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Rīga Stradiņš University

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Descemet Membrane Endothelial Keratoplasty: NGS Metagenomics-Optimized Tissue Preparation and Surgical Technique

Doctoral Thesis for obtaining a doctoral degree
"Doctor of Science (*Ph.D.*)"

Sector – Clinical Medicine
Sub-Sector – Ophthalmology

Riga, 2021

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Abbreviations

16S	16 Svedberg
18S	18 Svedberg
AC	Anterior Chamber
ANOVA	Analysis of Variance
BSS	Balanced Salt Solution
C	Calcein AM
C3F8	Perfluoropropane
CDM	Conventional Diagnostic Method
DE	Dry Eye
DMEK	Descemet Membrane Endothelial Keratoplasty
DMD	Descemet Membrane Detachment
DNA	DeoxyriboNucleic Acid
DNA-seq	DNA Sequencing
DSAEK	Descemet Stripping Automated Endothelial Keratoplasty
dsDNA	Qubit double-stranded DNA
E	Ethidium Homod imer
ECL	Endothelial Cell Loss
FBOV	Venice Eye Bank Foundation
gDNA	genomic DeoxyriboNucleic Acid
GDRB	Graft Detachment and Re-Bubbling rate
GMP	Good Manufacturing Practices
H	Hoechst 33342
HIV	Human Immunodeficiency Virus
HBV	Hepatitis B Virus
HEC	Hoechst / Ethidium / Calcien
HMP	Human Microbiome Project
HST	Horseshoe Shaped Tear
ITS	Internal Transcribed Spacer
MGD	Meibomian Gland Dysfunction
NGS	Next Generation Sequencing
OC	Organ Culture
OCT	Optical Coherence Tomography
OS	Ocular Surface
OUT	Operational Taxonomic Unit
P	P-value

PBDS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PKP	Penetrating KeratoPlasty
RNA	RiboNucleic Acid
rRNA	ribosomial RiboNucleic Acid
RT	Room Temperature
SAV	Illumina Sequence Analysis Viewer
SF6	Sulfur Hexafluoride
SS	Sjögren Syndrome
SubHyS	Submerged Hydro-Separation
TBPCs	Trypan Blue-Positive Cells
TCM	Tissue Culture Medium
TS	Target-Specific
UK	United Kingdom
USA	United States of America
VA	Visual Acuity
ZO-1	Zonula Occludens-1

Introduction

Individual contribution

The author of this work has taken part in all stages of the studies, has performed the metagenomics analysis and DMEK procedures. He has acquired scientific data and completed statistical analysis. The author has written all this work.

Ethical aspects

The research work was performed in accordance with Helsinki declaration and the permission of Rīga Stradiņš University Ethics Committee No. 49/26.11.2015 and No. 29/29.09.2016.

Volume of the Doctoral thesis

The Doctoral Thesis was written in English. Background and Abstracts were written in English and Latvian. It consists of five chapters: introduction, background of literature, novelties, the studies and conclusions. The list of references consists of 112 sources. The volume of the Doctoral Thesis covers 124 pages, including 9 tables, 28 figures and 7 Studies. The Doctoral Thesis is supported by 30 indexed publications.

Structure of Doctoral thesis

Hypothesis of the study

DMEK surgery with NGS metagenomics-optimized tissue preparation and modified stripping technique has better outcomes than standard DMEK surgery and CDM.

Aim of the study

To improve outcomes of DMEK surgery.

Tasks of the study

1. To improve the storage media of human donor corneas with the use of NGS.
2. To evaluate the role of metagenomics in the preservation of donated cornea.
3. To create an improved protocol for DMEK graft preparation.
4. To compare different DMEK graft preparation methods.
5. To evaluate the efficacy, safety, preparation time, ECL and failure rate of a novel technique to prepare DMEK grafts.
6. To evaluate if the speed of stripping a DMEK graft influences the graft scroll width.
7. To evaluate the rebubbling rate and its management.

Innovations

1. NGS provides a full taxonomic profiling and delivers a wider microbiological diagnostic approach than CDM.
2. Metagenomics improves preservation of donated cornea.
3. The new protocol for DMEK graft preparation would help new surgeons to decide the instruments and improve their tissue preparation skills also in challenging cases.
4. Graft preparation using Sinskey hook (M2 technique) and donor punch (M3 technique) are reliable methods in terms of efficiency and quality with acceptable range of ECL.
5. This new DMEK graft preparation technique provide shorter preparation time and low failure rates independent of surgeon's experