissues have highlighted how ECD probably overestimates true cell density^{13,14}, however these methods, even though they allow a global evaluation of endothelial status, are not feasible in theatre as they are destructive to the graft.

PURPOSE

In this dissertation, we propose, validate and implement a simple and effective method to evaluate the pan-corneal endothelial viability of donor tissue. On behalf of an international collaboration of cornea services, our group proposes a study protocol whose aims are to validate a novel method for the peri-/intra-operative assessment of endothelial viability of donor corneal grafts for keratoplasty, and to verify its correlation with clinical outcomes in a cohort of patients receiving those grafts. This method takes advantage of the ability of Trypan Blue 0.05%, a vital dye extensively employed in ophthalmic surgery, to stain areas of denuded Descemet membrane and endothelial cell death or loss.

As shown in **Figure 3**, staining with Trypan Blue provides a clear picture of the overall endothelial cell loss and of suitability of the donor tissue for transplantation. In two of the cases shown, both scheduled for endothelial keratoplasty, the surgeon decided not to proceed with transplantation due to the excessive proportion of areas of Trypan Blue staining (**Figure 3A-B**). In both cases the ECD poorly reflected the effective endothelial status of donor corneas.



Figure 3. Trypan Blue 0.05% staining of three corneoscleral rims suitable for transplantation. The declared ECD for the three tissues was 2700, 2600 and 2700 cells/mm², respectively. Both case A and case B, originally scheduled for endothelial keratoplasty, were postponed and the donor tissues discarded due to excessive Trypan Blue staining. Case C did not have any trypan blue staining and underwent successful surgeon-stripped DMEK.

Quality assurance (QA) is any systematic process of determining whether a product or service meets the specified requirements. Currently, corneal surgeons do not have an objective method to verify these requirements, i.e., the corneal endothelial status, even if the surgeon is the only figure responsible for the actual transplantation.¹¹ With the development and validation of the method proposed in the presented works., we aim to provide surgical teams with an effective and independent method to check the viability of the tissues before implanting, hopefully improving the outcomes and survival of the grafts delivered to our patients.

Chapter 2

CORNEAL ENDOTHELIAL EVALUATION – WHY DOES IT MATTER?

The long-term success of human allogeneic corneal transplantation fundamentally depends on the viability and functional reserve of a single, non-regenerative monolayer, the corneal endothelium. This is true for all transplants involving corneal endothelium such as penetrating keratoplasty (PK) and endothelial keratoplasty (EK), thus excluding anterior lamellar keratoplasties (ALK) which represent 10% or less of all transplants.^{15,16} Quality control of donor tissue destined to keratoplasty is therefore of paramount importance to ensure both safety and efficacy of transplantation.¹¹ Among all preoperative tissue parameters, endothelial health status has consistently emerged as the principal determinant of long-term graft survival.^{7,17}

From a clinical standpoint, the corneal endothelium maintains corneal stromal deturgescence through its barrier and pump functions. Because human endothelial cells exhibit limited proliferative capacity in vivo, the transplanted endothelial cell density (ECD) represents a finite functional reserve. Progressive endothelial cell loss—whether immune-mediated, surgically induced, or related to donor factors—can culminate in endothelial decompensation, stromal edema, and graft failure.

Corneal edema represents the final common pathway of endothelial failure after keratoplasty. In the Cornea Preservation Time Study (CPTS), graft failure has been classified either as the need for regrafting or the persistence corneal cloudiness, either immediately after transplantation or after an initial clearing of the donor cornea (i.e.,

early and late failure, respectively).¹⁸ Late endothelial failure may occur following immune events (i.e. rejection)¹⁹ or non-immune mechanisms, with late endothelial decompensation playing a central role.²⁰ Clinically, failed grafts exhibit signs of endothelial dysfunction such as diffuse stromal haze, Descemet membrane folds (stromal ripples), and increasing central corneal thickness.^{21,22} As edema progresses, epithelial microcysts and bullae may develop, leading to pain, recurrent epithelial breakdown, infection risk, and, in chronic cases, subepithelial fibrosis and stromal scarring that limit visual recovery even after surgical resolution of the endothelial dysfunction.^{23,24}

Long-term data following PK demonstrate substantial endothelial cell loss even in clear grafts at 10 years, with greater loss associated with older donor age and lower early postoperative ECD.⁸ Importantly, six-month postoperative ECD has emerged as a powerful predictor of graft survival across surgical modalities. In PK, lower six-month ECD was predictive of failure, whereas morphometric indices such as coefficient of variation (CV) and hexagonality fluctuated, potentially reflecting endothelial instability.²² Similarly, after Descemet membrane endothelial keratoplasty (DMEK), low six-month ECD is associated with significantly increased five-year graft failure risk.²⁵ In DSAEK, although preoperative ECD was not directly correlated with graft failure in the CPTS, lower ECD at six months strongly predicted late failure.²⁶

These findings underscore a crucial concept: while preoperative ECD alone may not fully determine outcome, early postoperative endothelial survival—strongly influenced by donor quality and tissue handling both before and during transplantation—critically defines long-term success. Sequential specular microscopy in failing grafts demonstrates continued ECD decline and progressive thickening preceding late

decompensation.²⁰ Specific morphological features, such as intracellular dark spots on specular microscopy, have recently been associated with increased endothelial cell loss and graft failure in both PK and DMEK.^{27,28}

Furthermore, early postoperative events can also significantly impact endothelial survival. For instance, eyes requiring rebubbling after DMEK exhibit higher endothelial cell loss and lower graft survival, particularly when multiple rebubbings are necessary.²⁹ These data reinforce the need to transplant tissue with maximal viable cell reserve to buffer against surgical trauma and postoperative complications.

However, measurements of donor ECD only estimate the cellular density of selected areas of the endothelium and thus cannot adequately represent the full picture of corneal endothelial health.³⁰ Conventional specular microscopy samples limited areas and may not capture regional endothelial mortality.^{13,14} Estimates of pan-corneal evaluation of endothelial viability tend to return higher mortality values compared to standard eye bank assessment tools such as specular microscopy or visual assessment under light microscopy, with studies on viability after DMEK grafts preparation frequently reporting mortality values exceeding the 15-20% mark.^{31,32} This is possibly due to the fact that pan-corneal evaluations explore a larger area compared to ECD or manual counting, which might miss areas of mortality, but also because standard counting by eye banks overestimates the actual pool of viable endothelial cells.^{13,33} Advanced approaches—including global viability mapping and deep learning—assisted analysis—allow broader evaluation of endothelial integrity and may help exclude tissues exhibiting high mortality prior to transplantation.^{33,34} For instance, this is particularly relevant given evidence that eye bank—preloaded DSAEK grafts may

exhibit substantially higher primary graft failure rates compared to surgeon-prepared grafts, likely reflecting additional endothelial trauma during preparation and storage.³⁵

Therefore, meticulous endothelial evaluation translates directly into reduced risk of primary graft failure, improved long-term clarity, fewer regrafts, and improved visual prognosis. However, thorough viability assessment of donor corneas is not only relevant for patients, but it also has a role in the standardization of keratoplasty outcomes. In fact, corneal transplantation outcomes vary across centers and techniques. Variability in donor tissue selection criteria—particularly regarding endothelial parameters—can confound comparative effectiveness analyses. Standardized endothelial evaluation is essential for harmonizing outcomes across eye banks, cornea clinics and keratoplasty techniques.

The Cornea Donor Study demonstrated that donor age influences long-term endothelial cell loss⁸, yet graft survival was not significantly different between donors aged 12–65 and 66–75 years in earlier analyses.⁷ This apparent paradox highlights the complexity of endothelial biology: ECD alone does not fully capture functional competence, and morphometric stability may matter. Nonetheless, standardized assessments (ECD, polymegathism, pleomorphism) are needed to reduce inter-eye bank variability. Furthermore, automated cell counts often require interactive correction for accuracy.³⁶ The need for harmonized methodologies across different eye banks and in the operating theatre, which are critical for reproducibility and quality control, leave an open window for pan-corneal endothelial assessments to be seamlessly integrated into a shared algorithm of perioperative donor corneal evaluation by technicians and surgeons.

Finally, not all corneas unsuitable for endothelial keratoplasty are unusable. Lamellar procedures such as deep anterior lamellar keratoplasty (DALK) preserve recipient endothelium and do not require viable donor endothelial cells. Thus, corneas with low ECD may still serve as valuable tissue for anterior lamellar indications. Accurate endothelial assessment enables appropriate triaging: high-ECD tissues for DMEK, DSAEK and PK, and low-ECD for DALK or tectonic applications. This stratification maximizes utilization and minimizes waste, improving overall graft availability without compromising outcomes. Newer approaches might be capable of combining low-count tissues from paired donations to optimize endothelium-insufficient donor corneas but require highly specialized eye bank processing not yet available to most centers.

In this review, we systematically evaluate the clinical, logistical, and regulatory dimensions of donor corneal endothelial assessment in the context of eye banking and corneal transplantation. We synthesize current evidence on the impact of endothelial parameters on graft outcomes, examine international eye bank guidelines (EEBA and EBAA) and their suitability criteria, and analyze reported rates of corneal acceptance and discard related to endothelial evaluation. We highlight the different methods available for endothelial evaluation, and we focus on strategies to reduce its impact on endothelial viability. By bridging a knowledge and information gap between eye bank and corneal surgeon, we aim to clarify the role of standardized endothelial assessment in optimizing tissue utilization and improving transplantation outcomes.

Chapter 3

EYE BANK PRESERVATION METHODS

The initial efforts in transplanting corneal tissue trace their origins to the late 19th century, culminating in the first documented transplant in 1906.³⁷ Because preservation techniques were entirely absent during this early period, surgical teams had to perform transplants by moving the tissue immediately from the donor to the recipient. This necessity imposed considerable logistical and practical challenges. Throughout the initial decades of the 20th century, various approaches were investigated for the storage of whole donor eyes. A significant advancement occurred in the 1940s with the implementation of a feasible method for keeping ocular tissue in moist chambers.³⁸ This development led to the establishment of the world's first eye bank: the Eye Bank for Sight Restoration in New York, which was funded to provide surgeons with preserved eye globes. Since their establishment, eye banks have progressed together with the surgical practice, including advancements in sterile practices, refinement of surgical methods as well as the preservation methods of the donor tissues. These factors together contributed to make corneal transplantation increasingly accessible and currently eye banks have become a solid and essential partner to support surgeons in their activities.

The objective of eye banks regarding corneal tissue storage is to maintain endothelial cells viability and density unchanged until transplantation. This goal is accomplished through two distinct preservation methods: hypothermic storage, mostly used in the United States³⁹, and Organ culture⁴⁰, the preferred method for European eye banks.

Hypothermic storage

In hypothermic storage, human corneoscleral discs are kept in specifically designed media at 2-8°C for up to 14 days. In this type of preservation, the low temperature slows down endothelial cell metabolism according to the Arrhenius equation⁴¹, which states that cellular energy demand is reduced by approximately 2-3 folds every 10°C of temperature reduction, meaning that cellular metabolic activity is reduced but not entirely eliminated. Consequently, hypothermic storage vials typically contain a modest media volume of 20 mL, as corneal cells require only minimal nutrients to preserve cellular viability. During the preservation period, as cells are kept in an inactive state, there is reduced endothelial cell pumping activity and endothelial cell death is more common compared to the organ culture preservation method (see **Table 1**).⁴²

Table 1. Advantages and disadvantages of cold storage in Eye Banking for corneal tissue

Cold storage in Eye Banking	
Advantages	Disadvantages
Maintenance of corneal thickness (no edema)	Slowed down cellular metabolism
Single cold storage medium (less manipulations)	Antibiotics less effective
Can perform specular microscopy from viewing chamber	No microbiology screening
	Short preservation (14 days)

509

510 The most common commercial media currently available in the market are Eusol-C,
511 Kerasave and XTRA4 (AL.CHI.MI.A. SRL. Italy), and Optisol-GS⁴³ (Bausch&Lomb,
512 United States).

513 These media are formulated enriched with nutrients such as minerals, amino acids
514 and energetic substrates in order to preserve as much as possible endothelial cell
515 viability. In addition, they are provided with substances exerting oncotic pressure on
516 the stroma able to counteract corneal swelling (edema) during the preservation, like
517 dextran, chondroitin sulfate, and Poloxamer 188.^{44,45} Nonetheless, a slow but
518 progressive increase in corneal thickness is usually observed due to the reduced
519 endothelial pump function⁴⁶ and lack of intraocular pressure (IOP), which normally
520 limits water influx into the corneal stroma.

521 Additionally, despite the low temperature generally slowing microbial growth, the
522 hypothermic media contain antibiotics (Gentamicin, Streptomycin) and antifungals
523 (Amphotericin-B, only Kerasave) to guard against potential contaminations.⁴⁷

524 Overall, hypothermic preservation is able to give a short-term, easy, safe and relatively
525 cheap strategy to maintain corneal quality mostly unchanged until transplantation.
526 Length of tissue preservation remains the most critical issue, as reports of the Eye
527 Bank Association of America highlight of tissue expiry is the leading cause of wastage
528 of corneal tissues in cold storage (see **Table 2**).

529

530 **Table 2.** EBAA reports for unused released tissues.

Reasons Released Tissues Were Not	2021	2021	2022	2022	2023	2023
Transplanted		(%)		(%)		(%)
Transportation Issue	469	7.10%	247	4.10%	192	2.30%
Surgeon Issue	124	1.90%	80	1.40%	82	1.00%
Recipient Issue	36	0.50%	38	0.60%	33	0.40%
Returned and Unable to Place Again	507	7.60%	432	7.20%	590	6.90%
Donor Information Not Available at the Time of Tissue Release	54	0.80%	20	0.30%	58	0.70%
Expired or Unable to Place Tissue	3,539	53.30%	2,922	48.90%	4,584	53.80%
Tissue Damaged During Processing	1,416	21.30%	1,529	25.60%	1,927	22.60%
Other Reason After Release of Tissue	1,096	16.50%	2,985	50.00%	4,079	47.80%
Total eyes/corneas released for transplant but not used for transplant	6,634		5,974		8,527	

531

532

533 **Organ culture**

534 Organ Culture (OC) consists in the storage of corneas at 28-37°C for a maximum of
535 34 days.³⁶ This preservation technique was developed in 1976⁴⁸ with the goal of
536 keeping corneas close to their physiological body temperature (approximately 35°C)
537 and providing them with micro- and macro-nutrients to sustain their ongoing metabolic
538 processes. Although this method is more complex than hypothermic storage and faces
539 some challenges (such as corneal swelling, higher manipulation, higher infection risk),
540 Sperling, Pels, and colleagues improved its effectiveness, leading organ culture to
541 become the main corneal preservation technique in Europe (see also **Table 3**).

This preservation strategy generally involves a three-steps protocol:

Initial Hypothermic shipment to the eye bank: In the first phase of the process, once the corneoscleral disc is excised from the donor, the tissue is placed in hypothermic storage media at 2-8°C to guarantee an optimal preservation from the donor site (morgue) to the processing facility (eye bank). Typically, this phase should be kept as short as possible in order to avoid endothelial cell stress and mortality.^{36,42}

- **Corneal Organ Culture Phase:** once the donor cornea is deemed suitable for transplantation by eye bank's evaluation, it is transferred into an organ culture medium to keep the cornea viable for up to 28 days at 31°C. This formulation is a cellular culture mix containing variable concentrations of animal (typically bovine) serum, along with essential components like amino acids, minerals, antibiotics, antifungal agents, and buffers. A crucial characteristic of this organ culture media is the absence of dextran, as extended preservation in its presence can induce endothelial cells loss.^{49,50} The absence of a deswelling agent such as dextran means that tissues will incur in corneal edema during this preservation phase, with an increase in central corneal thickness typically up to 1050-1200 µm.^{51,52} Volume solution of organ culture media are typically greater (100 mL) than hypothermic storage media, due to higher metabolic demand of cells at physiological temperatures. The only currently CE marked medical device for corneal organ culture is TISSUE-C (AL.CHI.MI.A. SRL. Italy).

- **Deswelling Transport Phase:** during this stage, the cornea is transferred in a deswelling medium to reduce corneal edema resulting from the previous phase. Deswelling media are formulated similarly to organ culture media, with the

addition of polymers with oncotic properties able to restore the physiological thickness (deswelling effect) of the cornea, that was temporarily lost during corneal organ culture. Typically, deswelling media are effective after 24 hours at room temperature. After the deswelling phase the cornea can be shipped in deswelling medium to the operation theatre or can be subjected to DMEK or DSAEK graft preparation before the shipment. Indeed, the restoration of physiological thickness makes the graft preparation procedure easier and safer. The only currently CE marked medical device for corneal deswelling and transport is CARRY-C (AL.CHI.MI.A. SRL. Italy).

Table 3. Advantages and disadvantages of organ culture in Eye Banking for corneal tissue

Organ culture in Eye Banking	
<i>Advantages</i>	<i>Disadvantages</i>
Long preservation (28 days)	Corneal edema (900-1400 µm)
Active cellular metabolism	Endothelial mortality on corneal folds due to edema
Larger window for tissue processing	Need for deswelling
Effective antibiotics	Multiple media passages (more manipulations)
Can perform microbiological analysis	No specular microscopy

CORNEAL ENDOTHELIUM EVALUATION METHODS

Regardless of the preservation method, eye banks must examine corneas before transplantation to ensure they meet the biological criteria needed for successful outcomes.

The primary indicator of adequate corneal quality for successful transplantation is the endothelium's morphological and functional condition. The most critical factors affecting corneal quality are (i) the number of endothelial cells, indicated by the parameter endothelial cell density (measured in cells/mm²), (ii) their viability and (iii) and endothelial cells morphology, that should be a regular, mosaic-like arrangement of flattened, uniformly sized hexagonal cells.

Following the EEBA guidelines, corneas deemed suitable for transplantation must possess a viable and regular endothelium (lacking cell degenerations, dystrophies or substantial pleomorphism or polymegathism), with an Endothelial Cell Density (ECD) above 2000 cells/mm².^{36,42}

Corneal endothelia are evaluated using different microscopy techniques, either specular microscopy (most common in the hypothermic storage eye banks), light microscopy (most common in corneal organ culture eye banks) and vital dye staining (e.g. Trypan Blue staining) to evaluate cells viability, density and morphology.

Light Microscopy

Light microscopy (LM) is the preferred imaging technique used for organ cultured corneas, offering a quick evaluation of the endothelium. For LM evaluation, endothelia

are usually immersed into a sterile container (typically a Petri dish) containing a hypotonic solution with the endothelium oriented toward the microscope's objective. Hypotonic solutions are used to induce swelling in the intercellular spaces, which makes the endothelial cells (ECs) visible.⁵³ This quick evaluation is usually coupled with Trypan Blue (TB) vital dye staining to quantify the percentage of cells that are dead or damaged.^{54–56} A major drawback of this protocol is the lack of standardized procedures for ECD determination, which results in significant inter-operator variability.⁵⁷ Some eye banks often use a calibrated grid/reticule (10x10 mm) mounted on one of the microscope's eyepieces to calculate ECD. Others have adopted existing software (such as REA XDL from Robin GmbH) to assist in semiquantitative methods to calculate ECD, thereby aiming to reduce the operator-dependency of the measurements. Similarly, the evaluation of EC morphology remains subjective, despite prior attempts to semi-quantitatively grade it using scoring systems.^{58,59}

Specular Microscopy

Specular microscopy (SM) is a microscopy technique that utilizes non-contact, computerized approach to analyze human corneas kept in hypothermic storage.

Specular microscopy gives the possibility to evaluate the human cornea on all of its features directly within its original preservation vial, which must have an optically clear base, or within a specialized viewing chamber. In this way, the handling of the tissue, with its potential for mechanical damage, is minimized and the risk of microbiological contamination is reduced.

The specular microscope is able to effectively visualize the endothelial layer, provided the cornea is optically transparent. The principle of how this imaging works is by

628 exploiting light reflection: light is directed onto the cornea from the bottom and is
629 specifically reflected by the endothelial layer^{60,61}, producing a high-quality image. Once
630 the image is captured, the technician is able to both observe pathologies such as
631 Fuchs' dystrophy and corneal guttae and to evaluate the endothelial cells in a semi-
632 automatic manner, using the built-in software. This process requires a technician to
633 manually select the center of the endothelial cells (ECs) by clicking, which then allows
634 the software to recognize them and quantitatively determine the ECD and
635 morphological parameters. These parameters include the hexagonality index (HEX%)
636 and the coefficient of variation of cell size (CV%), both expressed as percentages.
637 Pleomorphism is inversely indicated by HEX%, while polymegathism is directly
638 indicated by CV%. Normal criteria are generally defined as a HEX% above 50% and
639 a CV% below 40%.^{36,42}

640 All these evaluations together are able to give the technician an overall evaluation of
641 the tissue, without the need of manipulations keeping it in a closed system.

642 The evaluation process is often lengthy, typically necessitating that the cornea be
643 warmed to +35°C for up to 90 minutes to reactivate endothelial cell metabolism.⁶² This
644 procedure enhances the visibility of endothelial cells during image acquisition by
645 restoring Na⁺/K⁺-ATPase pump function, which promotes clear cell borders and
646 optimal light reflection at the endothelium-aqueous interface for effective specular
647 microscopy visualization.⁶¹ Furthermore, factors like stromal or Descemet membrane
648 folds or endothelial mortality can non-planarly reflect the light, thus hindering the
649 imaging process. This potential for inadequate examination can lead to the
650 unnecessary discarding of otherwise suitable corneas due to examination issues.

651 Finally, acquiring quality endothelial images via SM demands experienced
652 technicians.

653 **Alternative techniques**

654 A newer approach, known as the Hybrid Method, was recently developed to combine
655 the image capture of LM with the analytical capability of SM software. This method
656 has been shown to be effective for determining ECD and morphological parameters
657 like Coefficient of Variation of cell size (CV%) and Hexagonality index (HEX%), with
658 minimal, non-clinically relevant bias compared to standard LM and SM analyses.⁶³

659

Chapter 4

VITAL DYES

The corneal endothelium is the determinant of graft clarity after transplantation. Small absolute losses in viable endothelial cells during preparation or handling translate into clinically meaningful reductions in postoperative endothelial cell density (ECD), increased graft failure risk, and higher re-bubbling rates. Eye banks therefore must provide accurate, reproducible information on ECD and focal endothelial damage so surgeons can match tissue to indication and anticipate intraoperative handling. For the eye banks that follow organ-culture (OC) protocol, trypan blue exclusion assay protocols have been used routinely^{64,65}, whereas hypothermic-storage eye banks rely more heavily on specular microscopy and cell counts.⁶⁶ Validated eye-bank prepared tissue (full thickness, pre-stripped/preloaded) allows shorter operating time and wider dissemination of DMEK, with generally comparable clinical outcomes when adequate QA is performed; but preparation-related endothelial cell loss (ECL) during processing and staining contributes materially to early and sometimes late postoperative cell counts and re-bubbling risk. Systematic quantification at the eye-bank level (and standard reporting to surgeons) improves selection and surgeon decision-making. To achieve this, eye banks use a mix of non-destructive imaging (specular microscopy, slit-lamp/phase imaging) and vital-dye based methods in conjunction with light microscopy to evaluate corneal endothelium.⁶⁷

OC (typically 31–37°C culture after procurement) method allows longer storage and importantly permits post-injury endothelial recovery processes that are not observed during hypothermic storage.^{68,69} This has implications for interpreting viability assays, a damaged area may demonstrate some repair/regression under OC, so timing of assessment relative to injury matters. OC also makes some dye-based assessments (easier to wash, precondition) relatively easier. On the other hand, cold storage (4°C, e.g., Optisol-GS) is simpler and ubiquitous in many aspects. It demands minimization of time out of cold during any bench testing.⁷⁰ Non-contact/rapid specular central ECD measurement is therefore commonly used; full-surface staining approaches are possible but must be balanced against additional cold-exposure and manipulation. Practical consequence is that organ-culture banks have more flexibility to perform laboratory viability assays and to accommodate fluorometric tests; hypothermic banks often favor rapid, non-destructive central ECD measurement plus selective staining when needed.

Vital dyes and whole-graft visualization

Vital dyes are a practical bridge between eye-bank QA and surgical needs, as they permit visualization of dead/damaged cells over the *entire* graft surface. Several dyes/approaches are in routine or emerging use.

Trypan blue exclusion assay

Trypan blue (TB) staining is currently the most widely validated, pragmatic method in eye-bank workflows for whole-graft endothelial visualization (particularly in DMEK preparation) and can be quantitative when combined with image analysis. It can further

shorten processing time vs full specular imaging and visualize the entire graft surface. However, technique and exposure time matter because excessive exposure/contact can add stress.^{71–78} Quick “bath” staining or selective staining of Descemet’s membrane to reveal denuded areas and cell loss has been widely used to assess DMEK grafts after preparation to enhance visibility during loading. Eye-bank studies show TB staining combined with image analysis (ImageJ/FIJI) yields quantifiable ECL estimates comparable to manual specular measures and can assess the whole graft.⁶⁶ Implementation of TB-based whole-graft inspection can reduce processing times and cold-exposure compared with full specular mapping. TB staining further facilitates ECL mapping from preloaded grafts after the shipment (studies report values from approximately 13 – 19%).^{65,79} However, dye concentration, contact duration and technique (selective staining vs bath) influence both visualization and potential toxicity. Some surgical techniques that avoid direct endothelial contact with TB (selective staining/liquid bubble methods) report lower postoperative ECL than bath-staining approaches, while bath staining may improve double-scroll formation beneficial for implantation, as also reported in a clinical study.⁷⁸

Calcein-AM staining

Calcein-AM / fluorescence “live-cell” assays provide higher sensitivity for live/dead mapping and can be adapted for pre-stripped graft validation but needs fluorescence imaging capacity and careful optimization to avoid dye toxicity and incompatibility with downstream immunostaining. Recent work has improved calcein-AM protocols specifically for DMEK tissue, however, due to the toxicity of the dye, it has not yet been fully exploited by the eye-banks for routine use.^{13,69,76,80–82}

Calcein-AM is a cell-permeant ester cleaved by intracellular esterases to fluorescent calcein in live cells; dead cells remain unstained (often used with ethidium homodimer or propidium iodide as counterstain). It allows higher sensitivity for live/dead mapping and can be quantified across the graft. Recent optimizations addressed dye washout, photobleaching, and compatibility with post-assay immunofluorescence. Calcein assays are increasingly used in laboratory validation of pre-processed DMEK grafts. Limitations for routine eye-bank quality assurance include the requirement of fluorescent imaging equipment, potential dye toxicity if not optimized, and compatibility issues with releasing tissue for clinical use (unless dye is fully washed away and validated safe). Alternative vital stains / augmenting agents like Lutein/Trypan combinations have been proposed to improve visualization while potentially lowering toxicity, while early pilot work shows promise for DMEK graft inspection.

Destructive staining - not for clinical use

These types of staining are usually used for end-stage analysis, some of which include TUNEL for apoptosis⁸³, ethidium homodimer for staining the dead cells, Alizarin Red stain⁸⁴ to detect cellular borders, and triple-labeling methods that uses a combination of Hoechst, ethidium homodimer, and Calcein-AM staining.^{13,80} These staining methods remain gold-standard for mechanistic/quantitative lab studies of cell death, apoptosis and intercellular border integrity but are not compatible with clinical release of tissue.³³

For mechanistic or quantitative research on endothelial cell death, or for validation studies that sacrifice tissue, the following are used. *Alizarin Red S* - stains intercellular borders and can be used with TB to visualize both borders and dead cells (classic

dual-stain method).⁸⁵ This technique is good for morphological assessment but is destructive. *Hoechst 33342 + Ethidium homodimer + Calcein (HEC- triple labelling)* - allows discrimination of total nuclei, dead nuclei and live cells. HEC staining is used in research to determine apoptotic vs necrotic fractions.¹³ *TUNEL / caspase immunostaining* - identifies apoptosis for mechanistic studies.^{13,85-87} These assays provide high-resolution, biologically specific data but are unsuitable for tissues intended for transplantation.

How eye-bank assessments influence surgical practice & patient outcomes

Preprocessing (eye-bank) to surgeon decision making

Eye banks that provide whole-graft ECL maps (via dye imaging + image analysis) enable surgeons to match higher-quality grafts to complex eyes, anticipate intraoperative behavior (e.g., fragility, scroll tightness), and decide on using preloaded vs surgeon-prepared tissues.⁸⁰ Validation studies show prestripped/preloaded tissues with documented ECL can be shipped and used safely with comparable clinical outcomes when QA thresholds are met.^{66,88}

Effects on intraoperative workflow and outcomes

Operating room efficiency / wider uptake of DMEK can be achieved by using eye-bank prepared and preloaded tissues. These techniques have shown to reduce surgeon preparation time in theatre and lower the barrier to DMEK adoption which could further shorten anesthesia and OR time and possibly reduce intraoperative handling-related ECL.^{65,89} Meta-analyses and multi-center series show comparable visual outcomes

and variable impacts on re-bubbling and early ECL; but processing-related ECL (documented at eye bank) contributes to the total early postoperative ECL.

Staining technique used at preparation (eye-bank or surgeon) affects postoperative ECD

Selective TB techniques that avoid direct endothelial contact show lower ECL in some series compared to bath staining, but may produce different scroll behaviors that affect ease of implantation.⁷⁸ Surgeons thus use eye-bank staining reports to plan graft handling.

Quantified impacts

Preloaded DMEK studies report mean processing-related ECL to approximately 10–20% depending on technique and shipping/storage including mean ECL ~16.8% after overnight storage and shipping studies reporting ~18.5% ECL.^{65,66,89,90} Postoperative ECL (early postoperative period) frequently adds another ~20–30% depending on technique and re-bubbling events. In clinical cohort comparisons, differences in 3-month ECD of the order of hundreds of cells/mm² (approximately 5–10% relative) have been reported between staining/preparation methods. These percent ranges are consistent across multiple validation and clinical outcome studies.

Practical recommendations for eye banks (operational takeaways)

It is advisable to adopt a multilayered QA approach, such as a) central specular ECD for baseline reporting in addition to whole-graft visualization (trypan blue + image

analysis) for pre-stripped/preloaded tissues to detect focal damage to improve speed and reduce mortality; b) standardize TB protocols (concentration, exposure time, wash steps, image capture conditions) and ECL reporting with a standard image-analysis pipeline (e.g., FIJI/ImageJ); c) validate methods to reduce inter-processor variability and shorten processing time³³; d) optimize Calcein-AM protocols for lab validation of novel processing/transport conditions (especially in OC banks) for clinical release; e) provide structured tissue reports to surgeons (pre- and post-processing ECD, whole-graft ECL map, storage/shipping conditions), these data materially inform graft selection and operative plans; and f) minimize dye contact on fragile grafts where possible; for surgeons, consider the trade-off between improved visibility (bath staining) and potential incremental toxicity in marginal tissues.

Limitations in the literature and knowledge gaps

Lack of universally standardized protocols for staining, imaging, and ECL quantification across eye banks makes cross-study comparisons challenging. Comparative long-term clinical outcome data linking specific eye-bank QA methods (e.g., calcein vs trypan vs specular alone) to 1- and 5-year graft survival are limited. Most studies focus on early postoperative ECD and rebubbling. Toxicity thresholds and cumulative stress (mechanical + chemical) for endothelial cells during processing vs clinical behavior remain incompletely characterized. Hence, more mechanistic work (TUNEL/caspase, live imaging) is needed to identify modifiable steps.

Future directions (research & practice)

Automated whole-graft image analysis (AI/ML) to standardize ECL quantification from TB or calcein images.^{33,91} Improved non-toxic dyes or staining methods that yield excellent visibility without compromising cell function (e.g., lutein-enhanced dyes). Standardized multi-center trials correlating eye-bank QA metrics with long-term graft survival and patient-reported outcomes. Optimized calcein-AM workflows for validation of pre-stripped/preloaded tissues to support broader adoption of preprocessed grafts where appropriate.

Conclusions

Eye-bank endothelial assessment is a critical bridge between tissue procurement/processing and surgical outcome. A pragmatic hybrid approach, specular microscopy for central ECD + whole-graft vital dye imaging (principally trypan blue) with standardized image analysis, currently provides the best balance for clinical tissue release and surgeon information needs. Fluorescent live/dead assays (calcein-AM) and destructive triple-label techniques remain indispensable in validation and research but are not yet first-line routine release assays for clinical tissue without rigorous safety validation. Eye-bank reporting of quantified, whole-graft ECL directly impacts surgical planning, operative technique choice, and ultimately early postoperative cell counts, and therefore should be integrated into standard QA practice and surgeon communication pathways.

Chapter 5

MINIMIZING ENDOTHELIAL DAMAGE DURING CORNEA MANAGEMENT

Each procedural step from donor cornea procurement to keratoplasty may carry risk of iatrogenic corneal damage, especially to the endothelium, which cannot regenerate. This chapter focuses on factors representing risks for endothelium health from procurement to corneal transplantation and suggests consequent best practices for eye banks for managing corneas during their route from the donor to the recipient.

Procurement

All personnel involved in the management of corneas must follow proper procedures to preserve corneal health immediately following donor death. Placing ice directly on the donor's eyelids is to be avoided, as it can cause cold shock tissue damage and induce endothelium necrosis due to formation of intracellular and extracellular ice crystals.^{92,93} Therefore, hospital staff taking care of the donor body should receive training on appropriate methods to support donor corneal endothelial health.

Procurement should take place as soon as possible in a sterile environment. There is currently no universal consensus regarding the maximum allowable death-to-explant (DEI) time. For example, the Joint United Kingdom Blood Transfusion Services Professional Advisory Committee (JPAC) recommends a maximum interval of 24 hours from death to enucleation⁹⁴, while the EEBA Technical Guidelines for Ocular

867 Tissues (TGOT) suggest a maximum DEI time of 48 hours (§1.4), leaving the final
868 decision to individual eye banks.³⁶ Additionally, some studies report that a maximum
869 DEI time of up to 72 hours does not compromise corneal transplant quality.⁹⁵ It is,
870 however, generally recommended that corneal procurement should occur as soon as
871 possible after donor's death.⁹⁶

872 Taking into account that post-mortem ocular tissue is to be considered contaminated³⁶,
873 it is crucial to perform thorough decontamination of the donor eye globe before
874 excision, to reduce microbial contamination that may affect endothelial viability during
875 preservation. Povidone-iodine with concentrations ranging between 0.5% and 5% w/V
876 applied on donor globes before explantation is the most often employed antiseptic
877 solution for its recognized wide-spectrum activity against bacteria, fungi and viruses.^{97–}

878 101

879 After decontamination, the donor corneoscleral button can be excised from the donor's
880 eye by the harvesting surgeon. This step is delicate for endothelium, as excessive
881 traction during excision can result in iatrogenic damage or lysis of corneal endothelial
882 cells with a typical pattern of central striae or stretch marks of degenerated
883 endothelium. Therefore, the surgeon must be trained to perform procurement with
884 utmost care.¹⁰²

885 The shipment from the procurement site to the eye bank should be carried out in
886 accordance with validated procedures that ensure proper use of medical devices
887 under their intended use and maintain controlled transport conditions (i.e. validated
888 transport box), as stated in EEBA TGOT (§1.5).³⁶ Of note, the EEBA TGOT
889 recommends using only reagents and materials from suppliers that meet CE marking
890 / pharmacopeia requirements during all processing and storage steps of the cornea

(§2).³⁶ The aim of this step is to deliver the tissue to the eye bank as promptly as possible while preserving appropriate and standardized transport and temperature conditions. Shipment of corneas at uncontrolled room temperature, though practiced by some eye banks^{57,13} is less effective for preserving corneal endothelium viability.^{103,104} Hypothermic shipping is therefore preferable.

Preservation at the Eye Bank

Upon arrival at the eye bank, two primary preservation methods are employed, as described above: corneal organ culture and hypothermic storage. These approaches correspond to distinct evaluation techniques and potential impacts on endothelial health.

One major difference between corneal organ culture and hypothermic storage is that the former requires more manipulations of the donor cornea, involving multiple media (typically a long-term organ culture medium followed by a short term deswelling medium) and more extensive tissue handling for evaluation. In contrast, hypothermic storage uses a single mid-term medium containing at least one deswelling agent from procurement to the operation theatre, resulting in reduced manipulation.^{39,57}

Another key distinction relates the metabolic status of the cornea. During hypothermic storage, metabolic activity is minimal, whereas in corneal organ culture cellular metabolism is active with ongoing nutrient consumption and metabolic waste production. The volume of organ culture medium is limited (usually 100 mL) and not replenished as in vivo, making it exhaustible during prolonged organ culture¹⁰⁵, lasting up to maximum of 34 days.³⁶ A previous study indicated that decreasing the organ culture medium volume below 80 mL exacerbates endothelial cell loss during storage

of human corneas at 31°C for up to 30 days.¹⁰⁶ Consequently, maintaining an adequate medium volume is critical for long term organ culture, and procedures that involve withdrawal of storage medium (such as for microbiological testing) should consider this factor and minimize medium removal whenever possible. Alternatively, renewal of the medium every 1–2 weeks during storage is a feasible approach to replenish nutrients supply and remove metabolic wastes.³⁹

The positioning of the cornea within the storage vial during organ culture is another critical parameter to be considered. During organ culture, the corneal epithelium undergoes continuous regeneration¹⁰⁷, resulting in the shedding of cells and tissue debris into the culture medium. These cellular remnants, which contain proteolytic enzymes such as cathepsins and matrix metalloproteinases, settle by gravity over the bottom of the vial where accumulate and can exert toxic effects to the endothelium by extracellular matrix degradation.^{108–110} To minimize such detrimental effects, specific methods have been developed to suspend the cornea in the culture medium, thereby avoiding direct contact with the vial's bottom. This suspension maintains a cleaner environment around the cornea, reduces exposure to potentially harmful degradative components, and supports the preservation of the endothelium health during organ culture.

The first adopted technique involved the insertions of a suture thread into the corneal scleral rim securing it with a knot, with the opposite end of the suture being passed through a specific opening in the vial cap. This procedure should be considered obsolete and avoided as it introduces risks of microbial contamination through the opening in the vial cap and along the suture thread, which can harbour bacterial biofilms.^{111–113}

The use of sterile, floating suspension devices made of biocompatible plastic and affixed to the scleral rim via a claw, without direct endothelial contact, represents instead a safe method for maintaining corneal separation from the vial bottom without posing microbiological risks to tissue health. In addition, the use of floating suspension devices enables easier application and removal of the device, as well as more straightforward retrieval of the cornea from the vial for examinations. Therefore, this approach simplifies handling procedures and reduces risk of iatrogenic corneal damage associated with manipulation.¹¹⁴

Endothelium evaluation at the eye bank

Assessing corneal quality often requires manipulation that can risk iatrogenic endothelial damage. The procedures required for evaluating corneal quality are more significant in organ-cultured corneas. In contrast, hypothermic storage vials feature optical bottoms, allowing tissue evaluation via specular microscopy without removing or handling the cornea.^{39,57} Nevertheless, hypothermic stored corneas are also subject to iatrogenic risks during evaluation procedures. For example, some eye banks employ viewing chambers, specialized containers designed to transport the cornea and compatible with specular microscopy evaluation due to their two optical fundi. Size compatibility between the chamber and the scleral rim size is crucial, because mechanical stress can damage the endothelium once the chamber cap is closed if the scleral rim is too large for the chamber. A previous report showed that different viewing chambers currently available in the market have different compatibility ranges with scleral rim sizes¹¹⁵, so selecting the appropriate chamber is important for each eye bank based on the typical scleral rim dimensions handled.

For organ cultured corneas, endothelial examination involves four main steps: (i) rinsing the tissue, (ii) staining the endothelium with Trypan Blue (TB) staining solution, (iii) immersing the endothelium into a hypotonic solution, and (iv) imaging with a light microscope. These procedures carry the risk of iatrogenic insult if not conducted with precision and care. Adherence to validated and evidence-based protocols is therefore essential to minimise endothelial damage.

Rinsing the cornea prior to examination

The first step needed for endothelium examination is the rinsing of the cornea to remove storage medium residues from the tissue that may interfere with TB staining and hypotonic solution effect. Isotonic solutions such as Balanced Salt Solution (BSS), or CE-marked medical devices solutions designated for corneal rinsing like PSS-L (AL.CHI.MI.A. SRL, Ponte San Nicolò, Italy), should be used.¹¹⁶

During this step, the operator should avoid damaging the cornea with excessive solution flow or mechanical force. The recommended method is to fully immerse the cornea into rinsing solution and gently rinse it for one minute at room-temperature with delicate movements using a sterile forceps.

Staining with Trypan Blue solution

During the process of endothelium staining with TB solution, factors such as TB purity, concentration, and exposure time should be considered carefully to minimise potential endothelium damage.

985 The first parameter to consider is the purity of the raw TB material. TB (CAS number:
986 72-57-1) is a synthetic bis-azo dye, manufactured through chemical synthesis
987 involving azo coupling reactions of aromatic amines derived from toluidine
988 compounds. This process can result in the formation of mono-azo by-products (the so
989 called “purple fraction”), which may be present as contaminants alongside with TB.¹¹⁷
990 Mono-azo compounds are the most toxic impurities found in TB dye.^{117,118} Impurities
991 in TB preparations have been associated with toxic anterior segment syndrome and
992 complete endothelial cell loss.¹¹⁹ Research-grade TB is subject to less stringent quality
993 controls and may therefore pose increased risks to endothelial integrity; therefore, its
994 use is not recommended on donor corneas, and eye banks should use CE-marked TB
995 solutions that comply with ICH Q3A and Q3B contaminant guidelines.^{120,121}

996 The second and third TB-related aspects, i.e. concentration and exposure time of the
997 TB staining solution, should be considered and both minimized, as TB is inherently
998 cytotoxic. An in vitro study on human ARPE19 cells found that TB concentration
999 decreased cell viability in a concentration-dependent manner and that TB 0.4%
1000 increased significantly apoptosis-related gene p53 expression.¹²² The work by van
1001 Dooren and colleagues investigated the effect of combination of different TB
1002 concentration (range: 0.001% - 0.1%) on ex vivo donor human corneal endothelium,
1003 showing that exposure to 0.1% TB for 30 minutes is detrimental for endothelial
1004 morphology.⁵⁹

1005 Considering that TB concentrations used significantly differ among eye banks, with
1006 EEBA “Endothelial Evaluation Report 2020” (38th ed., §3.7.1) reporting concentrations
1007 between 0.06% and 0.6%, it may be concluded that the impact of TB concentration in
1008 endothelial toxicity may be underestimated by eye banks.¹²³

A more recent study performed on endothelial cells on DMEK grafts suggested the combination of concentration and duration of application of TB dye to be 0.06% up to 5 minutes, to enhance visibility of the graft while minimizing damage to the endothelium, while a longer staining time resulted in a decrease in ECD.¹²⁴

Based on abovementioned considerations, it can be speculated that given a certain level of TB purity (with higher purity being preferable), the safety of TB staining depends on the combination of the concentration of the solution and the duration of exposure.⁵⁹ By tuning these two factors, effective and safe staining with different strategies can be achieved. For example, lower TB concentrations can be used with longer exposure, while shorter exposure times can take advantage of higher concentrations.

A final practical consideration on TB solution is that it should be applied slowly, drop-by-drop, directed toward the corneal periphery rather than the central zone, in order to minimize flush and shear stress on the endothelium.

Hypotonic solution and microscopy examination

After TB staining, the corneal endothelium must be immersed into a solution to perform microscopy analysis. Literature reports different strategies, commonly using hypotonic sucrose solutions in the range of about 1.4% to 1.8% or hypotonic balanced salt solution (BSS) obtained by dilution in water, and sometimes sodium chloride solutions with concentrations around 0.45% (hypotonic saline) to 0.9% (physiologic [isotonic] saline).^{57,125,53,126–129}

Hypotonic solutions enhance the visualization of endothelial cells and facilitate microscopy analysis. However, exposure duration is critical and should be as limited

as possible to avoid cytotoxicity induced by osmotic stress. Of note, a study performed in porcine corneas showed that exposure of endothelium to 1.8% sucrose hypotonic solution for 4 minutes resulted in a significant endothelial cell loss.¹²⁸

Short exposures (a few minutes) to hypotonic solutions are recommended to avoid damage to corneal endothelial cells. Therefore, hypotonic solution osmolality and exposure time should be balanced to minimize osmotic stress.

During microscopy analysis, maintaining aseptic conditions of the cornea is also crucial, in order to minimize microbial contamination risk. The ideal setup is performing microscopy under a laminar flow hood, but this is not always feasible. An effective alternative is transferring the cornea within a closed system, such as a Petri dish, allowing safe handling outside the hood guaranteeing aseptic conditions.

Shipping to the operating theatre

As with the transfer of corneas from the procurement site to the eye bank, transporting the cornea from the eye bank to the operating theatre carries potential risks of iatrogenic endothelial damage. It is therefore essential to use medical devices specifically intended for cornea shipment and follow validated procedures and standardized control of temperature.³⁶ When using viewing chambers for transport, it is important to note that a previous study using stress leakage tests demonstrated significant differences in hermetic sealing performance among marketed chambers.¹¹⁵ Noteworthy, failure of hermetic sealing increases the risk of microbial contamination, which can compromise endothelium safety and overall tissue quality during transit.

Chapter 6

TISSUE QUALITY AND ENDOTHELIAL EVALUATION BY THE CORNEAL SURGEON

Endothelial viability assessment has emerged as a critical quality control step in modern corneal transplantation, particularly for endothelial keratoplasties such as DMEK and DSAEK. While eye banks perform initial evaluations, surgeons' intraoperative checks can address post-release cell losses, ensuring only functional endothelium is transplanted. This surgeon-led evaluation complements standard metrics like ECD, revealing hidden damage that predicts graft outcomes.

Why Should the Surgeon Evaluate the Endothelium?

ECD, measured via specular microscopy, serves as the primary benchmark for donor cornea suitability across eye banks worldwide. Typical thresholds range from 2,000 to 2,500 cells/mm², reflecting decades of data linking higher ECD to improved graft survival. Landmark studies, including the Cornea Donor Study, have established that donor age and ECD independently influence long-term outcomes in penetrating keratoplasty, with lower ECD accelerating cell loss over 10 years post-transplant.^{7,9} These findings underscore ECD's prognostic value, yet its limitations demand supplementary surgeon assessments.

First, ECD measurements are geographically biased toward the central cornea, typically sampling a 0.5-1 mm² area, while surgical grafts utilize peripheral

endothelium prone to handling artifacts, folds, and trauma.¹³⁰ This central-peripheral mismatch becomes critical in DMEK and DSAEK, where grafts cover 8-9 mm diameters, amplifying the impact of unseen peripheral damage. Pipparelli et al. quantified this discrepancy using pan-corneal viability assays on eye bank-pre-dissected grafts, revealing that the actual number of live, functional endothelial cells was 12-20% lower than ECD reports, depending on tissue preparation and storage conditions.¹³ This gap can contribute to the rapid early postoperative ECD decline, which is common after all corneal transplantations and can often be in the range of 20-40% within the first year¹³¹, suggesting fewer viable cells are transplanted than anticipated.

Second, eye bank ECD reporting exhibits systematic biases. Munir et al.'s analysis of 48,207 donor reports identified unnatural clustering of ECD values just above common clinical cutoffs (e.g., 2,500 cells/mm²), with a sharp discontinuity below thresholds.¹³² This pattern implies subjective human influence during semi-automated counting, where technicians may favor supra-threshold reports to maximize tissue utilization amid donor shortages. Such overestimation risks releasing suboptimal corneas, contributing to primary graft failures.

Third, endothelial cell loss (ECL) continues post-eye bank release due to storage, transportation, and preparation stresses. Preloaded DMEK grafts, increasingly common for surgical efficiency, experience 10-20% ECL from scrolling, insertion, and media changes.^{76,80} International shipments may exacerbate this: Imafuku et al. reported variable damage in US-to-Japan corneas based on transit duration and donor factors.¹³³ Wojcik et al. found an average of around 20% ECL in preloaded DMEK grafts shipped from Italy to the UK.¹³⁴

Finally, ECD quantifies density but not viability or function. Morphological metrics like polymegathism (cell size variation) and pleomorphism (hexagonality loss) offer clues to stress but fail to differentiate live from apoptotic cells. Functional assays, such as pump leakage or barrier integrity, are impractical preoperatively. Surgeon evaluation bridges these gaps, enabling real-time rejection of poor tissues, reducing waste, and aligning transplants with patient-specific needs.

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1129 parts":[["2024",5,1]]}},{"id":2364,"uris":["http://zotero.org/users/4010603/items/6XED
1130 XC7Y"],"itemData":{"id":2364,"type":"article-journal","abstract":"In the current issue of
1131 BMJ Open Ophthalmology , Downward et al ,1 on behalf of the UK National Health
1132 Service Blood and Ocular Tissue Advisory Group and Contributing Ophthalmologists,
1133 investigate long-term graft survival and rejection rates in patients who received
1134 corneas from donors who donated both corneas for Fuchs' endothelial dystrophy or
1135 pseudophakic bullous keratopathy (PBK), defined as 'paired' donors. The aim was to
1136 assess, whether there was an association between recorded donor characteristics
1137 such as endothelial cell density (ECD), age and sex, and endothelial graft failure and
1138 rejection. A total of 1536 paired and 9302 unpaired corneal transplants were included.
1139 While for these endpoints, no clear differences were found between paired and
1140 unpaired patients in terms of endothelial graft failure and rejection rates, the authors
1141 were able to confirm a lower ECD in unpaired single eye donors. In addition, based on
1142 their results, they suggested that unpaired single cornea donors had slightly
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1159 "author":[{"family":"Steger","given":"Bernhard"},{"family":"Kaye","given":"Stephen
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1164

1165 **Risk of Overlooking Poor Quality Tissues and Impact on Graft Failure**

1166 Primary graft failure (PGF) remains a devastating complication of keratoplasty,
1167 characterized by profound endothelial depletion leading to corneal edema within
1168 weeks postoperatively. Clinicopathologic series confirm PGF stems from preoperative
1169 or intraoperative cell loss, rarely rejection, with decompensated grafts showing near-
1170 total ECL on histopathology.¹³⁵ Overlooking poor-quality tissues—via reliance on ECD
1171 alone—amplifies this risk.

1172 In our cohort of UT-DSAEK eyes in the V-CHECK study, TB staining revealed
1173 fingerprint-like endothelial stains in most lenticules, strongly associating with early
1174 graft failure. These cases lacked rejection episodes (only one reverted successfully)
1175 or late complications, implicating pre-/perioperative damage. TB-positive areas
1176 (TBPA) exceeding 10-15% marked high-risk grafts, uncaptured by eye bank ECD.

Similarly, preloaded DMEK grafts in non-scrolled, endo-out configurations showed 25% TBPA yet proved viable upon detailed assessment, highlighting how unassessed tissues risk discard despite transplantability.¹³⁶

Large-scale data reinforce these findings: 4.1% of 194 internationally transported corneas exhibited >10% TBPA, stratifying donors into high/low damage groups via multivariate models.¹³³ Post-preparation analyses consistently report 15-20% mortality in DMEK tissues, far exceeding central ECD estimates.¹³⁷ The failure cascades ensue: low viable cells trigger ECL, edema, and retransplant needs, inflating costs and waitlists.

Quality assurance surveys note that donor shortages drive marginal tissue use, yet PGF rates (1-5%) persist, partly from undetected damage.¹¹ Surgeon-led checks mitigate this by identifying biomarkers like fingerprint stains, correlating TBPA directly with outcomes where ECD fails.

State of the Art of Perioperative Evaluation of Endothelial Viability

Perioperative endothelial assessment has evolved from visual inspection to standardized, quantifiable protocols, driven by imaging and automation advances. Trypan blue (TB) staining dominates due to its simplicity, selectivity for dead/damaged cells, and intraoperative feasibility.

Scherer et al. used an image analysis protocol to identify patterns of endothelial damage in a semiautomated fashion, defining categories of damage and causal hypothesis—street-like (preparation trauma), peripheral dots (handling), focal (folds)—using 36 donor corneas before a no-touch liquid bubble DMEK preparation

technique.³⁴ They employed a semi-automated approach in which endothelial damage was manually traced and quantified on captured images, enabling the characterization of the distinct injury patterns.

Although their meticulous work adds valuable evidence to the growing interest of cornea specialists in quality assurance of donor tissues, we would aim to underscore specific technical aspects that may have reduced the accuracy of the imaging analysis and to contextualize the authors' findings within recent efforts to standardize preoperative assessment of endothelial viability. Specifically, pooling of the dye can mask the underlying endothelial damage, and it makes automated or semi-automated analysis of TB-positive areas more challenging. In the V-CHECK protocol, we have proposed a similar method to quantify the proportion of TB-positive areas from surgical images of donor cornea to assess the pan-corneal endothelial viability and suitability for transplantation.^{33,91} To optimize image acquisition, the cornea should be rinsed thoroughly with BSS to remove any residual conservation medium such as Modified Eagle Medium (MEM), as its presence can reduce the effectiveness of TB staining due to its viscosity and a dilution effect. Additionally, we recommend that the cornea is filled up to the Schwalbe's line with BSS after rinsing, to capture images without any light reflexes from the surgical microscope. Blocs or trephines without suction holes should be used to avoid optical disturbances in the acquired images. Finally, we suggest moving from a manual and/or semi-automated protocol, which can introduce bias due to intra- and inter-reader variability, to a fully automated analysis of endothelial viability.

Szkarlat et al. developed a TB-based, pan-corneal viability assessment after the processing of pre-loaded DMEK tissues in an eye bank setting.³² Interestingly, they found that visual assessment by experienced eye bank technicians produced similar

estimations of global endothelial cell mortality compared to automated image analysis, while specular microscopy overestimated the ECD after DMEK preparation, again suggesting that ECD represents an inaccurate and poorly reproducible measure of endothelial viability. It is also important to note that estimation of endothelial mortality through automated image processing in Szkarlat et al. as well as in other similar works analyzing DMEK tissues after preparation frequently exceeds mortality values of 15-20%, underlying how the evaluation of the whole endothelium might frequently yield larger estimates of endothelial damage compared to ECD or visual inspection.^{31,32,137}

Yoon et al. presented a comparable method to evaluate the damage to corneal endothelium after preparation of corneal tissues for transplantation.¹³⁸ In their analysis, images of donor corneas stained with 0.06% Trypan Blue were processed in an image analysis software using manual selection of stained areas. Ghafarian et al. used novel light with 0.6% TB to assess a non-scrolled "endo-out" preloaded DMEK graft, revealing 25% cell damage but confirming ultimate cell viability via ImageJ.¹³⁶

Jardine et al. utilized trainable segmentation software (Fiji) to analyze calcein AM-stained DMEK grafts, demonstrating that this automated method accurately and reproducibly quantified an average endothelial cell loss exceeding 18%. This established a novel, standardized quality control measure for eye banks to evaluate the safety of their preparation techniques.³¹

Subsequent research expanded on software-based quantification by evaluating preloaded DMEK tissues stored for 24 hours, finding no significant difference in endothelial cell loss (19.2%) compared to freshly prepared grafts (22.0%).¹³⁹ Furthermore, the study validated MetaMorph software as a highly efficient and

reproducible alternative to Fiji for quantifying vital dye staining, significantly reducing the time required to process images.

More recently, the application of artificial intelligence has revolutionized accuracy of pan-corneal viability assessment, as exemplified by the V-CHECK validation study in which a deep learning model, trained on foundation architectures, automated TBPA segmentation across full corneoscleral rims, reporting 6.5-9.5% higher mortality than manual gold standards with inter-operator reproducibility.³³

Moreover, to our knowledge the V-CHECK protocol is the only image analysis protocol aiming to be included in the operating theatre for a perioperative assessment of tissue quality.

Possible Options for Endothelial Assessment in the Eye Bank

Currently, most global eye banks adhere to protocols established by organizations such as the Eye Bank Association of America (EBAA) or the European Eye Bank Association (EEBA), which generally mandate an endothelial cell density (ECD) of >2,000 cells/mm², cell mortality below 5%, and the maintenance of a normal hexagonal morphologic profile.³⁶ However, the methodology used to verify these metrics varies significantly, categorized into non-destructive clinical assessments and destructive research-grade assays.

Non-destructive methods are mandatory for tissue intended for transplantation as they preserve the graft. Specular microscopy remains the universal gold standard, though it is increasingly being augmented by artificial intelligence (AI) to automate cell counting and reduce the subjective human bias that can be seen in manual counting methods.¹³²

With regards to safety concerns, it is recognized that higher concentrations and prolonged exposure times to TB are cytotoxic to corneal endothelial cells. TB, a synthetic di-azo dye, relies on a compromised lipid bilayer to enter the cell membrane and bind to intracellular proteins. In vitro and ex vivo studies demonstrate that TB concentrations commonly used in ophthalmic surgery (e.g., 0.06%–0.15%) and exposure times of 1–5 minutes do not result in significant corneal ECL or functional impairment and are considered safe for clinical use.⁵⁹ Short-term exposures (≤ 5 minutes) to 0.06%–0.15% TB do not impair mitochondrial function or reduce ECD.^{124,140} The V-CHECK protocol employs staining with 0.05% TB for 30–45 seconds, well below the cytotoxic thresholds.

Conversely, cytotoxicity becomes evident at higher concentrations (as low as $\geq 0.1\%$) and/or extended exposure (as low as ≥ 30 min) in an inversely proportional fashion, leading to significant endothelial cell loss and morphological changes.^{59,124} Despite these known thresholds, 0.25% TB is commonly used by eye banks during multiple stages (stripping, evaluation and loading) of membrane preparation for DMEK surgery, and no toxicity issues have been reported during validation.⁷¹

In contrast, destructive pan-viability assays provide a comprehensive, "bird's-eye" view of the entire endothelial monolayer, though they render the tissue unusable for clinical transplantation. Triple staining—using a combination of Hoechst, ethidium homodimer, and calcein-AM—allows for the differentiation between live, apoptotic, and necrotic cell populations. However, research suggests that this method may over-index on "stressed" cells. In preloaded Descemet Membrane Endothelial Keratoplasty (DMEK) grafts, triple staining often flags cells that are calcein-low but not yet membrane-permeable, potentially overestimating iatrogenic damage.⁸⁰ Alternatively,

1296 Trypan Blue (TB) exclusion assays are increasingly utilized after graft preparation to
1297 detect true denuded areas of Descemet membrane or dead cells with compromised
1298 lipid bilayers.³²

1299 Advanced quantification now employs trainable segmentation software, such as Fiji or
1300 MetaMorph, to measure endothelial cell loss (ECL) with high reproducibility.³¹
1301 Furthermore, applying calcein-AM specifically after tissue deswelling has been shown
1302 to yield a "viable ECD" (vECD) that aligns more closely with routine counts, offering a
1303 more functional metric for eye bank quality control.¹⁴¹

Bank Method	Viability Metric	Pros	Cons	Utilization
Specular ECD	Density/morphology	Standard	Central bias	Universal
Triple Stain	Live/apoptotic/dead %	Detailed	Overestimation, destructive	Select labs, research
TB Pan-Assay	TBPA %	Simple, accurate	Dye safety	Growing (Europe)
AI Segmentation	Automated TBPA	Scalable	Validation needed	Emerging
vECD Calcein	Viable density	Functional	Post-prep	Research/optimized prep

1304

1305 **Role of the V-CHECK assessment**

1306 The V-CHECK platform represents a paradigm shift in corneal transplantation by
1307 uniquely empowering surgeons with an objective, real-time quality assurance tool. The

1308 process is fully automated via free deep-learning models, which significantly reduce
1309 the inter-reader variability and subjectivity inherent in manual and semi-
1310 automated.^{32,34,138} Evidence confirms the protocol is safe, utilizing sub-cytotoxic TB
1311 concentrations that preserve endothelial function.⁵⁹

1312 Implementation is designed for global accessibility, requiring minimal infrastructure
1313 beyond emerging smartphone microscope applications and open-source AI.³³ By
1314 standardizing the synergy between surgeons and eye banks, V-CHECK aims to curb
1315 tissue waste and harmonize keratoplasty outcomes.

1316

Chapter 7

PREOPERATIVE SURGEON EVALUATION OF CORNEAL ENDOTHELIAL STATUS: THE VIABILITY CONTROL OF HUMAN ENDOTHELIAL CELLS BEFORE KERATOPLASTY (V-CHECK) STUDY PROTOCOL

Published Paper

Airaldi M, Zheng Y, Aiello F, et al. Preoperative surgeon evaluation of corneal endothelial status: the Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK) study protocol.

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1329 **Authors:** Matteo Airal^{1,2}, Yalin Zheng³, Francesco Aiello⁴, Bjoern Bachmann⁵, Lamis
1330 Baydoun^{6,7}, SORCHA NI DHUBHGHAILL^{8,9}, Mor Dickman^{10,11}, Stephen B Kaye^{2,3}, Luigi
1331 Fontana¹², Kunal A Gadhvi², Antonello Moramarco¹², Marina Rodriguez Calvo De
1332 Mora^{13,14,15}, Carlos Rocha de Lossada^{13,14,5,16}, Vincenzo Scordia¹⁷, Pietro Viola¹⁸,
1333 Stefano Calza¹, Hannah J Levis³, Mohit Parekh¹⁹, Alessandro Ruzza²⁰, Stefano
1334 Ferrari²⁰, Diego Ponzin²⁰, Francesco Semeraro²¹, Vito Romano^{2,3,21}.

1335 **Affiliations:**

- 1336 1. Department of Molecular and Translational Medicine, Università degli Studi
1337 di Brescia, Brescia, Italy
- 1338 2. St. Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, UK
- 1339 3. Department of Eye and Vision Science, Institute of Ageing and Chronic
1340 Disease, University of Liverpool, Liverpool, UK
- 1341 4. Ophthalmology Unit, Department of Experimental Medicine, University of
1342 Rome Tor Vergata, Rome, Italy
- 1343 5. Department of Ophthalmology, University of Cologne, Faculty of Medicine
1344 and University Hospital Cologne, Cologne, Germany
- 1345 6. Department of Ophthalmology, University Medical Center Munster,
1346 Munster, Germany
- 1347 7. ELZA Institute Dietikon/Zurich, Switzerland
- 1348 8. Department of Ophthalmology, University Hospital of Brussels, Jette,
1349 Brussels
- 1350 9. Department of Translational Neurosciences, University of Antwerp, Wilrijk,
1351 Belgium

- 1352 10. University Eye Clinic Maastricht, Maastricht University Medical Center,
1353 Maastricht, the Netherlands
- 1354 11. MERLN Institute for Technology-Inspired Regenerative Medicine,
1355 Maastricht University, Maastricht, the Netherlands
- 1356 12. St. Paul's Eye Unit, The Royal Liverpool University Hospital, Liverpool,
1357 United Kingdom
- 1358 13. IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy
- 1359 14. Hospital Regional Universitario de Málaga, Plaza del Hospital Civil, S/N,
1360 29009, Malaga, Spain
- 1361 15. Qvision, Department of Ophthalmology of VITHAS Almería Hospital, 04120
1362 Almería, Spain
- 1363 16. Ophthalmology Department, VITHAS Málaga, 29016 Malaga, Spain
- 1364 17. Surgery Department, Ophthalmology Area, University of Seville, 41013
1365 Seville, Spain
- 1366 18. Department of Medical and Surgical Sciences, University "Magna Græcia",
1367 Catanzaro, Italy
- 1368 19. Ophthalmology Unit, San Bortolo Hospital, Vicenza, Italy
- 1369 20. Massachusetts Eye and Ear, Department of Ophthalmology, Harvard
1370 Medical School, Boston, MA, USA
- 1371 21. Fondazione Banca degli Occhi del Veneto, Venezia, Italy
- 1372 22. Eye Clinic, Department of Medical and Surgical Specialties, Radiological
1373 Sciences, and Public Health, Università degli Studi di Brescia, Brescia, Italy
- 1374

ABSTRACT

Introduction: The success of keratoplasty strongly depends on the health status of the transplanted endothelial cells. Donor corneal tissues are routinely screened for endothelial damage before shipment, however surgical teams have currently no means of assessing the overall viability of corneal endothelium immediately prior to transplantation. The aim of this study is to validate a preoperative method of evaluating the endothelial health of donor corneal tissues, to assess the proportion of tissues deemed suitable for transplantation by the surgeons, and to prospectively record the clinical outcomes of a cohort of patients undergoing keratoplasty in relation to preoperatively defined endothelial viability.

Methods and analysis: In this multicentre cohort study, consecutive patients undergoing keratoplasty (PK, DSAEK, UT-DSAEK or DMEK) will be enrolled and followed-up for 1 year. Before transplantation, the endothelial viability of the donor corneal tissue will be evaluated preoperatively through trypan blue staining and custom image analysis to estimate the overall percentage of Trypan Blue Positive Areas (TBPA), a proxy of endothelial damage. Functional and structural outcomes at the end of the follow-up will be correlated with preoperatively assessed TBPA values.

Ethics and dissemination: The protocol will be reviewed by the ethical committees of participating centres, with the sponsor centre issuing the final definitive approval. The results will be disseminated on ClinicalTrials.gov, at national and international conferences, by partner patient groups and in open access, peer-reviewed journals.

Study registration number: NCT05847387

INTRODUCTION

Endothelial cell viability is one of the most important determinants of long-term graft survival in corneal transplantation.^{7,17} Higher Endothelial Cell Density (ECD) values, both before transplantation and in the early postoperative period, have been associated with better long-term survival rates of lamellar and perforating keratoplasties.^{142,143} However, ECD represents only an estimation of total corneal endothelial health and, although ECD loss after keratoplasty has been accurately reported and modelled,^{8,144} it is a less than ideal proxy for future endothelial failure.^{7,8,145}

In the current chain of supply of corneal tissues for keratoplasty, the surgical team performing the transplantation has no means to independently evaluate the global endothelial cell viability of the graft. This task is in fact relied entirely upon the eye bank providing the tissue. Pan-corneal evaluations of corneal endothelium viability using triple staining (Hoechst/ethidium homodimer/calcein-AM combination) suggest that central ECD as measured by eye banks might overestimate the true cell density.^{13,14,81} However, triple staining methods are not reproducible routinely in the operating theatre, as they require the Endothelium-Descemet Membrane complex to be peeled, incubated with appropriate stains and mounted in imaging chambers or microscope slides for examination, making the tissue no longer fit for transplantation.

The Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK) is a cohort study that aims to validate a preoperative method to assess the global corneal endothelial viability of donor tissue. The V-CHECK method only employs trypan blue 0.05%, a vital dye widely available in operating theatres which is capable of staining areas of denuded Descemet membrane and areas of endothelial cell damage or

1421 mortality.^{59,146} Stained donor corneas will be preoperatively assessed with a custom
1422 image analysis tool to check for areas of endothelial damage, and the cohort of
1423 transplanted patients will be followed over 1 year postoperatively to evaluate the
1424 correlation of the V-CHECK method with long-term ECD measurements and graft
1425 survival.

1426 By validating this new preoperative assessment of global corneal endothelial viability,
1427 we aim to provide eye banks and surgical teams with an accessible tool to perform a
1428 final quality assurance before transplantation of the donor tissue and to identify
1429 potential issues in their chain of supply.

1430

1431 **METHODS AND ANALYSIS**

1432 The Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK)
1433 study protocol has been established according to the Strengthening the Reporting of
1434 Observational studies in Epidemiology (STROBE) checklist for cohort studies.

1435

1436 **Study design and setting**

1437 The V-CHECK study is a multicentre, prospective, single-arm cohort study of patients
1438 affected by corneal disease requiring keratoplasty. The purposes of this study are to
1439 validate a simple and accessible method of image analysis designed to preoperatively
1440 evaluate the viability of the donor corneal graft endothelium in the operating theatre,
1441 to report the proportion of tissues that are deemed suitable for transplantation by the
1442 surgical teams, and to investigate the relationship of preoperatively measured
1443 endothelial viability with the long-term ECD and overall survival of transplanted
1444 corneas. The study described adheres to the tenets of the Declaration of Helsinki.

1445

1446 Consecutive patients undergoing keratoplasty at participating sites will be evaluated
1447 for inclusion in the cohort study starting from September 1st, 2023. After Institutional
1448 Review Board (IRB) approval, patient recruitment will last until September 30th, 2024,
1449 date of primary completion of the study. The start of the follow-up will correspond to
1450 the day of surgery. The follow-up of recruited patients will last 1 year, and secondary
1451 outcomes will be evaluated at 1, 3, 6 and 12 months after keratoplasty. The study
1452 completion date will be September 30th, 2025.

1453

Treatment decisions, such as the indication for corneal transplantation, the choice of corneal graft, the timing of surgical treatment, and concurrent systemic and/or topical medications administered will be at the discretion of the treating practitioner in consultation with the patient with no intervention by the investigators, reflecting routine clinical practice.

Participants

Patients undergoing either full thickness corneal transplantation (i.e., perforating keratoplasty [PK]) or endothelial keratoplasty (i.e., Descemet Stripping Automated Endothelial Keratoplasty [DSAEK], ultra-thin [UT] DSAEK or Descemet Membrane Endothelial Keratoplasty [DMEK]) satisfying eligibility criteria will be asked to participate in the study. To avoid the enrolment of patients at higher risk of endothelial cell loss, only patients undergoing procedures considered to be at low risk for graft rejection (i.e., keratoconus, Fuchs endothelial dystrophy) will be included, as indications for low-risk surgeries account in fact for the majority of keratoplasties.

The investigators will confirm the patients' ability to understand the study and their willingness to participate. Only one eye per patient will be included. In cases where both eyes of a patient are scheduled for corneal transplantation, only the first eye undergoing the procedure will be considered.

Inclusion criteria:

- Age older than 18 years old
- Planned corneal transplantation: PK, DSAEK, UT-DSAEK or DMEK

- 1478 - Indication for corneal transplantation: keratoconus, Fuchs endothelial
1479 dystrophy
1480 - Ability to provide written informed consent.
1481

1482 *Exclusion criteria:*

- 1483 - Prior corneal transplantation
1484 - Planned preloaded DSAEK or preloaded DMEK
1485 - Planned keratoplasty not involving corneal endothelium transplantation
1486 (i.e., superficial/deep anterior lamellar keratoplasty)
1487 - Evidence of concurrent microbial keratitis
1488 - Evidence of non-infectious or autoimmune keratitis
1489 - Impending or frank corneal perforation
1490 - Uncontrolled, elevated intraocular pressure (IOP)
1491 - Previous glaucoma surgery
1492 - Acute or chronic inflammatory/infectious anterior segment uveitis
1493 - Presence of an anterior chamber intraocular lens (IOL) for phakic or
1494 aphakic purposes
1495
1496

1497 **Data measurements and variables**

1498 *Preoperative endothelial viability evaluation*

1499 The ECD, overall mortality,¹⁰ and mortality on corneal folds of corneal tissues destined
1500 for transplantation as declared by the eye bank providing the tissue will be recorded
1501 at baseline (i.e., the day of surgery). Corneal tissues will then be assessed

preoperatively for endothelial viability before transplantation following the method hereby described.

In the operating room, donor corneas will be rinsed in balanced salt solution (BSS) to remove excess transport medium. The corneal endothelium will be stained for 30 to 45 seconds with 0.05% trypan blue, then the dye will be gently washed away with BSS. Trypan blue is a vital dye commonly used in anterior segment surgery, which stains the nuclei of severely damaged and dead endothelial cells of donor corneas, as well as areas of Descemet membrane denuded of endothelial cells.⁵⁹

The corneal tissue will then be placed face-up on a custom trephine base designed with no suction holes (e.janach, Como, Italy) in order to provide a smooth, concave surface to rest the cornea on, with no background optical disturbance that could prevent optimal image analysis of the central endothelium. The corneoscleral rim will be filled with BSS to avoid the formation of reflexes of the microscope lights on the central endothelium.

Images of the stained corneal endothelium will then be acquired and exported. In order to improve reproducibility of the acquisition process and limit variability across different sites, magnification levels of images obtained through a surgical microscope will be adjusted to make sure that the entirety of the Schwalbe line is visible just inside the image frame. Illumination levels will be adjusted to avoid areas of hypo/hyper-exposure of the image and to provide a uniform, diffuse illumination of the corneal tissue. An example image can be seen in **Figure 4**.

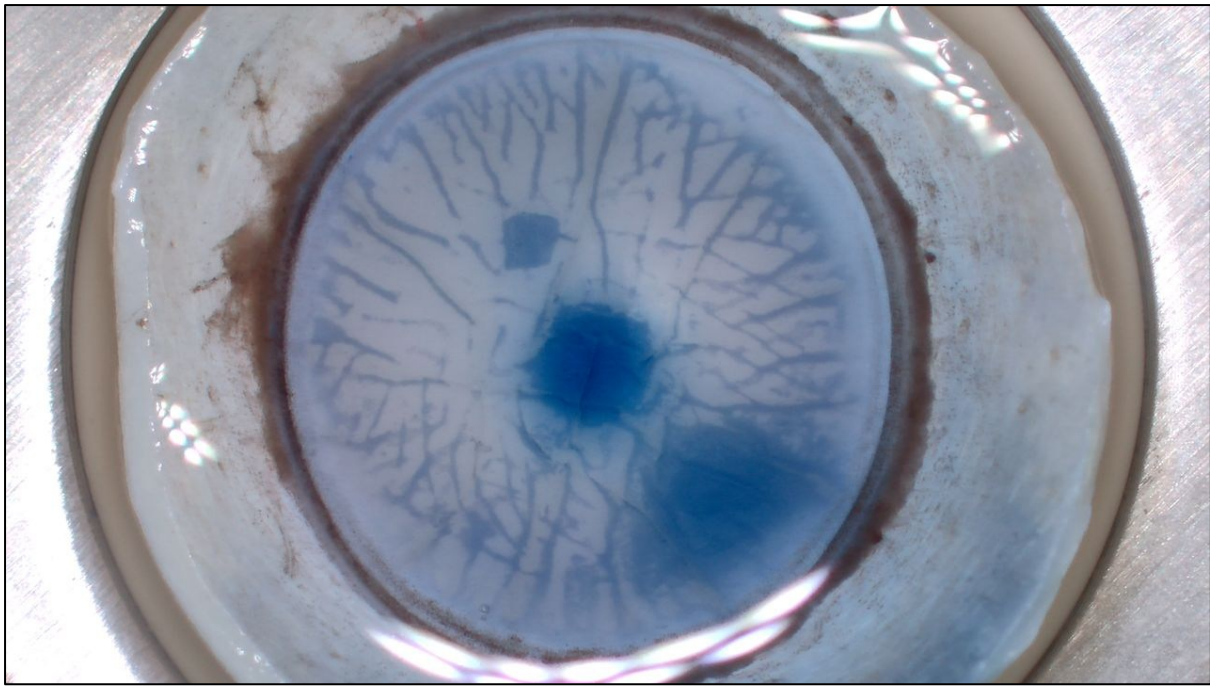


Figure 4 Example image acquired with surgical microscope showing correct positioning of the tissue and lighting. The corneoscleral rim is filled with BSS to avoid the formation of central reflexes. Several linear streaks of TBPA are visible on corneal folds, along with circular areas of TBPA corresponding to mechanically induced endothelial damage. The cornea is resting on a custom trephine base with no suction holes to improve the visibility of the central endothelium (e.janach, Como, Italy). BSS, balanced salt solution; TBPA, trypan blue-positive area.

A custom algorithm will be developed to analyse the images in real time. All the images will be automatically enhanced with Contrast Limited Adaptive Histogram Equalization (CLAHE). A weakly supervised segmentation model will be used to segment the cornea from the enhanced images.¹⁴⁷ An unsupervised clustering method will be applied to the pixels within the cornea so to group pixels into different regions according to the colour and detect the Trypan Blue-Positive pixels. The percentage of Trypan Blue-Positive Areas (TBPA) over the whole area of the corneal endothelium will be calculated based on the segmentation results.

1544

1545 *Patient data*

1546 Demographic data, indication for corneal transplantation, type of graft transplanted,
1547 and other surgery-related variables – including intraoperative times and complications
1548 - will be recorded at baseline.

1549 Clinical data will be recorded by the treating practitioner at each follow-up visit at 1, 3,
1550 6 and 12 months postoperatively. Recorded data will include the number of letters
1551 read on a logarithm of the minimum angle of resolution (LogMAR) visual acuity chart,
1552 presence of clinically definite corneal edema, measurements of IOP, central corneal
1553 thickness, corneal tomography, and endothelial cell density (ECD), and occurrence of
1554 clinically defined postoperative complications such as graft rejection, infective keratitis,
1555 endophthalmitis.

1556

1557 **Outcomes and variables**

1558 *Primary outcome*

1559 The primary outcome of this study will be the comparison of the endothelial mortality
1560 of included donor corneas measured preoperatively (TBPA) and the overall endothelial
1561 mortality as declared by the eye bank providing the tissue. TBPA will be estimated
1562 preoperatively with the above described novel method. TBPA and nominal overall
1563 endothelial mortality will be expressed as mean and 95% confidence interval.

1564

1565 *Secondary outcomes*

1566 Secondary outcomes will include the correlation of preoperatively measured TBPA
1567 and nominal ECD as declared by the eye bank providing the tissue, the correlation of

TBPA with mortality on corneal folds as provided by the eye bank, the final ECD of transplanted corneas at 12 months, the rate of change of ECD over the follow-up, the incidence of endothelial graft failure (i.e., the occurrence of clinically definite corneal edema and/or need for endothelial keratoplasty), and the risk of endothelial graft failure associated with TBPA.

Study size

Sample size was estimated based on the width of the confidence interval for the LOA (limit of agreement) of a Bland-Altman plot.¹⁴⁸

Anticipating a sample difference (TBPA vs. overall mortality) with mean and standard deviation of 0 and 1%, respectively, a sample of 108 subjects achieves 80% power to detect agreement when the confidence level of the limit of agreement (LoA) is 95% and the confidence level of the confidence intervals about the LoAs is 95%, given a maximum allowable difference of 2.5%.

Accounting for a 25% drop-out rate for the estimation of 12-month secondary outcome, we calculated a final sample size of 135 subjects.

Statistical methods

Data will be summarized using mean (standard deviation) or median (interquartile range) for quantitative variable, and counts (percentages) for qualitative variables.

Concordance between preoperative TBPA and nominal as ECD, as measured by the eye bank providing the tissue, will be estimated using Bland-Altman model on the log (or arcsine) transformed variables.

A Pearson correlation coefficient will be estimated for preoperatively measured TBPA and nominal ECD as measured by the eye bank providing the tissue. Pearson correlation coefficients will also be reported for the correlation of TBPA with mortality on folds and ECD of the donor cornea.

A Generalized Linear Mixed Model, with random intercepts (practice and patient) and suitable family distribution will be used to evaluate the within patient trend of ECD over 12-months ECD. The model will include visits from baseline through 12 months from all eyes, regardless of whether 12 months of follow-up have been completed, as well as age and eye bank ECD values.

Time to endothelial graft failure as a function of preoperatively measured TBPA will be evaluated using Cox proportional hazards model. Cumulative incidence curve will be used to display the trend of endothelial graft failure over 12 months.

Raw, unadjusted estimates for the other secondary outcomes will be computed using the last observation carried forward in case of subjects lost to follow-up.

All models will be evaluated assuming a 5% significance level and two-sided tests. All analyses will be conducted using R Statistical Software (v4.3.0 or higher, R Core Team 2023).

Patient and public involvement

International and national organ donors' associations will be involved in the assessment of the patients' burden of recruitment to and participation in the cohort study. Dissemination of the study results is expected to involve trial participants, patient groups, and organ donors' associations in addition to the traditional routes of publications outlined in the "Ethics and dissemination" section.

1616

1617 **ETHICS AND DISSEMINATION**

1618 Local IRB approval for participation will be sought by all centres willing to participate
1619 to the study. The study protocol is currently undergoing registration as observational
1620 study at ClinicalTrial.gov, and the results from this study will be submitted and
1621 published on ClinicalTrials.gov.

1622 In addition, every attempt will be made to publish results in open access, peer-
1623 reviewed journals and to present these data at national and international meetings.
1624 Consistent with the collaborative nature of the proposed research, the Principal
1625 Investigator anticipates sharing all data generated by the study with collaborators.
1626 Furthermore, data generated by the study will be made available upon reasonable
1627 request.

1628

Chapter 8

VALIDATION OF A DEEP LEARNING-ASSISTED EVALUATION OF TOTAL CORNEAL ENDOTHELIAL CELLS VIABILITY

Published paper

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Authors: Matteo Airaldi, MD^{1,2,3}, Filippo Airaldi, MSc⁴, Zhuangzhi Gao, MSc³,
Alessandro Ruzza, BSc⁵, Mohit Parekh, PhD⁶, Diego Ponzin, MD⁵, Stephen Kaye,
MD, PhD^{2,3}, Francesco Semeraro, MD^{7,8}, Stefano Ferrari, PhD⁵, Yalin Zheng, PhD³,
Vito Romano, MD^{3,7,8}.

Affiliations:

1. Department of Molecular and Translational Medicine, University of Brescia,
Brescia, Italy
2. St Paul's Eye Unit, Liverpool University Hospitals NHS Foundation Trust,
Liverpool, United Kingdom
3. Department of Eye and Vision Sciences, Institute of Life Course and Medical
Sciences, University of Liverpool, Liverpool, UK
4. Delft Center for Systems and Control, Delft University of Technology, Delft, The
Netherlands
5. Fondazione Banca degli Occhi del Veneto, Venezia, Italy
6. Schepens Eye Research Institute of Massachusetts Eye and Ear, Boston, MA,
USA
7. Ophthalmic Unit, ASST Spedali Civili di Brescia, Piazzale Spedali Civili, 1,
25123, Brescia, Italia
8. Department of Medical and Surgical Specialties, Radiological Sciences, and
Public Health, University of Brescia, Brescia, Italy

ABSTRACT

Purpose: To describe the validation of a novel automated analysis of preoperative pan-corneal endothelial cell viability.

Methods: Pre-clinical experimental study. Dead endothelial cells and denuded areas of Descemet membrane of corneoscleral rims were stained with Trypan Blue (TB) 0.05%. Endothelial mortality was estimated by an experienced eye bank technician ("gold standard") and by deep learning-aided automated segmentation of TB Positive Areas (TBPAs) on images of the stained corneas ("V-CHECK method"). V-CHECK mortality was calculated for the whole cornea and for concentric 2-mm rings. The agreement in the estimation of endothelial mortality between the two methods was assessed with Bland-Altman analysis and correlation tests.

Results: Nineteen corneas deemed unsuitable for transplantation were used for the experiment. The automated V-CHECK method was able to accurately segment the corneal endothelium and the TBPAs. The gold standard and the V-CHECK method showed strong positive correlation for all rings (Pearson's ρ , range [0.76 – 0.81], all $p < 0.001$). The V-CHECK method resulted in higher average estimated endothelial mortality (mean difference range [+6.5% - +9.5%]).

Conclusion: The V-CHECK method enables reproducible estimation of endothelial cell viability in donor corneas. Incorporating this technique into the preoperative assessment of donor corneal tissues (in the eye bank and in the operating theatre) can provide a reliable evaluation of endothelial health, thereby improving the consistency of tissue quality and further supporting optimal surgical results.

1682 **Translational relevance:** The V-CHECK deep-learning assisted computer vision
1683 protocol will allow surgeons and eye bank technicians to perform an independent,
1684 preoperative assessment of global corneal endothelial viability.

1685

INTRODUCTION

Quality control of donor tissue destined to keratoplasty is of utmost importance to ensure the safety and efficacy of corneal transplantation.¹¹ Endothelial health status in particular is a major determinant of long-term success of keratoplasty.¹⁷ Higher endothelial cell density (ECD) values have been associated with lower rates of long-term graft failure.⁷ Nevertheless, ECD only estimates the cellular density of selected areas of the endothelium, and thus cannot adequately represent the full picture of corneal endothelial health.^{13,14}

Although numerous pre-operative assessments and graft selection criteria are employed to ensure a successful outcome prior to any kind of transplantation³⁶, corneal surgeons usually do not have any means to directly measure the endothelial cell density or viability of a donor graft in the operating theatre, even if the responsibility for determining whether a donor cornea is suitable for transplantation ultimately rests with them.¹¹

The development of a reliable and reproducible assessment of global endothelial health status of donor corneas would represent a powerful tool for corneal surgeons, and it might not only contribute to the exclusion of poor-quality tissues but also lead to a greater standardization of keratoplasty outcomes.^{11,91}

In this paper, we validate an automated software for the preoperative evaluation of the corneal endothelial cell viability that can be used to obtain reliable descriptors of global corneal endothelial health. This software can be used for further analysis of large image datasets to correlate preoperative evaluated endothelial mortality with well-

1708 established parameters of corneal tissue quality such as endothelial cell density and
1709 eye bank declared endothelial mortality.

1710 The protocol described in this paper will be used for the image analysis of the Viability
1711 Control of Human Endothelial Cells before Keratoplasty longitudinal study (V-CHECK
1712 Study, NCT05847387)⁹¹, a cohort study that aims to correlate the preoperative
1713 evaluation of endothelial viability with one-year clinical outcomes of patients
1714 undergoing keratoplasty.

1715

MATERIALS AND METHODS

This preclinical experimental study for the validation of a new corneal endothelial cell viability assessment was conducted at the Veneto Eye Bank Foundation, Venice, Italy. Donor corneal tissues unsuitable for transplantation were used for the research purposes and the validation studies described in this manuscript, according to the realms of the Italian law 91/99 and after an informed consent form was signed by the donor's next of kin. The study was conducted in accordance with the tenets of the Declaration of Helsinki. This study has been evaluated by the Institutional review board of the Veneto Eye Bank Foundation and deemed not to require ethics approval. The human tissue experiments complied with the guidelines of the ARVO Best Practices for Using Human Eye Tissue in Research.

Live/dead staining

Exploratory live/dead assays aimed to verify an acceptable correlation between live/dead staining with Trypan Blue and Calcein acetoxymethyl (Calcein AM) in order to confirm the validity of the V-CHECK endothelial evaluation protocol.

Trypan blue is a vital dye commonly used in anterior segment surgery, which stains the nuclei of severely damaged and dead endothelial cells of donor corneas, as well as areas of Descemet membrane denuded of endothelial cells.⁵⁹ Calcein AM is a hydrophobic compound that permeates intact live cells. After incubation at 37°C for 20 minutes, hydrolysis by ubiquitous intracellular esterases produces a green fluorescent product. **Figure 5 (A-D)** shows areas of mortality from a whole cornea stained with 0.05% Trypan Blue for 45 seconds, demonstrating near perfect correlation with Calcein AM staining of the same tissue.

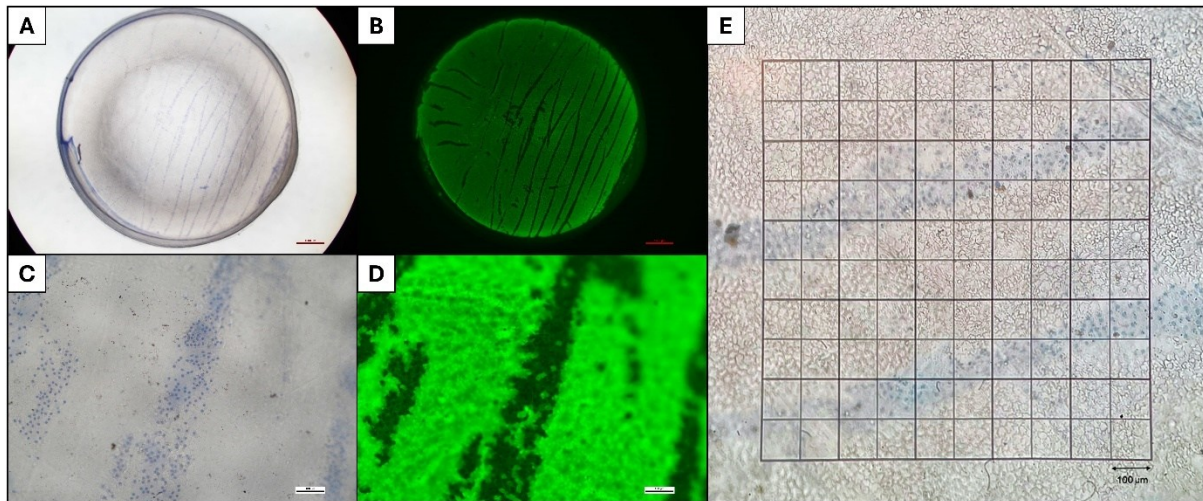


Figure 5. Live/dead analysis of the whole cornea. **A)** Staining with Trypan Blue 0.05% for 45 seconds highlights the presence of Trypan Blue Positive Areas (TBPA). **B)** After incubating the whole cornea with Calcein-AM at 37° for 20 minutes, the same linear areas of endothelial damage are visible. **C, D)** High magnification images of A and B highlight the near perfect inverse correlation between areas of endothelial cell death and of denuded Descemet membrane in C with metabolically active endothelial cells in D. **E)** The reticule used for manual counting of TBPA (i.e., gold standard method). The 10x10 reticule, observed with an inverted stereoscopic microscope with a 10x magnification, results in a 1 mm² overall area subdivided in 100 smaller squares, each with a 10⁴ μm² area. The percentage of TBPA in the gold standard method was estimated by counting the total surface of stained areas over the total area of the endothelium.

Gold Standard: manual endothelial viability assessment

A previously described light microscopy assessment was used as the gold standard for the estimation of endothelial mortality of included corneas by manually counting the percentage of TBPA over the entirety of the endothelium.¹³⁰ Once removed from their storage vessel, corneal tissues deemed unsuitable for corneal transplantation were rinsed in balanced salt solution (BSS) to remove excessive transport medium.

The endothelium was then stained for 30-45 seconds with Trypan Blue 0.05%. A 10x10 reticule was inserted into an inverted stereoscopic microscope with a 10x magnification, resulting in a 1 mm² overall area subdivided in 100 smaller squares, each with a 10⁴ μm² area, as shown in **Figure 5E**. The percentage of TBPA was estimated by counting the total surface of stained areas over the total area of the endothelium. Values of TBPA obtained with this method by an experienced eye bank technician (A.R.) served as the “gold standard” for the comparison with the V-CHECK method.

V-CHECK - Image acquisition

After manual counting, the corneal tissues were placed under a surgical microscope for the acquisition of images suitable for the V-CHECK analysis. The corneal tissue was placed face-up on a base without suction holes to avoid background optical disturbances that could prevent optimal image analysis of the central endothelium. The corneoscleral rim was filled with BSS to avoid the formation of reflexes of the microscope lights on the central endothelium. To acquire images suitable for analysis, the magnification level of the surgical microscope must be adjusted to make sure that the entirety of the Schwalbe line is visible just inside the image frame. Images of the stained corneal endothelium are then acquired and exported from the surgical microscope.

V-CHECK – Image analysis

Exported images were then analyzed with the V-CHECK method. The analysis consists of three sequential steps: 1) the segmentation of corneal tissue from each

surgical microscope image; 2) the segmentation of TBPAs in each corneal image; 3) the computation of its estimated viability. The computer vision algorithm described below was implemented and carried out in Python 3.12 with OpenCV.¹⁴⁹

Segmentation of Corneal Endothelium via SAM

The goal of the first step is to segment corneas from the original surgical microscope images, i.e., separate pixels belonging to the corneal endothelium from background pixels (e.g., trephine base, surgical tray).

To enable this segmentation in an automatic way, we adopted the Segment Anything Model (SAM)¹⁵⁰. SAM is a foundation model, trained on a large quantity of data, which has shown strong zero-shot generalization capabilities (i.e., handle tasks or make predictions for cases it was not explicitly trained for), sparking significant interest into its application to a wide range of tasks, including biomedical segmentation.^{151,152} SAM is also categorized as a promptable segmentation model, i.e., the user can provide, alongside the input image, one or more prompts specifying what needs to be segmented. Such prompts can be either sparse (text, bounding boxes, points of interest) or dense (masks).

In our case, SAM was consistently able to segment the corneal endothelium from the rest of the image when prompted with one or more points. The scleral rim and the image background detected by SAM were automatically removed from the images, thus yielding the endothelium image ready for further analysis in the next step. **Figure 6 (left panel, top)** showcases an example of the segmentation process.

Segmentation of TBPAs

Once the corneal endothelium surface has been isolated, the TBPAs can be segmented against the remaining regions of non-stained, healthy tissues via an automated computer vision algorithm. The algorithm consists of the following operations:

1. The input image is converted from RGB space to grayscale, and a Gaussian blur is applied to reduce noise and smoothen intensity variations (**Figure 6A**). For images with insufficient contrast, Contrast Limited Adaptive Histogram Equalization (CLAHE) is performed before the Gaussian filter in order to locally enhance contrast.¹⁵³
2. Adaptive thresholding is applied to the filtered grey image to coarsely identify potential TBPAs. This technique leverages variations in the intensity of pixels in local neighborhoods to generate a preliminary mask highlighting candidate stained regions. To enhance segmentation quality, small non-zero artifacts in the image are removed after thresholding via morphological opening, which helps isolate larger contiguous regions while removing smaller noise (**Figure 6B**).
3. A distance transform is computed for each non-zero pixel in the mask, transforming pixel values to represent their distance to the nearest background pixel (**Figure 6C**).
4. A second threshold is applied to the distance-transformed image, creating a binary map that distinguishes likely foreground regions (high-intensity areas, representing proximity to TB-stained regions) from background and unknown regions, likely non-stained healthy area (**Figure 6D**).

5. The segmented foreground is used as seed in a watershed algorithm.¹⁵⁴ Prior to watershed flooding, pixels outside the cornea are uniformly labelled to mitigate edge artifacts (otherwise, the boundary of the cornea could disrupt the segmentation), and unknown regions are grouped as a single region, enhancing segmentation precision. The watershed algorithm then iteratively refines region boundaries, resulting in well-defined segments for each candidate TB-stained area (**Figure 6E**).
6. Finally, contours of the regions segmented by the watershed algorithm are extracted by thresholding and filtering to retain only those within the corneal mask. These contours are drawn over a mask that serves as the final TBPA indicator, highlighting areas stained by TB within the corneal region (**Figure 6F**).

Viability Assessment

In the last step, given the segmented TBPAs, the V-CHECK viability assessment can be performed. To improve its interpretability, we divided the corneal endothelium segmented by SAM in five concentric rings, with radii approximately at 2, 4, 6, 8, and 10 mm from the centroid of the image. This centroid can be extracted from the image moments. The radius of the outermost ring is computed in a least-squares manner as the average of the distances between the centroid and each point on contour of the cornea. The other radii (i.e., at 2, 4, 6, 8 mm) are subsequently found as the corresponding fraction of the outermost radius. Given that the outer ring's radius is computed via least-squares and that the cornea is not a perfectly circular objective, a mismatch between the outer ring and the whole cornea is possible. For this reason,

subsequent analyses report the outcomes for both the 10 mm ring and the whole cornea.

For each ring, the percentage of TBPAs is calculated as the ratio of the number of positively segmented pixels lying within the disc, over the total count of corneal pixels in the area.

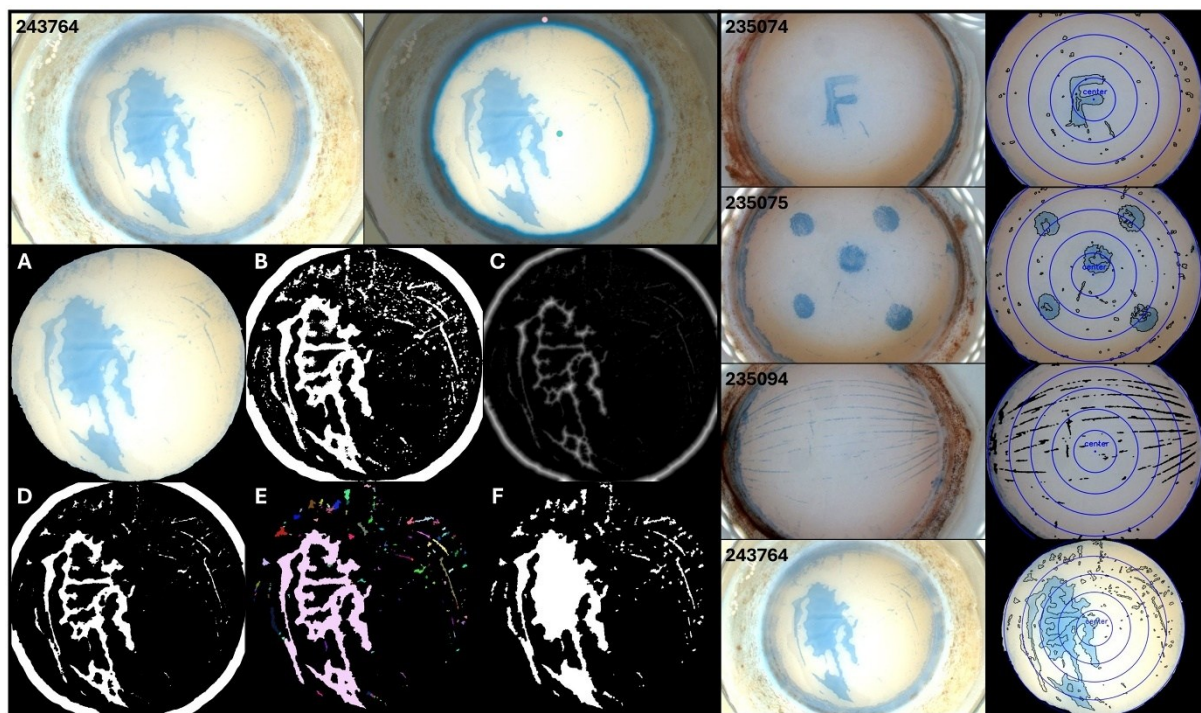


Figure 6. Left panel, top: example of segmentation of the corneal endothelium from a microscopic image via SAM. On the top left, the input image is shown. On the right, the user specified three points of interest: in green, the region to be segmented; in pink, regions to be excluded from the segmentation.

Left panel, bottom: stages of the TBPA segmentation algorithm. **A)** The final output of SAM endothelial segmentation, i.e. the input image. **B)** The filtered and contrast-adjusted grayscale conversion. **C)** The distance transform. **D)** The thresholded foreground, indicating likely TB-stained regions. **E)** The output of the watershed algorithm, where each TBPA is assigned a random color. **F)** Contouring and conversion of the watershed output as a mask of the TBPAs.

Right panels: examples of input images and processed images. Left column: surgical microscope view of donor corneal tissues after 45 seconds of staining with 0.05% Trypan blue, showing areas of endothelial damage in different configurations, some of which mechanically induced (top 2 images). Areas of endothelial damage are readily identified by Trypan Blue staining, indicating the presence of areas of endothelial cell death or denuded Descemet membrane. Right column: after deep learning segmentation of the corneal surface and automated isolation of the stained areas, the Trypan Blue Positive Area (TBPA) can be reliably quantified.

V-CHECK – validation

To investigate the reproducibility of the proposed V-CHECK method analysis, all available images were rotated by 90°, 180° and 270°, and then segmented and analyzed again with the aforementioned methodology. The goal was verifying the repeatability of the TBPA estimation, as well as testing its invariance and robustness with respect to rotations of the corneal images.

To further confirm the accuracy of the V-CHECK segmentation, a comparison with a manual ground truth was performed. In particular, the central, 2 mm ring of each image was segmented with the SAM tool. The image was then analyzed (1) with the V-CHECK method and (2) via a semi-automated approach by loading the segmented image into ImageJ (National Institutes of Health, Bethesda, Maryland, USA). The loaded image was converted into an RGB stack, and the green channel was selected for further processing. CLAHE was then performed in order to optimize the contrast (with a block size of 127 pixels, 256 histogram bins, maximum slope of 3), before obtaining a binarized mask through thresholding. The threshold value was manually adjusted for each image until the segmentation of TBPA was deemed adequate.

1896

1897 **Statistical analysis**

1898 Descriptive data were summarized using mean (standard deviation, [SD]), median
1899 (interquartile range, [IQR]), and 95% confidence intervals (CI) where appropriate.

1900 The distribution of V-CHECK measured mortality for each of the 0°, 90°, 180°, and
1901 270° rotations (with 0° being the original image) and for each of the ring segmentations
1902 was converted to Z-scores to allow for comparison of the different tissues on the same
1903 normalized scale. The distribution of V-CHECK mortality Z-scores was displayed by
1904 means of violin plots and boxplots, and Levene's tests were used to verify
1905 homoscedasticity (i.e., equal variance) of the values across the different rotations for
1906 each ring.

1907 The agreement between semi-automated segmentation of TBPA areas and V-CHECK
1908 segmentation was then evaluated by comparing the two binarized images of the
1909 central ring of each cornea. Various metrics, commonly employed in segmentation
1910 tasks, were calculated for each image pair and their distribution displayed via boxplots.
1911 These included precision and recall of TBPA areas, the balanced accuracy (i.e., the
1912 average of the recall of stained and non-stained areas), the Dice coefficient and
1913 Intersection over Union (also known as F1 score and Jaccard index, respectively), and
1914 the standard and modified Hausdorff distances (normalized by the size of each
1915 image).¹⁵⁵ Continuous performance metrics were computed to assess the agreement
1916 in predicted TBPA extent, including mean absolute error (MAE), root mean squared
1917 error (RMSE), and the coefficient of determination (R^2). Bootstrapping with 1,000
1918 resamples was used to estimate 95% confidence intervals for MAE, RMSE, and R^2 .

1919 Pearson's correlation coefficients were calculated to quantify the correlation between
1920 mortality as measured by an expert eye bank technician (gold standard) and mortality
1921 measured with the V-CHECK method. Scatterplots were employed to visualize the
1922 correlation between the two modalities.

1923 Bland-Altman plots were used to assess mean differences and the 95% limits of
1924 agreement between the two techniques.

1925 All statistical analyses were conducted using R software version 4.2.2 (R Project for
1926 Statistical Computing, Vienna, Austria).

1927

RESULTS

A total of 19 corneas deemed unsuitable for transplantation and destined for research purposes were included in the study.

The estimation of endothelial mortality with the V-CHECK method demonstrated good reproducibility, with constant variance across different rotations of the same images. As shown in **Figure 7** (top panel), Z-scores of V-CHECK mortality in each ring had homogenous variance across the different rotations (Levene's tests, all $p > 0.05$).

The V-CHECK method also demonstrated good overall agreement with the ground truth from semi-automatically segmented images of the central 2-mm corneal ring. Quantitative metrics comparing the two segmentations, along with representative binary masks, are shown in **Figure 7** (bottom panels). V-CHECK achieved high balanced accuracy (median [IQR]: 91.4% [84.4%, 96.6%]) and a good Dice coefficient (60.3% [54.3%, 75.6%]), reflecting substantial overlap between predicted and reference TBPA regions. The corresponding Intersection over Union (IoU) was 43.3% [37.7%, 62.1%]. The method showed strong recall (85.9% [69.4%, 95.1%]) with moderate precision (55.6% [41.6%, 77.5%]), suggesting slight over-segmentation. Spatial accuracy was supported by a low modified Hausdorff distance (median: 0.20 mm [0.11, 0.35]). In terms of continuous prediction accuracy, the model achieved a MAE of 0.019 (95% CI, 0.012-0.026), a RMSE of 0.024 (95% CI, 0.016, 0.030) and a R^2 of 97.4% (95% CI, 67.7%-98.9%) when comparing predicted and ground truth mortality fractions. The average (SD) mortality within the 2-mm ring predicted by the V-CHECK was slightly higher than the ground truth (11.8% [15.7%] vs 10.4% [15.2%]), suggesting a modest positive bias in the segmentation output.

The overall, pan-corneal endothelial mortality of the 19 tissues according to the V-CHECK method and that measured through manual counting under light microscopy by an experienced eye bank technician (i.e., the gold standard) are reported in **Table 4**. Example images of tissues analyzed with the V-CHECK method are displayed in **Figure 6** and **Figure 7**.

Table 4. Average mortality values and differences between V-CHECK method and gold standard (manual counting) method

Method		Average mortality – mean (SD)	Average difference GS v. V-CHECK – mean (95% LoA)
Gold standard	Whole cornea	9.1% (17.7%)	
	Whole cornea	15.9% (11%)	6.8% (-14.7%, 28.2%)
	Ring 1	18.7% (21.4%)	9.5% (-17.8%, 36.9%)
	Ring 2	15.7% (18.7%)	6.6% (-18.1%, 31.4%)
	Ring 3	15.6% (15.9%)	6.5% (-16.8%, 29.8%)
	Ring 4	16.7% (13%)	7.6% (-13.5%, 28.6%)
	Ring 5	16.1% (11.1%)	7% (-14.5%, 28.5%)

GS, gold standard; LoA, limits of agreement; SD, standard deviation.

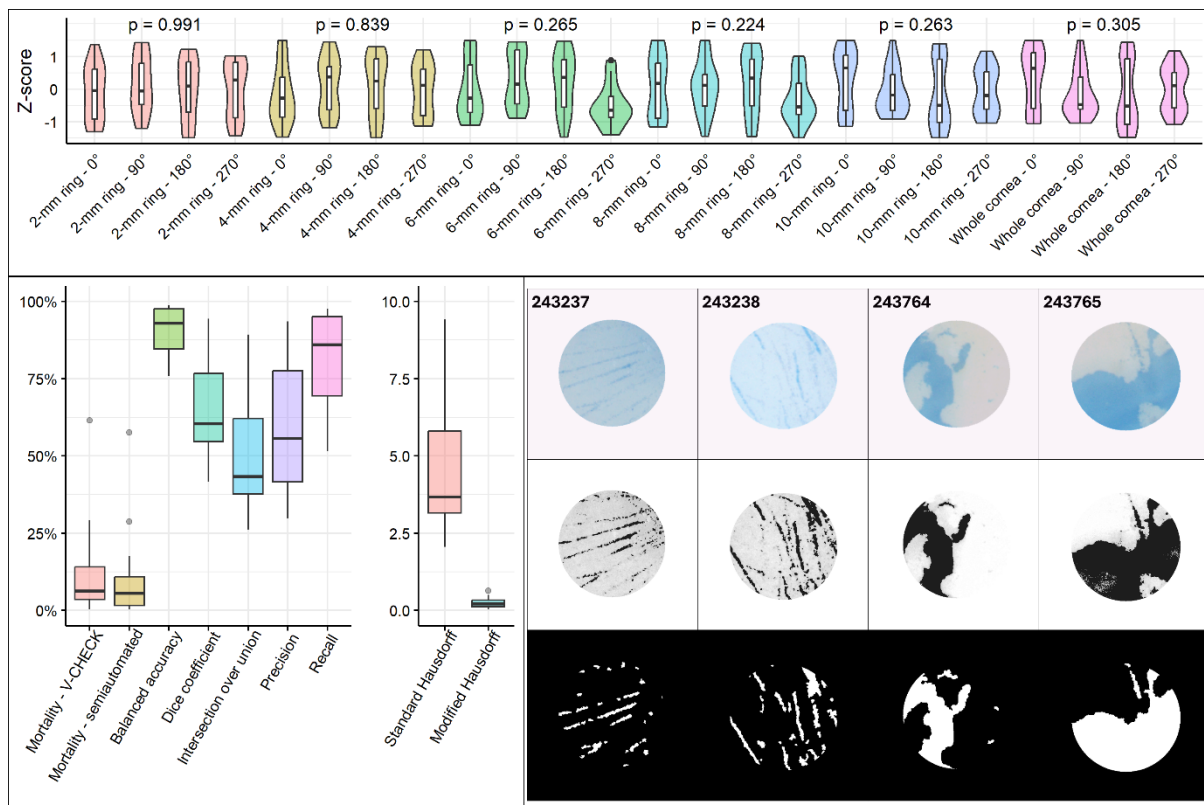


Figure 7. Top panel: violin plots and boxplots displaying the distribution of z-scores of endothelial mortality as measured by the V-CHECK method across all rings of the image for 0°, 90°, 180°, and 270° rotations. Levene's tests demonstrated homogenous variance of mortality for all rings and rotations, reflecting the repeatability of the V-CHECK method.

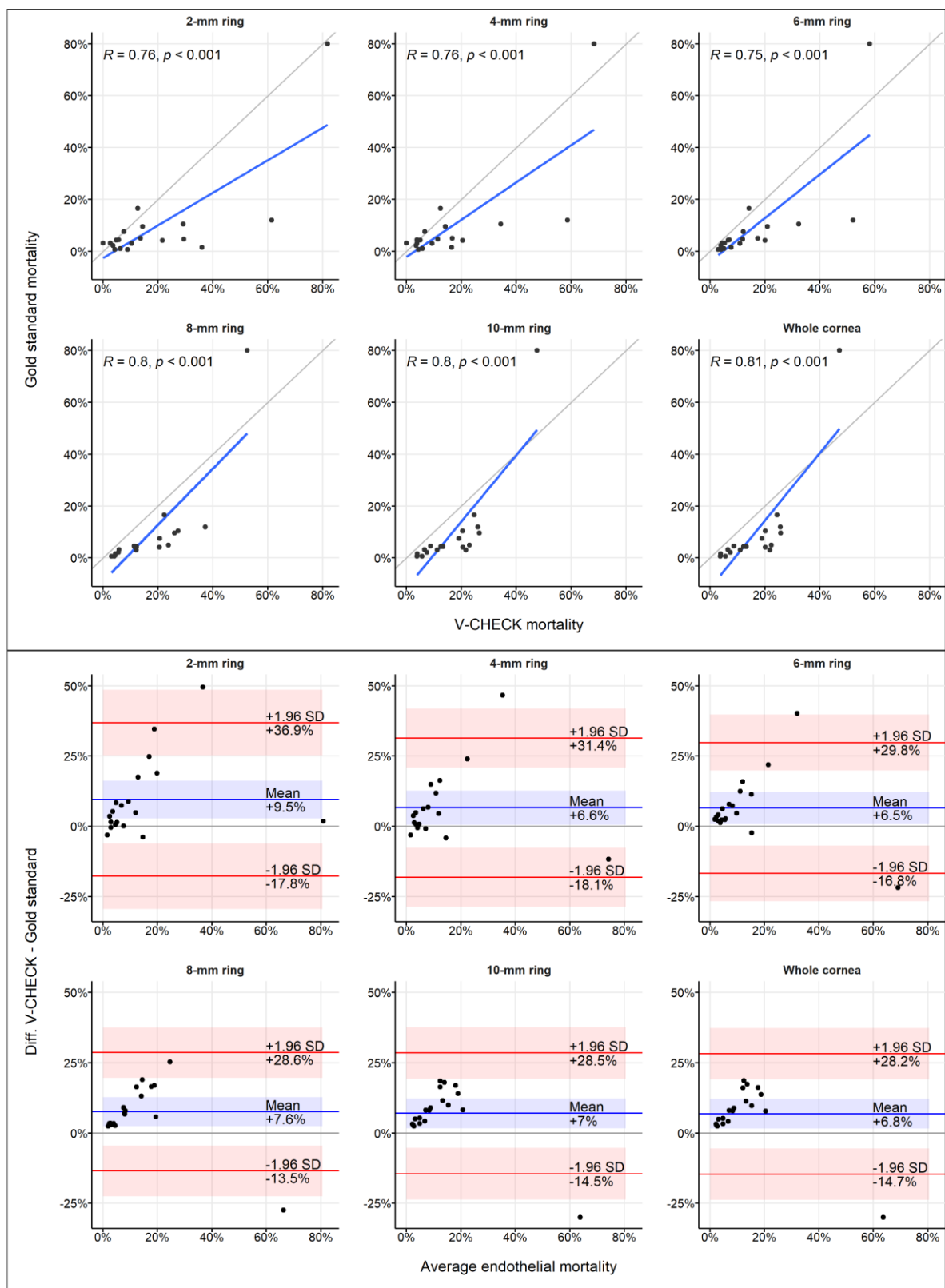
Bottom panel, left: boxplots displaying metrics of concordance between the semi-automated segmentation of TBPAs (ground truth) and the V-CHECK method, which performed with high balanced accuracy and good Dice coefficient, reflecting the overlap of the two segmented masks. Hausdorff distance scales are in millimetres.

Bottom panel, right: examples of central 2-mm segmented corneas (top strip), the semi-automated thresholding performed with ImageJ (middle strip), and the V-CHECK method segmentation (bottom strip).

1976 The gold standard and the V-CHECK method showed a strong positive correlation of
1977 mortality estimates in all rings (Pearson's ρ , range [0.76 – 0.81], all $p < 0.001$).
1978 Scatterplots displaying the positive correlation of V-CHECK mortality and gold
1979 standard mortality assessments are displayed in **Figure 8** (top panel).

1980 The gold standard and V-CHECK method also showed an overall acceptable
1981 agreement in the measurement of pan-corneal endothelial viability. The V-CHECK
1982 method resulted in higher average estimated endothelial mortality, with the average
1983 difference in TBPA estimation ranging from +6.5% to +9.5% across the different rings
1984 (**Table 4**). The results of the Bland-Altman analysis for each of the concentric rings,
1985 reporting the average difference between the estimated mortality by the two methods
1986 and the respective 95% limits of agreement, are displayed in **Figure 8** (bottom panel).

1987



1988

1989 **Figure 8. Top panel:** scatterplots showing the correlation between gold standard mortality
 1990 and V-CHECK mortality. The two methods showed strong positive correlation in all rings
 1991 (Pearson's ρ , range [0.76 – 0.81], all $p < 0.001$).

1992 **Bottom panel:** Bland-Altman analysis of the agreement between measurements of
1993 endothelial mortality from the gold standard method (i.e., manual counting of Trypan Blue
1994 positive cells) and the V-CHECK method. The V-CHECK method produced higher estimates
1995 of endothelial mortality than manual counting (mean difference range [+6.5% - +9.5%]). The
1996 blue line represents the mean of the differences between the two methods, while the red lines
1997 represent the 95% limits of agreement. The shaded ribbons represent the respective 95% CI.

1998

1999

2000 **DISCUSSION**

2001 In this study, we validated an automated software tool for assessing corneal
2002 endothelial cell viability in donor corneal tissues. The V-CHECK method provides eye
2003 bank technicians with an objective, comprehensive assessment of endothelial viability,
2004 which is essential for determining tissue suitability. Additionally, the V-CHECK method
2005 enables corneal surgeons to perform a final evaluation of endothelial health directly in
2006 the operating room prior to corneal transplantation. By providing a comprehensive,
2007 real-time assessment of endothelial health status, the V-CHECK method offers a
2008 valuable and robust quality assurance measure for donor tissues, supporting more
2009 informed surgical decision-making and potentially enhancing patient outcomes in
2010 corneal transplantation.

2011 Endothelial cell density (ECD) currently represents the single most important indicator
2012 of donor corneal tissue status, and its relevance as a determinant of long-term graft
2013 survival has long been proven.⁷ The minimum requirements for delivering a donor
2014 cornea might vary from eye bank to eye bank, however ECD is universally adopted to
2015 set a threshold for transplantation suitability. This metric is one of the few indicators of
2016 endothelial status available to the surgical team performing the keratoplasty, yet ECD
2017 does not necessarily reflect total corneal endothelial health.^{8,9} Pipparelli et al.
2018 compared ECD to viability assays and found that the actual number of living, functional
2019 endothelial cells of donor corneas was significantly lower than the ECD reported by
2020 standard eye bank methods, with a median reduction of approximately 12–20%
2021 depending on the preparation and handling of the tissue.¹³ This discrepancy might
2022 explain the rapid early postoperative decline in ECD observed after keratoplasty,
2023 suggesting that fewer viable cells are transplanted than the preoperative ECD would

indicate. Large-scale analyses of eye bank data have also revealed systematic anomalies in ECD reporting. An analysis of 48'207 eye bank reports by Munir et al. revealed a discontinuity in reported ECD values just below commonly used clinical thresholds (e.g., 2500 cells/mm²), with a clustering of values at or just above these cutoffs.¹³² This pattern is possibly explained by subjective human bias during the counting process, where supra-threshold values might be more likely to be reported to meet suitability criteria for transplantation. This further supports the claim that ECD as measured in the eye bank setting may be biased toward overestimating tissue viability. Given the inherent limitations of ECD, most eye banks, especially in Europe, also directly evaluate endothelial cells viability, specifying a 5% mortality threshold to be deemed suitable for transplantation.¹⁰

There is a need for a greater degree of standardization of outcomes, both functional and survival-wise, in corneal transplant surgery, a demand whose satisfaction is strongly dependent on developing metrics that better reflect global endothelial health of donor tissues.¹² Pan-corneal assessments of endothelial viability in donor tissues have highlighted how ECD probably overestimates true cell density,^{13,14} however these methods, even though they allow a global evaluation of endothelial status, are not feasible in theatre as they are destructive to the graft.^{13,14,31,66,76,137,139} With the possibility of a final endothelial health assessment in the surgical setting before transplantation, the V-CHECK method will instead allow corneal surgeons to estimate the true final endothelial viability of the graft. In fact, Endothelial Cell Loss (ECL) generated during the storage time between graft preparation in the eye bank and corneal transplantation, which can induce mortality especially if preserved for a longer period of time, is usually not accounted for.⁷⁶ This technique could potentially highlight true surgical outcomes since the ECL just prior to surgery will be accounted for. The

V-CHECK method therefore offers a reliable and comparable viability analysis, accounting for ECL just prior to transplantation and helping to determine the surgically induced ECL after the corneal graft is transplanted.

The feasibility of our method in the preoperative setting is not its only advantage. Compared to pan-corneal evaluation with the triple staining method, employing a Hoechst/ethidium-homodimer/calcein-AM combination^{13,76}, the simpler pan-corneal live/dead analysis that we described does not present the risk of overestimating the true damage to donor corneal tissues, as only dead cells or denuded areas of Descemet membrane get stained by Trypan Blue. For instance, it has previously been noted that under triple-staining coloration, preloaded Descemet Membrane Endothelial Keratoplasty (DMEK) tissues exhibit high levels of cells that are not actively converting the calcein AM to calcein, but whose membranes are not yet permeable to ethidium, meaning they have been damaged but are not yet apoptotic.⁸⁰ This population of cells will not be represented as TBPA in the V-CHECK method, guaranteeing that no overestimation of the true endothelial damage occurs and that donor tissues that might have been still employed for transplantation are excluded.

With regards to safety concerns, it is recognized that higher concentrations and prolonged exposure times to Trypan Blue are cytotoxic to corneal endothelial cells. In vitro and ex vivo studies demonstrate that TB concentrations commonly used in ophthalmic surgery (e.g., 0.06%–0.15%) and exposure times of 1–5 minutes do not result in significant corneal ECL or functional impairment and are considered safe for clinical use.⁵⁹ Cytotoxicity becomes evident at higher concentrations (as low as $\geq 0.1\%$) and/or extended exposure (as low as ≥ 30 min) in an inversely proportional fashion, leading to significant endothelial cell loss and morphological changes.^{59,124} Short-term

exposures (≤ 5 minutes) to 0.06%–0.15% TB do not impair mitochondrial function or reduce ECD.^{124,140} The V-CHECK protocol employs staining with 0.05% TB for 30–45 seconds, well below the cytotoxic thresholds.⁵⁹ In addition, 0.25% TB is commonly used by eye banks during multiple stages (stripping, evaluation and loading) of membrane preparation for DMEK surgery, and no toxicity issues have been reported during validation.⁷¹ Of note, impurities in TB preparations have been associated with toxic anterior segment syndrome and complete ECL¹¹⁹, therefore high purity commercial preparations are recommended.

It might seem contradictory that in our validation experiments the V-CHECK method produced higher estimated values of TBPAs compared to the gold standard, i.e. manual counting of Trypan Blue positive areas. The difference was relevant, with a range in average estimated mortality that was 6.5% to 9.5% higher in the V-CHECK method. However, the elevated correspondence in staining of endothelial cells between Calcein AM and Trypan Blue, and between the semi-automated ImageJ-aided segmentation and the V-CHECK method presented in this work, are indicators of a high reliability of the V-CHECK method in assessing endothelial mortality, as well as the strong positive correlation of mortality scores between the two methods. A crucial difference between the gold standard employed in this study and the V-CHECK method is that the latter aims to provide corneal surgeons with an overall evaluation of the global health of the endothelium, while manual counting in the eye bank is usually performed over a section of the central cornea (1 mm^2) such as in the present work.¹³⁰ This discrepancy in the analysed area might explain the difference in measurements between the two methods. It can be argued that the evaluation of a larger area of endothelium is more relevant to surgical outcomes of corneal transplant

than that of only a central square of tissue. The V-CHECK method can be easily applied to the whole surface of the corneal endothelium, or it can be limited to a certain area of endothelium, for example the 8-mm circle of a DMEK graft.

An approach similar to the V-CHECK method has been very recently proposed by Szkarlat et al., who developed a Trypan Blue-based, pan-corneal viability assessment after the processing of pre-loaded DMEK tissues in an eye bank setting.³² Interestingly, they found that visual assessment by experienced eye bank technicians produced similar estimations of global endothelial cell mortality compared to automated image analysis, while specular microscopy overestimated the ECD after DMEK preparation, again suggesting that ECD represents an inaccurate and poorly reproducible measure of endothelial viability. It is also important to note that estimation of endothelial mortality through automated image processing in Szkarlat et al. as well as in other similar works analyzing DMEK tissues after preparation frequently exceeds mortality values of 15-20%, underlying how the evaluation of the whole endothelium might frequently yield larger estimates of endothelial damage compared to ECD or visual inspection.^{31,32,137}

Finally, in a recent paper Yoon et al. presented a comparable method to evaluate the damage to corneal endothelium after preparation of corneal tissues for transplantation.¹³⁸ In their analysis, images of donor corneas stained with 0.06% Trypan Blue were processed in an image analysis software using manual selection of stained areas. Compared to this technique, the V-CHECK method presents several advantages. Firstly, the use of an automated, deep-learning based segmentation can allow the V-CHECK method to be applied to large datasets of images, and to be implemented in the operating theatre for real-time evaluation of endothelial damage.⁹¹

Furthermore, the use of a trephine base without suction holes avoids background disturbances that can affect the estimation of TBPAs. Likewise, the use of a BSS fill of the donor corneoscleral rim described in our image acquisition protocol reduces the glare and light reflexes from the surgical microscope, evident in the images from Yoon et al.¹³⁸, that again can affect the estimation of TBPAs.

A limitation of our current V-CHECK method is that we have not fine-tuned SAM, i.e., we employed the available pretrained model without adjustments to its weights. Despite the fact that SAM offers an adequate and reliable off-the-shelf tool, we hypothesize that additional training on even a small curated dataset of microscopy images via state-of-the-art fine-tuning methodologies, such as Conv-LoRA¹⁵⁶, could yield improved segmentation results. This would also have the benefit of streamlining and automating the process further, requiring fewer or no human prompt at all to detect corneal regions. Future developments of the V-CHECK method will therefore include these SAM optimizations. A further limitation of this study is the somewhat small sample size that might underrepresent the variability in mortality patterns, viability thresholds, or imaging strategies. However, given the high reproducibility demonstrated by the V-CHECK method in the present work we are confident in the generalizability of its results. Nevertheless, with the collection of a larger dataset through the longitudinal V-CHECK cohort study we may be able to implement more robust and better-tested iterations of the V-CHECK tool that are less susceptible to biases in the pre-trained, possibly fine-tuned model and misrepresentations in the corneal images.

In conclusion, the V-CHECK method provides an innovative and reliable approach for evaluating endothelial cell viability in donor corneas through automated, deep-

learning-based segmentation of Trypan Blue Positive Areas (TBPAs). This method demonstrated strong correlation with manual counting by experienced eye bank technicians, establishing its validity as a robust alternative for preoperative assessment. Unlike traditional methods, V-CHECK allows analysis of endothelial viability across the entire cornea — not limited to small, centralized samples of endothelium — providing eye bank technicians with a comprehensive tool for objectively reporting global endothelial health and enhancing quality assurance in donor tissue evaluation. Furthermore, the V-CHECK analysis enables corneal surgeons to directly assess endothelial status in the operating theatre — a critical capability not currently available to them, despite surgeons bearing sole responsibility for transplantation success.^{11,12} By incorporating the V-CHECK method into their preoperative routine, surgeons may be better equipped to identify any issues with the donor corneal tissue that could impact graft survival, evaluate their technique and compare with peers.

It is crucial to have accessible and reproducible methods for assessing endothelial status, as endothelial cell viability is one of the key factors in determining graft success and long-term outcomes. Prospective, large-scale studies are warranted to further validate the V-CHECK method and its role in enhancing donor corneal tissue quality assessments. In the ongoing V-CHECK study, a cohort of keratoplasty patients will be followed for 12 months to evaluate the correlation between preoperative endothelial viability, as measured by the V-CHECK method, and clinical outcomes.⁹¹ Integrating the V-CHECK analysis into routine preoperative evaluations in eye banks and operating theatres offers significant potential to standardize assessment protocols, optimize graft survival, and ultimately enhance patient outcomes in corneal transplantation.

Chapter 9

FINGERPRINT-LIKE ENDOTHELIAL STAINING OF PRECUT UT-DSAEK IS ASSOCIATED WITH EARLY GRAFT FAILURE

Paper under review

Matteo Airaldi, Filippo Airaldi, Sabrina Vaccaro, et al. *Fingerprint-like endothelial staining of precut UT-DSAEK is associated with early graft failure.*

2181 **Authors:** Matteo Airaldi, MD^{1,2}, Filippo Airaldi, MSc³, Sabrina Vaccaro, MD⁴, Zhuangzhi Gao,
2182 MSc², Mariacarmela Ventura, MD^{5,6}, Chiara Ancona, MD⁷, Beatrice Cesaro, MD⁶, Davide
2183 Romano, MD, PhD^{2,8}, Eliana Forbice, MD, PhD⁴, Francesco Semeraro, MD^{4,6}, Yalin Zheng,
2184 PhD², V-CHECK Study Group, Vito Romano, MD^{2,4,6}

2185

2186 **Author affiliations:**

- 2187 1. Department of Molecular and Translational Medicine, Università degli Studi di
2188 Brescia, Brescia, Italy
- 2189 2. Department of Eye and Vision Sciences, Institute of Life Course and Medical
2190 Sciences, University of Liverpool, Liverpool, UK
- 2191 3. Delft Center for Systems and Control, Delft University of Technology, Delft, The
2192 Netherlands
- 2193 4. Eye Unit, ASST Spedali Civili di Brescia, Piazzale Spedali Civili, 1, 25123, Brescia,
2194 Italy
- 2195 5. Department of Clinical and Experimental Sciences, Università degli Studi di
2196 Brescia, Brescia, Italy
- 2197 6. Department of Medical and Surgical Specialties, Radiological Sciences, and Public
2198 Health, Università degli Studi di Brescia, Brescia, Italy
- 2199 7. Eye Clinic, Ospedale Oftalmico, ASST Fatebenefratelli Sacco, Milan, Italy
- 2200 8. St Paul's Eye Unit, Liverpool University Hospitals NHS Foundation Trust, Liverpool,
2201 United Kingdom

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ABSTRACT

Purpose: To evaluate the association between a distinctive fingerprint-like trypan blue (TB) staining pattern in precut Ultrathin Descemet Stripping Automated Endothelial Keratoplasty (UT-DSAEK) grafts and the risk of postoperative graft failure.

Methods: UT-DSAEK grafts underwent preoperative TB staining and imaging using the V-CHECK protocol. Endothelial viability was quantified via a custom computer-vision algorithm and expressed as live/dead endothelial areas ratio. The presence of a fingerprint-like TB staining pattern, defined as concentric or adjacent linear ridges of TB staining, was recorded. A Cox proportional hazards model estimated the impact of preoperative mortality on graft failure.

Results: Twenty UT-DSAEK lenticules were evaluated. Two grafts with high endothelial mortality were excluded from transplantation but included in staining analysis. Eighteen eyes receiving UT-DSAEK were included in the survival analysis. Average (SD) endothelial cell density of the UT-DSAEK graft was 2588.9 (107.9) cells/mm². V-CHECK measured mortality was on average (SD) 14.2% (9.2%). Grafts with fingerprint-like staining (n = 18/20, 90%) showed significantly higher endothelial mortality (mean 16.8% vs. 2.2%, $p = 0.01$). Among the 18 transplanted eyes, the cumulative graft failure incidence was 44.4% (n = 8), corresponding to an incidence rate of 64 cases per 100 person-years (95% CI: 27.6, 126). No failures occurred in grafts without fingerprint-like staining. Cox regression revealed a significant association between preoperative V-CHECK mortality and failure (HR 1.26 [95% CI: 1.03, 1.53], $p = 0.02$).

2226 **Conclusions:** Fingerprint-like TB staining of UT-DSAEK grafts indicates areas of
2227 increased endothelial mortality and is associated with early graft failure. Preoperative
2228 endothelial viability assessment using the V-CHECK method may help identifying
2229 high-risk grafts.

2230

2231 INTRODUCTION

2232

2233 The preparation of precut Ultrathin Descemet Stripping Endothelial Keratoplasty (UT-
2234 DSAEK) tissues is a standardized procedure carried out by qualified technicians in the
2235 eye bank setting.¹⁵⁷ The key step in this process is the microkeratome pass, which
2236 guarantees a uniform cut of the donor cornea to produce an Ultrathin (i.e., less than
2237 100 μ m) DSAEK graft, which can then be shipped out for transplantation.^{158,159} After
2238 the cut, the UT-DSAEK graft can be examined with optical coherence tomography
2239 (OCT) to check for uniformity of the cut.¹⁶⁰ Careful handling of the tissue helps with
2240 preservation of the endothelial cell population of the precut graft.^{157,161}

2241

2242 Endothelial cell viability is in fact the single most important determinant of long-term
2243 survival of the UT-DSAEK graft.¹⁷ Estimates of the endothelial cell density (ECD) of
2244 precut grafts are reported by the eye bank, but ECD only reflects the status of a narrow
2245 window of endothelium and does not reflect the overall viability of the endothelial cells
2246 of the entire graft.¹² Some eye banks also report an estimate of overall mortality of
2247 endothelial cells, usually calculated via direct visualization under a light microscope,
2248 which can also serve as a threshold for transplantation suitability.¹⁰

2249

2250 Nevertheless, corneal surgeons transplanting such tissues have no means to
2251 independently verify endothelial mortality and guarantee a preoperative quality check
2252 of the grafts.¹¹ Vital dyes, such as Trypan blue (TB), can be used to stain dead
2253 endothelial cells and denuded Descemet membrane, and automated image analysis

2254 tools can provide corneal surgeons with preoperative estimates of endothelial
2255 mortality.^{33,91} In this paper, we present a cohort of UT-DSAEK grafts, dyed
2256 preoperatively with TB and analysed with a custom-made computer-vision algorithm,
2257 showing a peculiar, fingerprint-like endothelial staining pattern which was associated
2258 with early DSAEK graft failure.

2259

MATERIALS AND METHODS

Study design and setting

This cohort study included consecutive eyes undergoing UT-DSAEK at the *ASST Spedali Civili di Brescia* between April 2023 and December 2023 for which preoperative endothelial viability assessment was performed. Patients were included in the Viability Control of Human Endothelial Cells before Keratoplasty (V-CHECK) Study (NCT05847387).^{33,91} Local institutional review board approval was obtained (protocol number NP 6028), and the study adhered to the tenets of the Declaration of Helsinki. Patients expressed written informed consent for participation in the study.

Participants

The clinical data and demographic characteristics of consecutive eyes undergoing UT-DSAEK were identified from electronic health records, collected and analyzed.

Patients who experienced intraoperative complications which could affect the survival of the DSAEK graft were excluded.

Only donor lenticules for which an endothelial viability assessment was conducted were included in the study.

Endothelial viability assessment

Precut, cap-on donor lenticules prepared by the *Monza Eye Bank* were retrieved from their shipping vessels in organ culture medium. Endothelial viability assessment was performed before punching the donor lenticule as described in the V-CHECK

protocol.⁹¹ The analysis protocol has been described in detail and validated in a previous, preclinical study.³³ Briefly, after thoroughly rinsing the donor cornea from its transport medium, the corneal endothelium was stained for 30 to 45 seconds with 0.05% TB; then, the dye was gently washed away with balanced salt solution (BSS). The TB dye stains the nuclei of severely damaged and dead endothelial cells of donor corneas, as well as areas of Descemet membrane denuded of endothelial cells.⁵⁹ The corneal tissue was then placed face-up on a custom trephine base designed with no suction holes (e.janach, Italy) in order to provide a smooth, concave surface to rest the cornea on, with no background optical disturbance that could prevent optimal image analysis of the central endothelium. The corneoscleral rim was filled with BSS to avoid the formation of reflexes of the microscope lights on the central endothelium. Magnification levels of the microscope were adjusted to include the entirety of the Schwalbe line of the donor cornea just inside the image frame.

Images imported from the microscope were segmented with the Segment Anything foundation model to isolate the cornea from the background.¹⁵⁰ Adaptive thresholding, distance transform and a watershed algorithm were used to isolate TB-positive pixel in the cornea.^{33,162,163} The V-CHECK endothelial mortality was calculated according to the percentage of TB-positive area, that is, the ratio between the number of TB-positive pixels and the total pixel count corresponding to the whole area of the segmented corneal endothelium.

The presence of a fingerprint-like staining pattern, defined by concentric or adjacent linear ridges of TB staining, corresponding to areas of endothelial mortality or denuded Descemet membrane, was also recorded.

2306

2307 **Surgical technique**

2308 All surgeries were performed by experienced corneal surgeons (EF, VR). After
2309 endothelial viability assessment, the stromal cap was removed from the donor lenticule
2310 and an 8 mm Moria donor cornea punch was used to trephine the UT-DSAEK graft to
2311 size.

2312 A 2.5 mm clear corneal incision was created in the host eye, and the recipient
2313 Descemet membrane was stripped to 8.5 mm under BSS through an anterior chamber
2314 maintainer. Delivery of the graft was performed with a pull-through technique as
2315 described by Busin et al.¹⁶¹ Briefly, the donor lenticule was loaded endothelial side up
2316 onto a Busin glide or a Macaluso injector (e.janach, Italy) according to surgeon
2317 preference, and inserted using a single-use 25-gauge forceps. After the graft was
2318 unfolded and positioned centrally, a full air fill of the anterior chamber was maintained
2319 for 5 to 10 minutes. The corneal incision was closed with interrupted 10-0 nylon
2320 sutures. Enough air was removed and replaced with BSS to avoid pupillary block.

2321

2322 **Data measurement and variables**

2323 *Demographic and clinical data*

2324 Demographic data and clinical data of included subjects were extracted from electronic
2325 patient records. Recorded clinical data included preoperative best corrected visual
2326 acuity (BCVA), indication for DSAEK surgery, date of surgery, final follow-up date, and
2327 final BCVA. Whenever available, BCVA was also recorded for each follow-up visit after
2328 surgery.

2329 Anterior segment OCT (AS-OCT) and specular microscopy imaging were reviewed
2330 whenever available. Central corneal thickness (CCT) and central graft thickness were

2331 measured on AS-OCT raster scans (CASIA2, Tomey, Japan) with the built-in “Flap”
2332 caliper tool. Postoperative ECD as measured by specular microscopy (EM-3000,
2333 Tomey, Japan) was also included.

2334

2335 *Donor graft data*

2336 Donor graft data was extracted from the respective eye bank sheet and included donor
2337 age, donor gender, cause of death, time to preservation, UT-DSAEK thickness and
2338 ECD, and overall mortality and mortality on folds as determined by the eye bank.

2339

2340 *Graft rejection and failure*

2341 Graft rejection was defined as the presence of significant keratic precipitates, anterior
2342 chamber, endothelial or stromal inflammation, or stromal/epithelial oedema, according
2343 to the modified Collaborative Corneal Transplantation Studies classification of graft
2344 rejection.¹⁶⁴

2345 Endothelial graft failure was defined as irreversible corneal oedema in the presence
2346 of an attached donor lenticule, with evidence of corneal thickening, Descemet folds,
2347 or increased lenticular thickness as measured by AS-OCT.^{165,166}

2348

2349 **Variables and outcomes**

2350 The primary outcome was the incidence rate of graft failure in the cohort of eyes
2351 included, expressed in cases per 100 person-years.

2352 Secondary outcomes included measures of proportional relative risk of graft failure in
2353 relation to the extent of TB endothelial staining according to the V-CHECK method.

2354

2355 **Statistical Analysis**

2356 Descriptive data were summarized using the mean (standard deviation), median
2357 (interquartile range), and number (percentages) where appropriate.

2358 The incidence rate for the development of graft failure was calculated over the entire
2359 course of follow-up and reported as the number of cases per 100 person-months.

2360 A Cox proportional hazards regression model was used to estimate the hazard ratio
2361 (HR) for DSAEK failure according to the endothelial viability calculated with the V-
2362 CHECK method. Other covariates included in the model were age at surgery, donor
2363 age, donor ECD, lenticule thickness, and time from death to preservation. A Kaplan-
2364 Meier function was used to plot survival curves.

2365 A p value less than 0.05 was considered statistically significant. All analyses were
2366 conducted using R software version 4.2.2 (R Project for Statistical Computing, Vienna,
2367 Austria).

2368

RESULTS

A total of 20 precut UT-DSAEK grafts were analyzed in this study. For two grafts (10%) a decision not to proceed with transplantation was made because of the elevated endothelial mortality in the lenticule, as highlighted by the V-CHECK method, as well as due to the presence significant ocular comorbidities in the host eyes (multiple failed grafts and panuveitis, respectively). Therefore, a total of 18 eyes which underwent UT-DSAEK at the *ASST Spedali Civili di Brescia* Hospital between April 2023 and February 2025 were included in this cohort study.

Donor lenticule characteristics of all grafts ($n = 20$), including grafts rejected by the surgeon ($n = 2$), are summarized in **Table 5**. Average (SD) age at donation was 69.2 (14) years, with good ECD (mean [SD], 2588.9 [107.9] cells/mm²) and low average lenticule thickness (mean [SD], 74.3 [14.6] microns).

Overall eye bank-reported mortality was 0% in all cases, with limited (i.e., less than 2%) endothelial mortality on folds in 9 cases (50%).

Tissues, n	20
Donor age, years – mean (SD)	69.2 (14)
Donor gender, females – n (%)	3 (16.7%)
Cause of donor death, n (%)	
<i>Neoplasia</i>	9 (50%)
<i>Cardiac</i>	5 (27.8%)
<i>Cerebrovascular</i>	3 (16.7%)
<i>Respiratory</i>	1 (5%)
Time to preservation, hours – mean (SD)	13.9 (7.6)
Time to transplantation, days – mean (SD)	53.5 (14.4)
Lenticule thickness, micron – mean (SD)	74.3 (14.6)
Lenticule ECD, cells/mm ² – mean (SD)	2588.9 (107.9)
Eye bank-declared mortality	
Overall mortality, n (%)	
0%	18 (100%)
< 2%	0 (0%)
Mortality on folds, n (%)	
0%	9 (50%)
< 2%	9 (50%)
Fingerprint-like TB staining, n (%)	18 (90%)
V-CHECK mortality, % - mean (SD)	14.2% (9.2%)

Images of TB-stained donor lenticules according to the V-CHECK staining protocol are displayed in **Figure 9**. A distinctive fingerprint-like staining pattern, defined by the presence of concentric or adjacent linear ridges of TB staining, could be identified in 18 out of 20 tissues (90%). Both non-transplanted grafts showed fingerprint staining (100%).

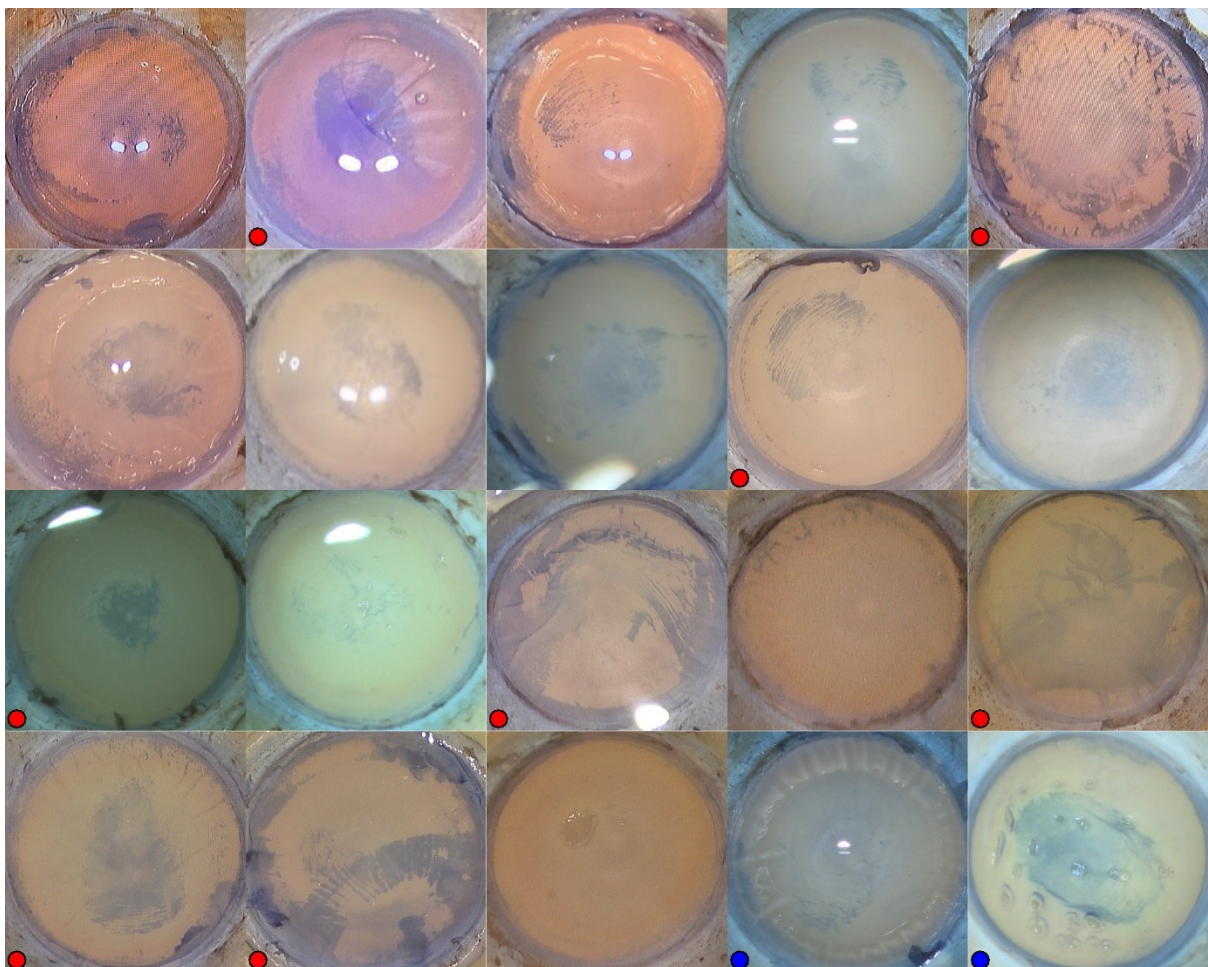


Figure 9. Images of TB-stained donor lenticules according to the V-CHECK method, in which donor corneas are stained with TB 0.05% for 30-45 seconds to allow for staining of areas of endothelial mortality and denuded Descemet membrane. Red dots signal cases which had an early graft failure. Blue dots signal tissues that were not transplanted due to elevated endothelial mortality in the lenticule as well as due to significant ocular comorbidities in the host eyes (multiple failed grafts and panuveitis, respectively).

Endothelial mortality as measured with the V-CHECK method was on average (SD) 14.2% (9.2%). Processed images with segmented endothelial areas and segmented TB-positive areas are displayed in **Figure 10**. V-CHECK measured endothelial mortality was significantly higher in grafts displaying fingerprint-like staining (mean [SD], 16.8% [9.2%] vs. 2.2% [3.0%], t-test p-value = 0.01).

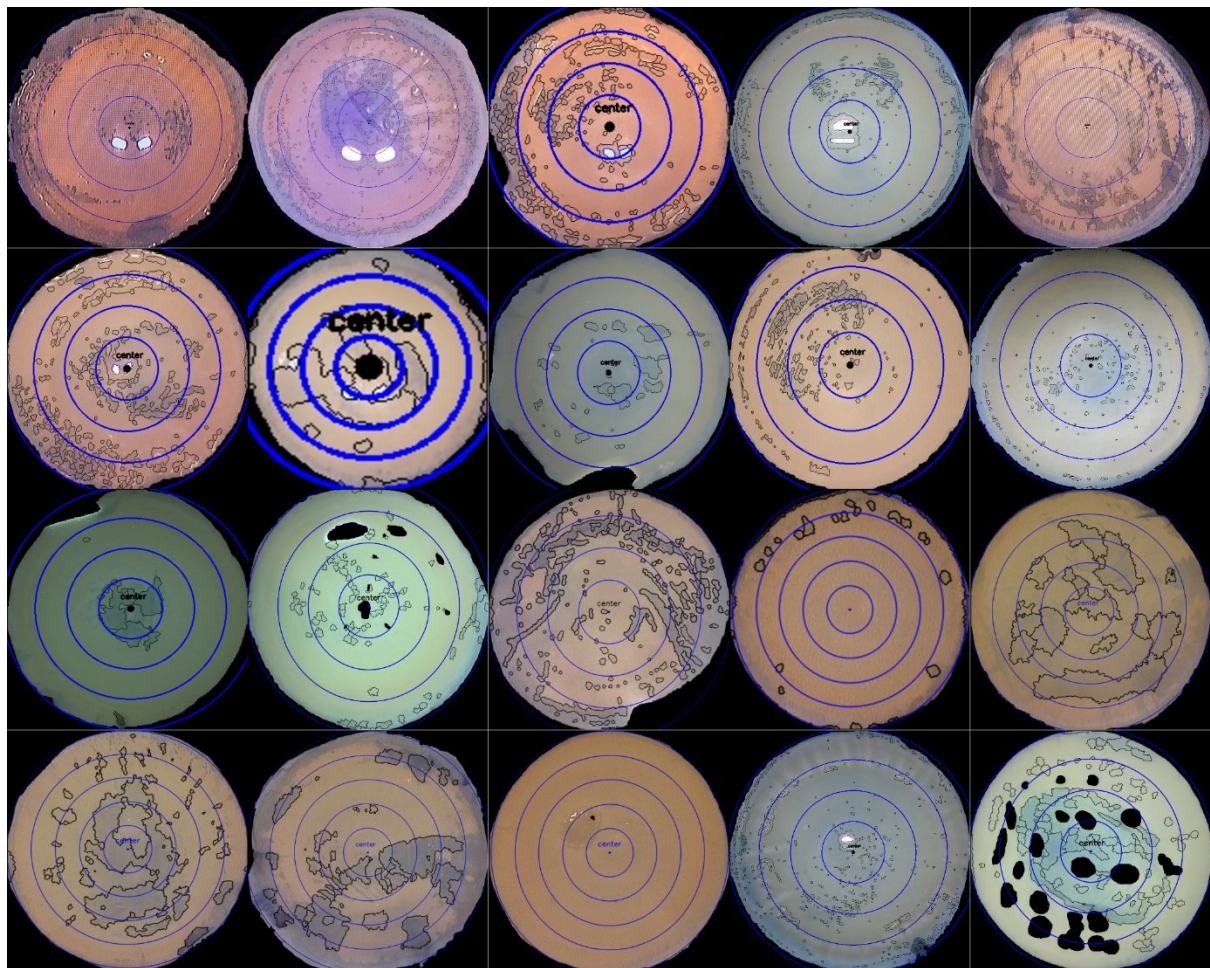


Figure 10. Processed images with segmented corneas and segmented areas of endothelial mortality, identified as TB-positive pixel. Corneal segmentation was performed using the Segment Anything foundation model. TB-positive areas were isolated using adaptive

2412 *thresholding, distance transform, and a watershed algorithm. Endothelial mortality (V-CHECK)*
2413 *was calculated as the percentage of TB-positive pixels over the total endothelial area.*

2414

2415 In the cohort of eyes included and successfully transplanted with an UT-DSAEK graft
2416 (n = 18), the average length of follow-up after the surgery was 10.1 (7.1) months.

2417 Patients were on average 73.8 (9) years old at time of surgery. Ten out of 18 patients
2418 were females (55.6%). The average preoperative BCVA and CCT were 1.6 (0.7)
2419 LogMAR and 786.5 (140) micron, respectively. The most common indication for
2420 DSAEK surgery were pseudophakic bullous keratopathy (n = 10, 55.6%), a failed
2421 previous DSAEK graft (n = 5, 27.8%), and Fuchs endothelial dystrophy (n = 3, 16.7%).

2422 The mean final visual acuity was 1.2 (0.8) LogMAR. The average final CCT was 722
2423 (137.4) microns, while the final central thickness of the lenticule was 116.7 (41.3)
2424 microns. The average final ECD was 732 (256.7) cells/mm². Clinical details are
2425 summarized in **Table 6**.

2426

Eyes, n	18
Females, n (%)	10 (55.6%)
Age, years – mean (SD)	73.8 (9)
Follow-up, months – mean (SD)	10.1 (7.1)
Indication for UT-DSAEK, n (%)	
<i>FED</i>	3 (16.7%)
<i>PBK</i>	10 (55.6%)
<i>Re-DSAEK</i>	5 (27.8%)
Ocular comorbidities, n (%)	
<i>Glaucoma surgery/drainage device</i>	4 (22.2%)
<i>Trauma</i>	3 (16.6%)
<i>Pars plana vitrectomy</i>	3 (16.6%)
<i>Iris/scleral fixated IOL</i>	2 (11.1%)
Preoperative BCVA, LogMAR – mean (SD)	1.6 (0.7)
Preoperative CCT, micron – mean (SD)	786.5 (140)
Final clinical outcomes	
<i>BCVA, LogMAR – mean (SD)</i>	1.2 (0.8)
<i>CCT, micron – mean (SD)</i>	722 (137.4)
<i>Lenticule thickness, mean (SD)</i>	116.7 (41.3)
<i>ECD, cells/mm² – mean (SD)</i>	732 (256.7)
Graft rejection and failure, n (%)	1 (5.6%)
<i>Graft rejection</i>	1 (5.6%)
<i>Endothelial failure</i>	8 (44.4%)

2428 The cumulative incidence of endothelial graft failure among all cases was 44.4% (n =
2429 8). This corresponded to an incidence rate (95% CI) of 64 [27.6, 126] cases of failure
2430 per 100 person-years. There were no instances of graft failure among grafts that
2431 showed no fingerprint-like staining. When considering only grafts displaying
2432 fingerprint-like staining (n = 16), the cumulative incidence of endothelial graft failure
2433 rose to 50%.

2434

2435 The Kaplan-Meier estimate of the survival curve can be visualized in **Figure 11** (left
2436 panel). Three patients (16.6%) were lost to follow-up before 12 months from surgery
2437 (right censored). A Cox proportional hazards model found a significant increase in risk
2438 of endothelial failure of the DSAEK graft in eyes with higher preoperative endothelial
2439 mortality as measured with the V-CHECK method (Hazard Ratio [95% CI], 1.26 [1.03,
2440 1.53], p = 0.02). This translated to a 26% increase in the risk of endothelial failure for
2441 each additional 1% increase in V-CHECK measured preoperative mortality. Hazard
2442 ratios and their respective p values for covariates included in the model are reported
2443 in **Figure 11** (right panel).

2444

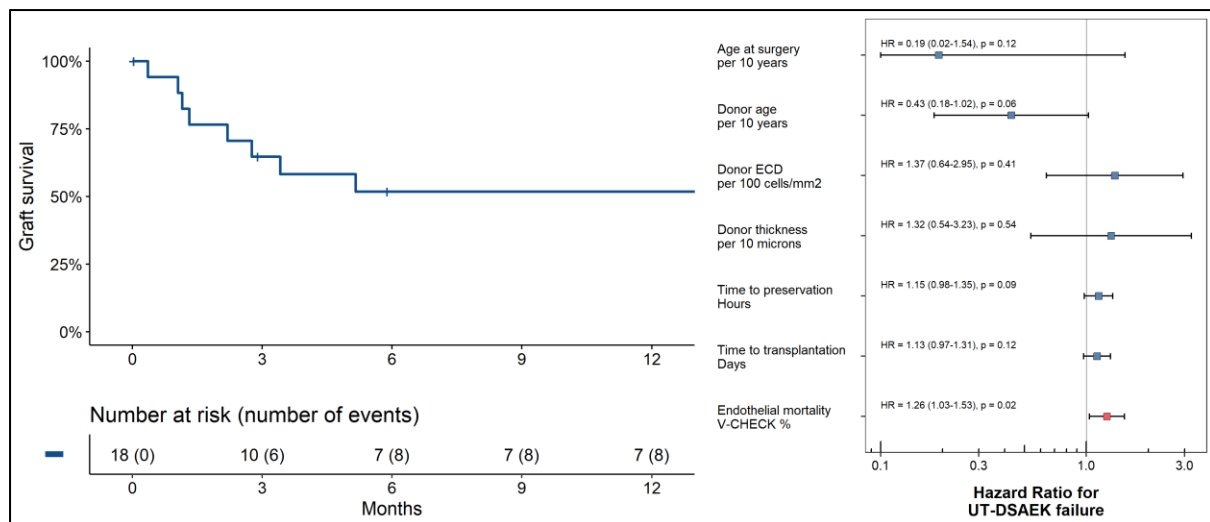


Figure 11. Left panel) Kaplan-Maier estimates of survival curve for DSAEK graft failure in the study cohort. **Right panel)** Forest plot of hazard ratios for DSAEK graft failure from a Cox proportional hazards model. Covariates with significant p-values are highlighted in red.

DISCUSSION

In this cohort of UT-DSAEK lenticules, we found that staining of the corneal endothelium with TB revealed a fingerprint-like stain in the majority of tissues analyzed. Fingerprint-like staining and endothelial mortality as measured by the V-CHECK method were associated with an increased risk of endothelial graft failure. These findings underscore the importance of preoperative endothelial viability assessment and suggest that the fingerprint-like stain may serve as a visible biomarker of endothelial damage.

Despite the increasing popularity of Descemet Membrane Endothelial Keratoplasty (DMEK), UT-DSAEK remains a highly relevant surgical option for patients needing endothelial keratoplasty, particularly for eyes with complex anterior segments, prior graft failure, significant comorbidities, or challenging anatomy (e.g., poor visualization, shallow anterior chambers, aphakia, iris or scleral-fixated intraocular lenses (IOLs), iris defects/loss, synechiae).^{167,168} The complexity of indications for UT-DSAEK is reflected in the present case series, where most patients had one or more significant ocular comorbidity (e.g., tube shunts, iris fixated/anterior chamber IOLs, trauma, previous pars plana vitrectomy) and/or previously failed UT-DSAEK grafts. Given the possible surgical challenge of delivering a graft in such eyes as well as the tendency for decline in ECD after DSAEK surgery^{131,169}, it is vital both to limit the endothelial cell loss associated with surgical manipulation but also to ensure that the viability of the endothelium is maintained before transplantation through the preservation, preparation and transport steps of the UT-DSAK graft.

2475

2476 In this study, we could identify a fingerprint-like staining of the endothelium after TB
2477 staining in 90% of tissues. The presence of TB positive areas on the corneal
2478 endothelium represents either areas of dead endothelial cells or denuded areas of
2479 Descemet membrane. However, the fingerprint pattern differs significantly from the
2480 classical findings of spoke-like, radial mortality on corneal folds caused by donor tissue
2481 swelling during organ culture storage.¹⁷⁰ We have defined fingerprint-like staining as
2482 concentric or adjacent linear ridges of TB staining, a morphology that does not
2483 resemble the radially oriented mortality on corneal folds. Instead, we hypothesize that
2484 the regular pattern of adjacent ridges of endothelial mortality might be linked to the
2485 movement or passage of the microkeratome head, possibly in the presence of a hyper-
2486 pressurization of the artificial anterior chamber.¹⁵⁷ Experimental laboratory
2487 investigations are warranted to further delineate the relationship of the setup for the
2488 preparation of UT-DSAEK lenticules and induced endothelial mortality in the form of
2489 fingerprint-like staining.

2490

2491 Regardless of the aetiology of the fingerprint stain, our results clearly show that
2492 endothelial mortality as measured with the V-CHECK method was strongly associated
2493 with early graft failure after UT-DSAEK. A profound depletion of endothelial cells is a
2494 well-known cause of primary graft failure of UT-DSAEK lenticules.¹³⁵ The absence in
2495 our case series both of episodes of rejection leading to failure (only one case,
2496 successfully reverted) or of late postoperative complications highlights how the
2497 endothelial cell loss must have happened primarily either before or during the surgery.

2498

In this regard, the discrepancy between the eye bank assessment of the UT-DSAEK grafts before shipping and the high mortality revealed by the V-CHECK preoperative analysis might explain the unexpectedly high failure rate reported in the present series.

In fact, the overall mortality of the graft declared by the eye bank was 0% in all cases, with only 9 cases (50%) showing less than 2% mortality on corneal folds, with elevated ECD in all cases (range [2400-2800] cells/mm²). The V-CHECK analysis however revealed an average pan-corneal endothelial mortality of 14.2% (range [0.1% - 34.1%]). Estimates of pan-corneal evaluation of endothelial viability tend to return higher mortality values compared to standard assessment tools such as ECD or visual assessment under light microscopy, with studies on viability after DMEK grafts preparation frequently reporting mortality values exceeding the 15-20% mark.^{31,32} This is possibly due to the fact that pan-corneal evaluations explore a larger area compared to ECD or manual counting, which might miss areas of mortality, but also because standard counting by eye banks overestimates the actual pool of viable endothelial cells as demonstrated by comparisons of direct counting with counting assisted by triple live/dead staining.¹³ We have also previously described how the V-CHECK method demonstrates good correlation with direct counting while consistently reporting higher mortality values (range [+6.5%-9.5%]), in line with other methods of pan-corneal endothelial viability evaluation.³³

The difference between eye-bank declared mortality and preoperatively assessed mortality both in this case series and in other studies also highlights the need for a thorough evaluation of the endothelial viability of the UT-DSAEK grafts at multiple points during their journey from retrieval to transplantation, in order to guarantee a

2523 suitable quality and density of endothelial cells for graft survival.^{26,135} The V-CHECK
2524 method is an inexpensive and easy tool that can provide technicians in the eye bank
2525 setting with an additional screening step both before and after tissue preparation.
2526 Likewise, the V-CHECK method can be adopted by corneal surgeons to preoperatively
2527 assess tissues before implantation, contributing to the exclusion of poor-quality tissues
2528 which expose the receiver to high risks of early graft failure.⁹¹

2529 The adoption in this patient cohort of the V-CHECK method led to the finding of a
2530 fingerprint-like stain of endothelial mortality in most of the UT-DSAEK grafts included,
2531 highlighting how issues in the retrieval, preservation, preparation and shipping chain
2532 can lead to poor quality tissues “slipping” through the quality checks already in place
2533 to guarantee their suitability for transplantation.¹¹ By demonstrating that V-CHECK
2534 measured endothelial mortality is associated with risk of early graft failure after UT-
2535 DSAEK, we encourage eye banks and especially corneal surgeons to adopt
2536 independent means of graft viability evaluation to provide transplanted patients with
2537 safer and more standardized outcomes after keratoplasty.

2538

Chapter 10

2539

2540

2541 **SURVIVAL OUTCOMES OF EYE BANK PRECUT UT-DSAEK: INSIGHTS FROM**
2542 **REAL WORLD, MULTISURGEON DATA**

2543

2544 **Paper under review**

2545 Sabrina Vaccaro, **Matteo Airaldi**, Erika Bonacci et al. *Survival outcomes of eye bank*
2546 *pre-cut UT-DSAEK: insights from real-world, multisurgeon data*

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2548

2549 **Authors:** Sabrina Vaccaro*¹, Matteo Airaldi*^{2,3}, Erika Bonacci⁴, Carlo Cagini⁵, Stefano
2550 Ferrari⁶, Paolo Fogagnolo⁷, Anna Limongelli⁸, Saverio Luccarelli⁹, Marco Messina⁵,
2551 Luca Pagano¹⁰, Emilio Pedrotti¹¹, Luigi Petronella¹⁰, Maria Poddi⁵, Alessandro Ruzza⁶,
2552 Francesco Semeraro^{1,12}, Alfonso Strianese⁷, Silvia Sonego⁷, Antonio Vagnoni⁷,
2553 Edoardo Villani^{13,14}, Riccardo Vinciguerra¹⁰, Paolo Vinciguerra¹⁰, Giuseppe
2554 Zuccarini¹⁰, Raffaella Mistò⁸, Francesca Pateri⁸, Diego Ponzin⁶, Vito Romano^{1,12}

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2556 *Sabrina Vaccaro and Matteo Airaldi contributed equally to this work as first co-authors

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2558 **Affiliations:**

2559 ¹ Eye Unit, ASST Spedali Civili di Brescia, Piazzale Spedali Civili, 1, 25123,
2560 Brescia, Italia

2561 ² Department of Molecular and Translational Medicine, University of Brescia,
2562 Brescia, IT

2563 ³ Department of Eye and Vision Science, University of Liverpool, Liverpool, UK

2564 ⁴ Ophthalmology Clinic, Department of Engineering for Innovation Medicine,
2565 University of Verona, IT

2566 ⁵ Department of Medicine and Surgery, Section of Ophthalmology, University
2567 of Perugia, S. Maria della Misericordia Hospital, Perugia, Italy

2568 ⁶ Fondazione Banca degli Occhi del Veneto ETS, Venice, Italy

2569 ⁷ Eye Clinic, ASST Santi Paolo E Carlo Hospital, University of Milan, Milan, Italy

2570 ⁸ Eye Bank of Monza, Azienda Ospedaliera San Gerardo, Monza, Italy

2571 ⁹ Eye Clinic, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico,
2572 Milan, Italy

2573 ¹⁰ Department of Ophthalmology, IRCCS Humanitas Research Hospital, Via
2574 Manzoni 56, Rozzano, Milan, Italy.

2575 ¹¹ Ophthalmology Clinic, Department of Surgery, Dentistry, Paediatrics and
2576 Gynaecology, University of Verona, IT

2577 ¹² Department of Medical and Surgical Specialties, Radiological Sciences, and
2578 Public Health, University of Brescia, Viale Europa 15, 25123 Brescia, Italy

2579 ¹³ Dipartimento di Scienze Cliniche e di Comunità, Dipartimento di Eccellenza
2580 2023-2027, University of Milan, Milan, Italy.

2581 ¹⁴ Eye Clinic San Giuseppe Hospital, IRCCS Multimedica, Milan, Italy

2582 ¹⁵ Humanitas San Pio X Hospital, Milan, Italy

2583

ABSTRACT

Purpose: To assess real-world survival outcomes of pre-cut ultrathin Descemet stripping automated endothelial keratoplasty (UT-DSAEK) grafts prepared by two Italian eye banks.

Methods: In this multicenter cohort study, clinical records of consecutive patients undergoing UT-DSAEK between January 2021 and October 2024 across eight tertiary centers in Italy were reviewed. Donor tissues were pre-cut by either Fondazione Banca degli Occhi del Veneto (FBOV) or the Monza Eye Bank. Graft survival was defined according to Cornea Preservation Time Study criteria and analyzed using Kaplan–Meier curves and mixed-effects Cox regression, adjusting for demographic, clinical, and surgical variables. Secondary outcomes included best-corrected visual acuity (BCVA), endothelial cell density (ECD), central corneal thickness (CCT), and graft thickness at baseline and follow-up visits.

Results: A total of 379 eyes were analyzed: 196 received FBOV grafts and 183 received Monza grafts. The cumulative incidence of graft failure was 18.9% for FBOV versus 36.1% for Monza grafts. Risk of failure was significantly higher in the Monza group (hazard ratio [95% CI], 6.58 [1.72–25.4]; $p=0.006$). BCVA improved in both groups, with superior outcomes in the FBOV group at all timepoints (final mean [SD], 0.6 [0.7] vs. 0.8 [0.8] LogMAR; $p=0.01$). Postoperative ECD remained higher in FBOV grafts (all $p<0.001$), while final CCT and graft thickness were comparable.

2606 **Conclusions:** UT-DSAEK survival varied markedly between eye banks despite
2607 comparable surgical expertise, underscoring the need for standardized, auditable eye
2608 bank protocols to optimize graft outcomes.

2609

INTRODUCTION

Descemet's stripping automated endothelial keratoplasty (DSAEK) is extensively employed for the treatment of corneal endothelial dysfunction.¹⁷¹ Notwithstanding significant progress in surgical procedures and postoperative care, graft rejection and failure continue to pose significant challenges.¹⁷² An immune response against the transplanted tissue is the hallmark of graft rejection, which, if not promptly and effectively treated, may result in failure.^{173–175} However, graft failure can also occur in the absence of rejection as a result of endothelial cell loss in aging grafts or mechanical factors.¹⁷⁶ Several variables have been identified as potential risks for graft failure, encompassing both graft preparation and preservation, as well as surgical factors and their indications.^{177–183} In particular, eyes undergoing endothelial keratoplasty for pseudophakic or aphakic corneal oedema have demonstrated a significantly higher risk of late graft failure compared to those treated for Fuchs endothelial corneal dystrophy.¹⁷⁹ Reported primary graft failure rates after DSAEK surgery range between 0-29 %.¹⁷² This wide range may be partially attributed to the diverse primary indications for DSAEK surgery. The preference for DSAEK in cases with complicated anterior chamber features and surgical complexities can contribute significantly to the risk of primary graft failure.¹⁸⁴ Significant manipulation of the graft in complex cases has also been associated with an increasing risk of graft failure.^{185–187}

The standard procedure for treating patients with endothelial disorders involves performing DSAEK using pre-cut tissue from eye banks.¹⁷¹ High-quality pre-cut donor tissue is essential for effective surgery and optimal visual acuity.¹⁸⁴ This shows that also graft preparation and conservation play an important role in the risk of graft failure.

2634 This study aims to investigate the survival outcomes of patients who received pre-cut
2635 Ultrathin DSAEK (UT-DSAEK)^{157,160} lenticules from two distinct eye banks in various
2636 hospitals across Italy, in order to gain a greater understanding of the factors
2637 influencing graft failure occurrence.

2638

MATERIALS AND METHODS

Study Design and Participants

This was a retrospective analysis of outcomes of UT-DSAEK using eye bank-prepared pre-cut tissue performed between January 2021 and October 2024. During the study period, pre-cut UT-DSAEK were prepared by two different eye banks: Fondazione Banca degli Occhi del Veneto (FBOV) and Monza eye bank. All surgeries were performed by expert corneal surgeons in 8 Italian clinics: “ASST Santi Paolo E Carlo Hospital”, Milan, “ASST Spedali Civili di Brescia”, Brescia, “Azienda Ospedaliera Universitaria Integrata”, Verona, “Humanitas Research Hospital”, Rozzano (MI), “Humanitas San Pio X Hospital”, Milan, “San Gerardo Hospital”, Monza, “San Giuseppe Hospital Multimedica”, Milan, and “S. Maria della Misericordia Hospital”, Perugia. The retrospective data collection followed the tenets of the Declaration of Helsinki and was approved by the local Institutional Review Board. We analyzed all patients with uncomplicated DSAEK and for whom preoperative and postoperative data were available.

Variables and data measurements

We examined the medical records of all patients, specifically considering information regarding the best correct visual acuity (BCVA), date of surgery, previous graft, combined surgery, central corneal thickness (CCT), graft thickness, rejection and failure.

Assessment of transplant failure followed the established definitions outlined by the Cornea Preservation Time Study (CPTS).¹⁸ In summary, grafts were deemed unsuccessful if: 1) the study eye underwent re-grafting for any reason; 2) the recipient

cornea exhibited edema on the first postoperative day which did not resolve within 8 weeks; or 3) the recipient cornea was initially clear postoperatively but subsequently became and remained cloudy for 90 days. A graft that failed within the first 8 postoperative weeks was classified as an early failure, instead a graft that failed thereafter was classified as a late failure.²⁶

Image Acquisition and Analysis

Data from AS-OCT scans were analyzed postoperatively at one, three, six, and twelve months where available. Data from the most recent follow-up, if exceeding 12 months post-surgery, was also collected. Pachymetry maps were created, offering corneal thickness data. High-resolution corneal scans focused on the corneal apex were chosen. The interface was manually identified by a corneal specialist, and the central corneal graft thickness was measured at the central point of the selected image using the caliper tool provided by the OCT software.

Graft preparation protocols

Monza eye bank

After arrival at the eye bank, corneas are aseptically isolated and examined by slit-lamp microscopy for abnormalities and screened with corneal specular microscopy (Cell check plus, Konan Medical, CA, USA). Tissues then tissues are stored in organ culture (Tissue-C, Alchimia, IT) for up to 28 days. Donor corneas considered suitable for UT-DSAEK preparation (transparent diameter ≥ 8 mm and scleral rim ≥ 2 mm, ECD ≥ 2500 cells/mm², corneal thickness 450-600 μ m, no opacities deeper than 300 μ m within the optical zone) are evaluated with Trypan Blue and transferred to a transport

medium (Carry-C, Alchimia, IT). After 24 hours, the first microbiological evaluation is conducted on the transport medium. After 48 hours, the corneal pachymetry is evaluated via AS-OCT (MS-39, CSO, IT) by mounting the donor cornea on an OCT adapter under a laminar flow hood. The microkeratome head (One Use-Plus mechanical microkeratome, Moria, FR) is selected by subtracting 150 μm from the AS-OCT measured central pachymetry. The selected microkeratome head is used to perform the cut, and the post-cut thickness is measured again. Precut flaps are considered suitable if their thickness is between 50 and 110 μm . The cap-on, pre-cut UT-DSAEK graft is transferred to a new transport medium (Carry-C) to be transplanted within a maximum of 4 days. A final microbiological check is conducted 24 hours after preparation, before shipment of the graft.

Venice eye bank

Upon arrival at the eye bank, corneas are aseptically isolated and examined by slit-lamp microscopy for abnormalities, then tissues are stored in tissue culture medium (TCM) for up to 28 days.

Suitability for UT-DSAEK is assessed per European and national guidelines¹⁸⁸: central ECD >2000 cells/ mm^2 , monomorphic endothelial mosaic without marked pleomorphism/polymegatism, and no more than minimal endothelial dystrophy, abnormal extracellular matrix deposition, or endothelial degeneration. ECD is estimated as the mean of five counts of cells within a 10 $\times$ 10 mm grid on light microscopy, including cells touching two contiguous sides.¹³⁰

Suitable corneas are placed in fresh TCM + Dextran at room temperature for at least 24 hours to restore physiological thickness and transparency, then mounted on an

2711 artificial anterior chamber (AAC) connected to a bottle filled with TCM positioned 110
2712 cm above and connected with an IV infusion set, resulting in a maximum pressure of
2713 90 mmHg as verified with a Barraquer tonometer (Ocular Instruments Inc.,
2714 Washington, USA). The epithelium is removed with a micro sponge. Guided by AS-
2715 OCT pachymetric maps (Casia SS-1000 OCT, Tomey Corp., Nagoya, Japan), the
2716 appropriate microkeratome head is selected (350 μ m for minimal corneal thickness
2717 >500 μ m, and 300 μ m if <500 μ m). Before cutting, pressure is increased to ~300
2718 mmHg as measured with a digital manometer (PCE-910, PCE Italia s.r.l., Lucca, Italy).
2719 A standardized dissection technique is used to separate the posterior corneal stroma
2720 and endothelium with a single-pass microkeratome head (Evolution-3 system, MORIA
2721 Ophthalmic Instruments, Antony, France). Target graft thickness is $\leq$ 100 μ m, in
2722 accordance with UT-DSAEK parameters. After cutting, the pressure is gradually
2723 restored to baseline values.

2724 After the cut thickness and regularity are confirmed with AS-OCT, and the stromal side
2725 is marked with an "F" outside the optical zone as well as the outer edges of the cut
2726 surface with a dermal pen. The anterior and posterior segments are then realigned.
2727 ECD and endothelial morphology (graded as regular, mild, moderate, altered) are
2728 reassessed. Mortality is estimated using 0,2% trypan blue (Merck KGaA, Darmstadt,
2729 Germany) under light microscopy (Axiovert 25, Carl Zeiss AG, Oberkochen,
2730 Germany).

2731 Prepared grafts are returned to TCM, shipped at room temperature, and transplanted
2732 within 3 days (maximum 4 days in dextran medium).

2733

2734

Statistical analysis

Descriptive statistics were presented as mean (standard deviation), median (25th–75th percentile), or counts (percentages), as appropriate. Comparisons of baseline characteristics between the two eye banks were performed using independent t-tests or Wilcoxon rank-sum tests for continuous variables and Fisher's exact test or chi-square test for categorical variables, as appropriate.

Cumulative incidence of graft failure was calculated as the number of events over the number of eyes at risk. Two-proportions tests were conducted to compare incidence across subgroups. Bonferroni adjustment was used for multiple pairwise comparison. Survival outcomes were assessed using Kaplan–Meier survival curves stratified by eye bank. The log-rank test was used to evaluate statistical differences in survival distributions. A mixed-effects Cox proportional hazards regression model was used to estimate the association between eye bank and risk of graft failure stratified between first UT-DSAEKs and redo grafts. The model included the clinic where surgery was performed ($n = 8$) as a random intercept to account for clustering of outcomes within centers. Other confounding variables included in the model as fixed effects were age at surgery, gender, indication for UT-DSAEK surgery and rebubbling. Rebubbling was included as a covariate since previous studies have shown that rebubbling rates can vary depending on whether tissue is prepared by the eye bank or by the surgeon.¹⁸³ A fixed-effects-only Cox model was also fitted with the same covariates to verify the homogeneity of risk among clinics using the same eye bank tissues.

Comparisons of secondary outcomes (BCVA, ECD, CCT and graft thickness) at each time point were performed using independent pairwise t-tests. Within-group changes from baseline to 12 months were assessed using paired t-tests. Survival data and secondary outcomes were collected at 0, 1-, 3-, 6-, and 12-months post-surgery ± 2

2760 months. Data from the last available follow-up, if greater than 12 months post-surgery,
2761 were also collected. No imputation was performed for missing data.

2762 A p-value < 0.05 was considered statistically significant. All analyses were performed
2763 in R software version 4.4.0 (R Foundation for Statistical Computing, Vienna, Austria)
2764 using the packages *survival*, *coxme*, and *epiR*.

2765

RESULTS

Demographic and clinical characteristics

A total of 379 eyes were included in the study. Precut UT-DSAEK grafts were provided by FBOV in 196 cases, while the Monza eye bank provided 183 tissues. Clinical features and descriptive statistics for the two groups are reported in **Table 7**. The group receiving the Monza graft was older than the group receiving the FBOV graft, while gender distribution was similar between the two groups. The prevalence of previous grafts was similarly distributed.

	FBOV	Monza	P value
Eyes, n	196	183	
Age, years – mean (SD)	67.3 (15.6)	73.7 (9.8)	< 0.001
Females, n (%)	111 (56.6%)	105 (57.4%)	0.96
Males, n (%)	85 (43.4%)	78 (42.6%)	
Follow up, months – median (IQR)	15.9 (8, 22.5)	8.5 (3.4, 13.2)	< 0.001
Diabetes, n (%)	13 (6.6%)	16 (8.7%)	0.56
Glaucoma, n (%)	53 (27%)	22 (12%)	< 0.001
Combined surgery, n (%)	0 (0%)	15 (8.2%)	< 0.001
Rebubbling, n (%)	18 (9.2%)	29 (15.8%)	0.07
Indication, n (%)			0.12
<i>FED</i>	45 (23%)	33 (18%)	0.29
<i>PBK</i>	49 (25%)	46 (25.1%)	1
<i>Redo DSAEK</i>	32 (16.3%)	40 (21.9%)	0.21
<i>Failed PK</i>	18 (9.2%)	24 (13.1%)	0.29
<i>Failed DMEK</i>	0 (0%)	3 (1.6%)	0.22
<i>Not reported</i>	52 (26.5%)	37 (20.2%)	0.18
Clinic, n (%)			
<i>Brescia</i>	0 (0%)	40 (21.9%)	
<i>Milano - Pio X</i>	0 (0%)	16 (8.7%)	
<i>Milano - Rozzano</i>	0 (0%)	20 (10.9%)	
<i>Milano - San Giuseppe</i>	0 (0%)	10 (5.5%)	
<i>Milano - San Paolo</i>	0 (0%)	34 (18.6%)	
<i>Monza</i>	0 (0%)	63 (34.4%)	
<i>Perugia</i>	60 (30.6%)	0 (0%)	
<i>Verona</i>	136 (69.4%)	0 (0%)	

2776 DSAEK, Descemet Stripping Automated Endothelial Keratoplasty; FBOV, Venice Eye Bank Foundation; IQR, Interquartile range;

2777 n, number; PK, penetrating keratoplasty; SD, standard deviation.

2778 ^aT-test; ^bChi-square test; ^cFisher test; ^dMann-Whitney U test.

Graft survival

The cumulative incidence of graft failure was 18.9% in the FBOV group (37/196) and 36.1% in the Monza group (66/183). Early and late graft failure were both more common in the Monza group (both $p < 0.05$). The cumulative incidence was lower in first UT-DSAEKs in FBOV group compared to repeat grafts (13.7% vs 34%, $p = 0.003$) but not in the Monza group (35.1% vs 37.7%, $p = 0.83$). Graft survival outcomes are summarized in **Table 8**.

	FBOV	Monza	P-value
Eyes, n	196	183	
Cumulative incidence of failure, n/tot (%)			
<i>Early (< 8 weeks)</i>	7/196 (3.6%)	19/183 (10.4%)	0.02
<i>Late (> 8 weeks)</i>	30/196 (15.3%)	47/183 (25.7%)	0.02
<i>Year one</i>	23/196 (11.7%)	59/183 (32.2%)	<0.001
<i>DSAEK type</i>			< 0.001
<i>Naïve</i>	20/146 (13.7%)	40/114 (35.1%)	< 0.001
<i>Redo</i>	17/50 (34%)	26/69 (37.7%)	0.83
<i>WG p-value</i>	0.003	0.85	
<i>Center:</i>			
<i>Brescia</i>	-	16/40 (40%)	
<i>Milano - Pio X</i>	-	10/16 (62.5%)	
<i>Milano - Rozzano</i>	-	7/20 (35%)	
<i>Milano - San Giuseppe</i>	-	2/10 (20%)	
<i>Milano - San Paolo</i>	-	13/34 (38.2%)	
<i>Monza</i>	-	18/63 (28.6%)	
<i>Perugia</i>	2/60 (3.3%)	-	
<i>Verona</i>	35/136 (25.7%)	-	
<i>Total</i>	37/196 (18.9%)	66/183 (36.1%)	< 0.001

The Kaplan-Meier curves relative to the survival distribution of tissues provided by the two eye banks are depicted in **Figure 12**. A log-rank test comparing the survival curves highlighted a significant difference in the overall probability of survival of the grafts ($p < 0.001$). Results from a mixed-effects Cox proportional hazards model showed that the risk of failure was over 6 times greater in the Monza group compared to the FBOV group (Hazard Ratio [95% CI], 6.58 [1.72, 25.4], $p = 0.006$).

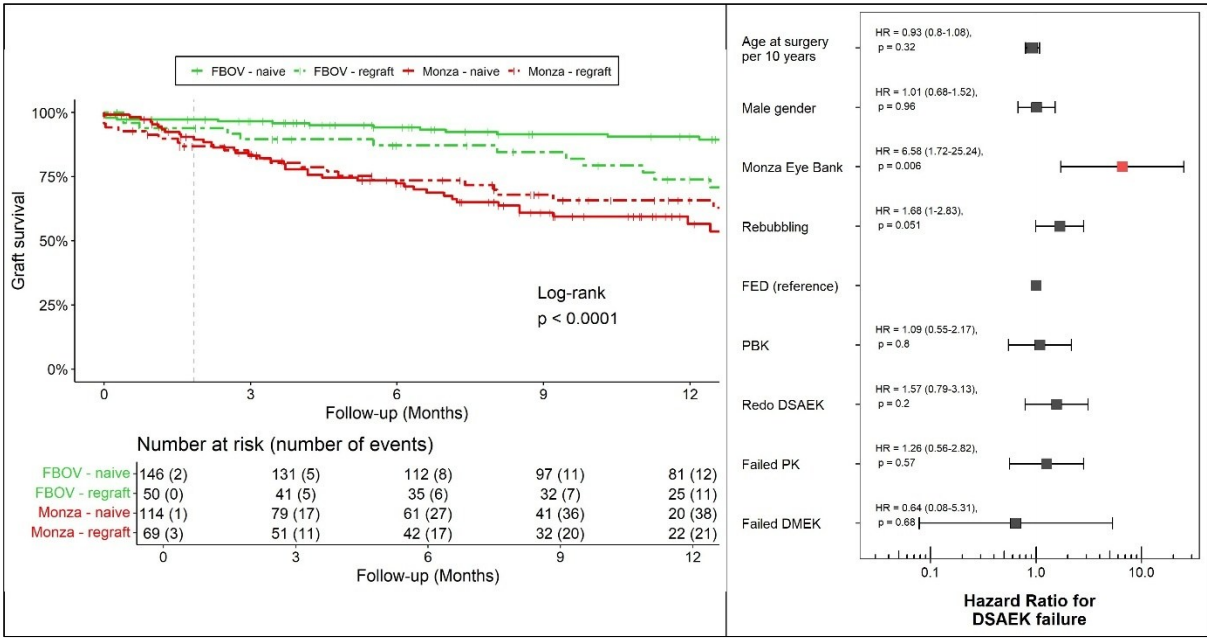


Figure 12. Left panel: Kaplan-Meier curve describing the survival of included UT-DSAEK grafts stratified by eye bank and by number of previous transplants (naïve vs re-grafts). A log-rank test comparing the survival curves highlighted a significant difference in the likelihood of survival among subgroups. The grey dashed line marks 8 weeks post-surgery (early vs late failure). **Right panel:** Hazard Ratios (HRs) from a Cox proportional hazard model for graft survival.

To investigate whether inter-clinic differences could explain a higher failure rate in the Monza group, a Cox model was fitted with the eye clinic where the procedure had been carried out as the main predictor. There were no differences in hazard ratio among the six clinics using tissues from the Monza eye bank (all $p > 0.05$), suggesting low variability of outcomes among centers. Kaplan-Meier survival curves stratified by clinic are reported in **Figure 13**.

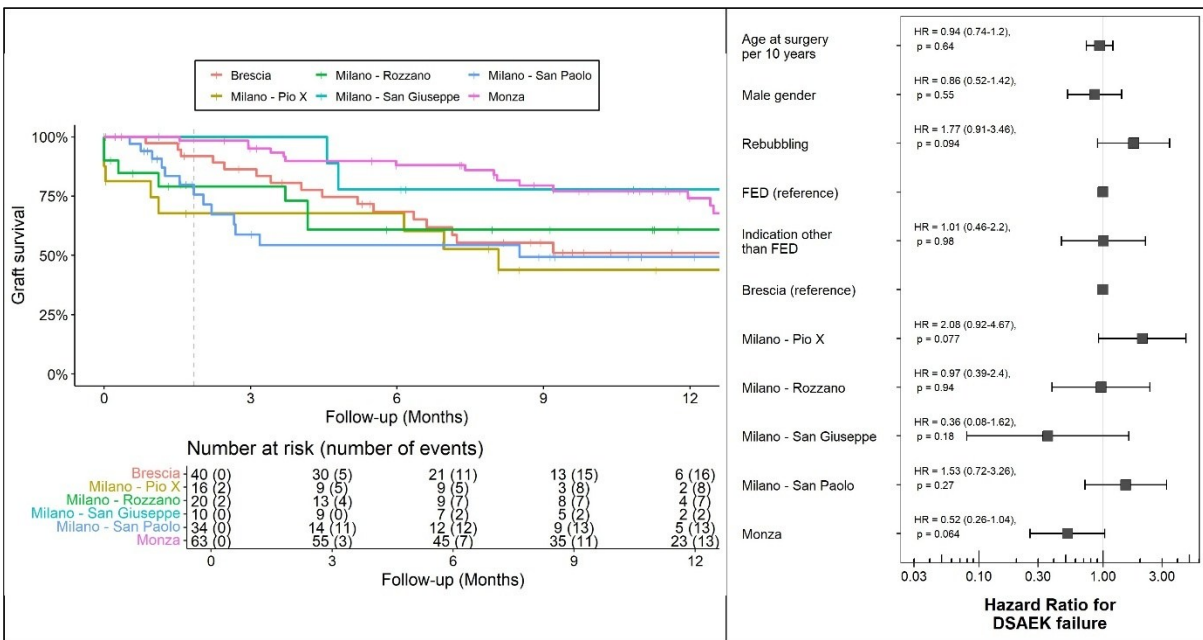


Figure 13. Left panel: Kaplan-Meier curve describing the survival of UT-DSAEK grafts prepared by the Monza eye bank and stratified by the eye clinic where the procedure was carried out. The grey dashed line marks 8 weeks post-surgery (early vs late failure). Right panel: Hazard Ratios (HRs) from a Cox proportional hazard model for graft survival.

Secondary outcomes

Trends in BCVA, ECD, CCT and graft thickness at 0, 1-, 3-, 6- and 12-months after surgery are visualized in **Figure 14**.

2821 Visual acuity at 12 months improved in both groups compared to baseline (paired t-
2822 test, both $p < 0.001$). BCVA was significantly higher in the FBOV group at baseline
2823 and at 1, 3, and 6 months. Final visual acuity was also higher in the FBOV group (mean
2824 [SD], 0.6 [0.7] vs. 0.8 [0.8] LogMAR, $p = 0.01$).

2825 Preoperative ECD counts of donor lenticules were greater in the Monza group (mean
2826 [SD], 2251.8 [261] vs 2547.7 [312.3], $p < 0.001$). However following DSAEK surgery
2827 ECD was higher in the FBOV group at all the other timepoints (all $p < 0.001$).

2828 Postoperative CCT was significantly higher in the Monza group at 3 and 6 months,
2829 however there was no significant difference in final CCT. Graft thickness was higher
2830 in Monza group at 1 month ($p < 0.01$), however final graft thickness was not significantly
2831 different between tissues provided by the two eye banks.

2832

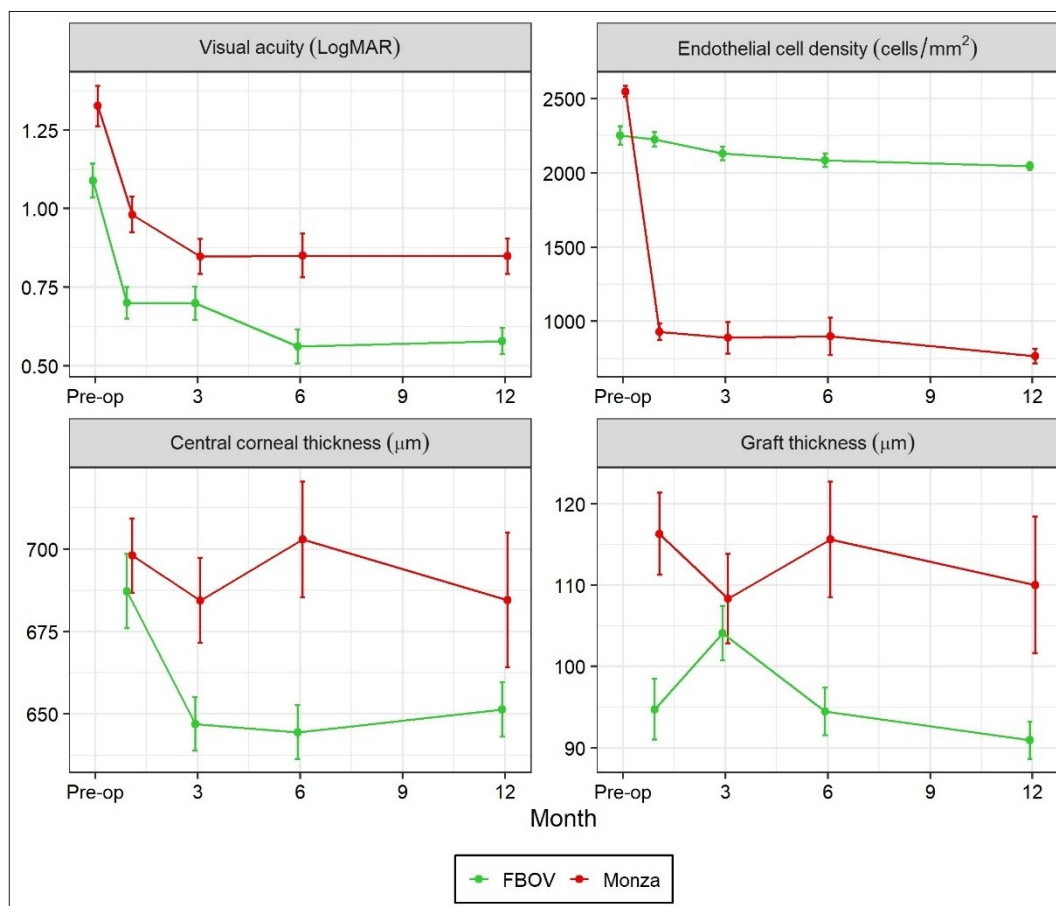


Figure 14. Errorbars displaying the average distribution of secondary outcomes at 0, 1, 3, 6 and 12 months of follow-up after UT-DSAEK surgery.

Secondary outcomes at each timepoint are summarised in **Table 9**.

2840 **Table 9.** Secondary outcomes by tissue provider

	Month	FBOV	Monza	BG p-value
		Mean (SD)	Mean (SD)	
LogMAR	Pre-op	1.1 (0.8)	1.3 (0.8)	0.004
	1	0.7 (0.6)	1 (0.7)	< 0.001
	3	0.7 (0.7)	0.8 (0.6)	0.06
	6	0.6 (0.7)	0.9 (0.7)	0.001
	12	0.6 (0.7)	0.8 (0.8)	0.01
	WG p-value	<0.001	<0.001	
ECD	Pre-op	2251.8 (261)	2547.7 (312.3)	< 0.001
	1	2224.4 (378.3)	930.8 (248.1)	< 0.001
	3	2131 (346.5)	888.7 (509.6)	< 0.001
	6	2083.8 (355.2)	898.4 (419.6)	< 0.001
	12	2044.6 (286)	766.8 (311.7)	< 0.001
	WG p-value	0.07	< 0.001	
CCT	1	687.2 (140.4)	698 (136.7)	0.05
	3	646.9 (107.1)	684.4 (132.1)	0.009
	6	644.5 (104.5)	703 (161.5)	< 0.001
	12	651.4 (129.3)	684.5 (170.7)	0.34
	WG p-value	0.004	0.40	
Graft thickness	1	94.7 (46.2)	116.3 (49)	< 0.001
	3	104.1 (45)	108.3 (44.2)	0.51
	6	94.4 (38.1)	115.6 (50.9)	0.002
	12	90.9 (35.3)	110 (48.1)	0.46
	WG p-value	0.49	0.72	

2841

2842 BG p-value, between-group p-value; CCT, central corneal thickness; ECD, endothelial cell density; FBOV, Venice Eye Bank

2843 Foundation; SD, standard deviation; WG p-value, within-group p-value (paired test).

DISCUSSION

In the present study we examined the survival outcomes of patients who received pre-cut UT-DSAEK lenticules from two separate eye banks in 8 different eye clinics in Italy. We found that the cumulative incidence of graft failure was 18.9% in the FBOV group and 36.1% in the Monza group. At the same time, the risk of failure was over 6 times greater in the Monza group compared to the FBOV group, independently from other risk factors and confounding variables.

Although surgical manipulation during DSAEK, such as graft handling and insertion, can contribute to endothelial cell loss and interface-related complications, potentially impacting graft survival¹⁸⁹, this factor is unlikely to explain the differences observed in our study. All procedures were performed by experienced corneal surgeons using standardized delivery techniques, and the surgical indications were comparable across groups. Likewise, the prevalence of eyes with previous grafts, a known risk factor for adverse outcomes, were evenly distributed between the study cohorts. In particular, in a previous study, the presence of a previously failed PK graft in patients who underwent DSAEK was identified as an independent risk factor for graft detachment.¹⁹⁰ At the same time, repeat grafts exhibit a higher failure rate compared to primary transplants. The reported 12-month failure rates with DSAEK-on-PK ranged from 11.7% to 36%.^{191–194} However, this is unlikely to explain the disparity in our findings, given the similar proportion of repeat grafts across both groups.

Considering corneal graft thickness, Fenech et al. reported that an increase in thickness observed after the first 6 months may indicate early graft decompensation.¹⁹⁵

In the same study, UT-DSAEK grafts showed a progressive thinning rate over 24 months, supporting the concept that graft thickness is a dynamic parameter and may serve as an indirect marker of graft health and survival. In our series, postoperative CCT was higher in the Monza group at 3 and 6 months, however there was no significant difference in CCT between the two groups at the last follow-up. Final graft thickness was also similar between tissues provided by the two eye banks.

In the reviewed literature, primary graft failure was reported as the third most prevalent DSAEK complication, with an average primary graft failure rate of 5% among all published studies and a range of 0% to 29%.¹⁷²

Several variables, including surgical factors and their indications, as well as graft preparation and preservation, have been identified as potential risks for transplant failure.^{177–182} The donor corneal endothelium's short- and long-term health and viability are crucial factors to consider in the surgical management of corneal endothelial disease. The short-term survival of donor corneal tissue is reflected in the primary graft failure rates.¹⁹⁶ Inadequate graft quality, persistent detachment, incorrect positioning, and excessive handling during graft preparation or surgery frequently result in primary graft failure.^{178,179,197} The CPTS study, including eyes receiving DSAEK, found that preoperative ECD was not correlated with endothelial graft failure, however a lower ECD at 6 months postoperatively was strongly associated with late failure.¹⁸ In our investigation, donor ECD was greater in the Monza group while it was higher in the FBOV group at all subsequent timepoints after surgery.

In fact, in this study preoperative donor ECD did not represent a significant risk factor for graft failure, highlighting how specular microscopy may lack the capacity to identify lower quality tissues, whereas manual assessment via light microscopy enables the operator to evaluate a wider endothelial area and to better identify tissues with borderline morphology or insufficient cell density.¹¹ Significantly higher ECD values have been reported when using transmitted light microscopy compared to specular microscopy.^{13,33,198} Furthermore, most clinical non-contact specular microscopes provide a field of view around 0.25–0.5 mm², a clear underrepresentation of pan-corneal endothelial viability.^{33,91,199} As a result, grafts that might be classified as suitable by automated specular microscopy could be excluded when examined manually, potentially contributing to the discrepancy in graft availability and outcomes.

Both eye banks use organ culture preservation methods followed by a deswelling period (24-48h) and transport medium that restores corneal transparency and preserves endothelial viability at room temperature, thus sharing the same preservation method and media composition. Therefore, while differences in survival outcomes were observed, it is unlikely that the preservation medium alone accounts for this disparity, as both approaches are validated and widely adopted in European practice.³⁶

Graft preparation is another critical determinant of DSAEK success, influencing endothelial cell viability, recovery of vision and graft survival. The evolution toward eye bank-prepared tissue offers logistical advantages and promotes procedural standardization. Standardizing graft preparation is particularly challenging with

ultrathin (UT)-DSAEK, which offers better visual outcomes than conventional DSAEK, but requires a more precise and reproducible graft preparation protocol. Achieving consistent UT grafts ($<100\text{ }\mu\text{m}$) depends on controlled dehydration and optimized intracameral pressure during the microkeratome pass.¹⁶⁰ As shown by Romano et al., variability in tissue dehydration, artificial anterior chamber (AAC) pressure, and timing can dramatically influence thickness reproducibility.²⁰⁰ Studies have shown that methods using a single microkeratome pass with peripheral manual dissection can reliably yield ultrathin grafts.¹⁶⁰ Another key variable in graft preparation is the pressure within the AAC. The use of an innovative ACP (Artificial Chamber Pressuriser) system allows consistent and elevated pressure (200 mmHg), which accelerates corneal thinning and enhances the reliability of ultrathin graft creation without compromising endothelial viability. In this context, high pressure ensures controlled dehydration and flatter stromal profiles, promoting smoother microkeratome passes. Data from Ruzza et al. demonstrated that maintaining 200 mmHg intracameral pressure achieves thinning rates over $25\text{ }\mu\text{m}/\text{min}$ and a graft thickness of around $83\text{ }\mu\text{m}$, with excellent clinical outcomes and no increase in endothelial cell loss or apoptosis.^{157,201} A UK-based study using tissue from the Fondazione Banca degli Occhi del Veneto reported excellent endothelial survival with large-diameter, preloaded UT grafts.²⁰² Similarly, comparisons between preloaded DMEK and UT-DSAEK found UT-DSAEK can reach outcomes as good as those of DMEK, reinforcing its practical value in certain clinical settings.^{131,203} To optimize outcomes, it is imperative that we implement standardized, transparent, and auditable protocols. Metrics such as microkeratome pass speed, pre- and post-cut thickness, intracameral pressure records, and endothelial viability assessments should become standard quality indicators. The preparation step, whether executed in the operating room or eye bank, remains the linchpin of

successful endothelial keratoplasty. The responsibility for the graft preparation learning curve has progressively expanded upstream to include eye banks, where standardization and expertise are essential. These insights emphasize the importance of harmonizing protocols to include precise trephination, dehydration techniques, microkeratome parameters, and high-pressure systems when aiming for UT-DSAEK grafts.

This study has several limitations. Importantly, although we observed a strong association between eye bank provenance and graft survival, the retrospective and observational nature of this study precludes definitive causal inference regarding supply-chain–related effects. The comparison reflects real-world practice rather than a randomized allocation of tissues, and therefore residual confounding cannot be excluded. While our mixed-effects Cox model adjusted for key demographic and clinical covariates and accounted for clustering at the center level, several potentially relevant variables were not captured or could not be fully standardized. These include unmeasured aspects of tissue processing (e.g., timing variability within preservation windows, intra-bank handling nuances, operator-dependent microkeratome dynamics), subtle differences in storage duration prior to cutting and prior to transplantation, and detailed transport conditions. A retrospective design implies potential risks of selection bias and insufficient data collecting. Variations in clinical decisions, surgical techniques, and postoperative treatment among surgeons and over time may have influenced graft survival outcomes. Likewise, surgeon-level factors—such as graft unfolding technique, intraoperative manipulation time, centration accuracy, and postoperative management strategies—were not granularly quantified and may vary despite comparable surgical expertise. Center-level practices, including

thresholds for rebubbling, postoperative steroid regimens, and criteria for defining or reporting failure in borderline cases, may also contribute to outcome heterogeneity. Consequently, although the association between graft source and outcomes is robust and clinically meaningful, it should not be interpreted as direct proof that specific supply-chain steps independently caused the observed differences. Prospective studies with harmonized perioperative protocols, standardized reporting of processing metrics, and ideally inter-center crossover use of tissues from multiple eye banks would be required to more definitively isolate causal effects within the preparation and distribution pathway.

Strengths of this study lie in the multicentre collection of a large case series of UT-DSAEK surgeries, closely reflecting the real-world outcomes and graft survival observed by multiple surgeons facing a wide array of indications for endothelial keratoplasty.

In conclusion, our study suggests that differences in donor tissue preparation, processing, and quality assurance may influence transplant survival. These findings underscore the importance of developing shared, standardized protocols and strengthening quality control measures in the preparation of pre-cut UT-DSAEK. Collaborative efforts between eye banks and surgical centers will be essential to further optimize tissue quality and, ultimately, surgical outcomes for patients.

Chapter 11

CONCLUSIONS AND FUTURE PERSPECTIVES

The success of corneal transplantation, particularly endothelial keratoplasty (EK), is fundamentally dependent on the health and viability of the donor endothelial cells. Endothelial Cell Density (ECD) is the primary, and often the sole, metric for determining the suitability of donor tissue for transplantation. However, ECD is an imperfect proxy; as it assesses only a small fraction of the endothelial surface and frequently overestimates the true functional capacity of the graft. This limitation creates a critical "blind spot" in the quality assurance chain, where grafts with high nominal cell counts but significant occult damage may be cleared for surgery, potentially leading to primary or early graft failure.

The general aim of the body of work presented in this dissertation was to bridge this gap by developing, validating, and implementing the V-CHECK (Viability Control of Human Endothelial Cells before Keratoplasty) method for corneal endothelial assessment. This method was designed to provide surgeons and eye banks with an accessible, independent, and objective tool for pan-corneal endothelial viability evaluation. This final chapter synthesizes the evidence presented in the preceding studies, highlighting the transition from a theoretical protocol to a clinically validated tool capable of predicting graft survival, and outlines the future trajectory for this research.

Summary of Key Findings

The V-CHECK study protocol

The foundation of this dissertation was the establishment of a robust protocol for visualizing endothelial damage and the institution of a large, multicentric cohort of patients for whom adequate imaging was available (*Chapter 5*). As detailed in the V-CHECK study protocol and validation papers, the use of 0.05% Trypan Blue (TB) for a short duration (30–45 seconds) proved effective in staining determining areas of endothelial mortality and denuded Descemet membrane without inducing toxicity, allowing a quick and reproducible evaluation of endothelial status.

Validation of a Novel Metric

The validation study (*Chapter 6*) demonstrated that the V-CHECK method, powered by a deep-learning segmentation foundation model (Segment Anything Model - SAM) and traditional computer vision algorithms, achieved high reproducibility and strong correlation with expert manual assessment (Pearson's $\rho \sim 0.8$). Crucially, the automated V-CHECK analysis consistently reported higher mortality estimates (approximately 6.5% to 9.5% higher) than standard manual counting.]This confirms the hypothesis that traditional methods, which often rely on counting cells in a small central window or subjective visual inspection, systematically underestimate the true extent of endothelial damage. By analyzing the entire graft surface, the V-CHECK method provides a more rigorous "stress test" of tissue quality, ensuring that damage located on the periphery or folds—often missed by specular microscopy—is accounted for.

3033 *The "Fingerprint" of Graft Failure*

3034 The clinical relevance of pan-corneal endothelial assessment was illustrated in a
3035 clinical study involving eyes undergoing Ultrathin Descemet Stripping Automated
3036 Endothelial Keratoplasty (UT-DSAEK) at the Eye Clinic of the Spedali Civili of Brescia
3037 (*Chapter 7*). The application of the V-CHECK method revealed a strong association of
3038 pan-corneal endothelial mortality with risk of early graft failure. We also described a
3039 previously unreported morphological marker of endothelial damage that became
3040 evidence through TB staining, a "fingerprint-like" staining pattern characterized by
3041 concentric or adjacent linear ridges of endothelial cell loss.

3042

3043 This pattern, present in 90% of the analyzed precut grafts, all coming from the same
3044 eye bank, was significantly associated with elevated endothelial mortality (mean
3045 16.8%) compared to grafts without the pattern (mean 2.2%), and was strongly
3046 predictive of failure. The cumulative incidence of graft failure in this cohort was
3047 unusually high (44.4%), and a Cox proportional hazard model confirmed that
3048 preoperative V-CHECK mortality was a significant predictor of early failure (Hazard
3049 Ratio = 1.26 for every 1% increase in mortality) This finding served as a proof-of-
3050 concept that pan-corneal dye assessments can identify high-risk grafts that standard
3051 eye bank screenings (ECD and slit-lamp exams) might have failed to detect.

3052

3053 *Real-World Implications of Supply Chain Variability*

3054 A multicenter survival analysis (*Chapter 8*) placed these findings into a broader
3055 context. By comparing outcomes of UT-DSAEK grafts from two major Italian eye banks

across 379 patients, the study uncovered a profound disparity in graft survival (18.9% failure rate for FBOV vs. 36.1% for Monza Eye Bank).

Despite comparable surgeon expertise and patient demographics, grafts from the Monza bank had a 6.58-times higher risk of failure. While both banks adhered to European safety standards, we hypothesize that slight differences in preparation protocols — possibly regarding microkeratome cutting pressure, dehydration, or quality control steps — might have resulted in varying degrees of endothelial trauma. The fingerprint-like staining pattern observed in *Chapter 7* is likely a manifestation of such preparation-induced stress. This study underscores that without standardized, rigorous preoperative viability assessments like V-CHECK, systemic issues in graft preparation can persist undetected, directly impacting patient vision and graft longevity.

Implications for Clinical Practice and Eye Banking

The findings of this dissertation argue for a paradigm shift in how donor tissue is evaluated.

For the Corneal Surgeon

Surgeons need to actively evaluate grafts before implantation, and the V-CHECK method empowers the surgical team to perform an independent "final check" in the operating theatre. Identifying a fingerprint pattern or high mortality stain allows the

surgeon to reject a suboptimal graft before implantation, mitigating the risks for the patient of graft failure, need for repeated surgeries and likely suboptimal outcomes.

For Eye Banks

The dissociation between high ECD values and high actual mortality rates in failed grafts indicates that cell density alone is an insufficient metric for both processed and unprocessed corneal grafts. Eye banks could benefit from an integration of pan-corneal viability assessments into their quality reports. Furthermore, the identification of preparation-related damage patterns might highlight the need of a re-evaluation of mechanical cutting protocols for UT-DSAEK.

Limitations and Future Directions

While this work establishes the V-CHECK method as a valuable tool, several avenues for refinement and expansion remain.

Automation and AI Refinement:

The current V-CHECK analysis uses a semi-automated pipeline requiring some user input (prompts) for the Segment Anything Model. Future research should focus on fine-tuning these models (e.g., using Conv-LoRA techniques) to create a fully automated "one-click" application. This would facilitate its integration into surgical or eye bank workflows, providing real-time feedback on tissue viability.

3100 *Investigating of the fingerprint-like staining mechanism*

3101 The precise mechanical cause of the fingerprint staining remains hypothetical. *Ex-vivo*
3102 laboratory studies are needed to replicate this pattern by varying microkeratome
3103 speeds, blade oscillation frequencies, and intracameral pressures. Standardized
3104 donor tissue models could systematically manipulate mechanical factors such as
3105 artificial anterior chamber pressure, microkeratome dynamics, and lamellar handling,
3106 as well as osmotic conditions during deswelling and exposure to vital dyes, to quantify
3107 their independent effects on endothelial viability. Such designs would minimize clinical
3108 confounding and allow precise control of hydration state and cutting parameters.
3109 Complementary histological and ultrastructural analyses—including light microscopy,
3110 transmission electron microscopy, and apoptosis assays—could determine whether
3111 subclinical endothelial trauma precedes clinically evident failure. Understanding the
3112 exact mechanism will allow eye bank technicians to employ safer cutting protocols.
3113 Ultimately, prospective multicenter studies using harmonized preparation protocols,
3114 ideally incorporating crossover use of tissues across institutions, will be required to
3115 disentangle preparation-specific effects from center- or surgeon-level influences.

3116

3117 *Results from the full V-CHECK cohort*

3118 Correlating laboratory findings with perioperative endothelial evaluation, postoperative
3119 endothelial cell density trends and keratoplasty outcomes will strengthen mechanistic
3120 inference and tie the project together. The full V-CHECK cohort analysis is the crucial
3121 next step, providing the robust statistical evidence needed to transform and integrate
3122 the quality assurance standards. This large, multicenter dataset will correlate
3123 preoperative pan-corneal viability (TBPA) with 12-month outcomes, allowing us to

3124 establish objective, clinically relevant thresholds for graft rejection based on pan-
3125 corneal endothelial viability assessment. Ultimately, the results will enable eye banks
3126 and surgeons to move beyond insufficient ECD metrics, implementing a definitive
3127 safety check that minimizes early graft failure and improves patient outcomes.

3128

3129 *Implementation and medico-legal considerations*

3130 Implementation of enhanced endothelial assessment strategies must also consider
3131 regulatory, organizational, and medico-legal factors. Integration within national and
3132 European tissue governance frameworks should complement existing accreditation
3133 systems while improving auditability through standardized reporting of preparation and
3134 viability metrics. Practical barriers may include modest increases in operative time,
3135 additional training requirements, and the need for workflow integration in high-volume
3136 settings. Clear protocols for image acquisition, interpretation, and documentation will
3137 be essential to ensure consistency.

3138 If surgeons adopt structured assessment tools that occasionally lead to rejection of
3139 eye bank–certified grafts, explicit policies defining acceptance criteria and shared
3140 accountability will be required. Strengthened collaboration between eye banks and
3141 surgical teams should be viewed as a quality-enhancement measure rather than a
3142 transfer of liability. Together, rigorous experimental validation and structured
3143 implementation planning will be essential to support causal interpretation while
3144 maintaining feasibility and regulatory coherence.

3145

3146 **Conclusion**

3147 By validating a simple and reproducible method to visualize and quantify pan-corneal
3148 endothelial damage, the presented work supports the use of the V-CHECK method as
3149 a safety net for corneal transplantation. This dissertation validates the use of deep
3150 learning to standardize tissue evaluation and highlights the urgent need for
3151 harmonized preparation protocols across eye banks. Ultimately, the integration of the
3152 V-CHECK method into routine practice promises to transform the "black box" of donor
3153 quality into a transparent, data-driven process, ensuring that every patient receives a
3154 graft with the highest probability of restoring their sight.

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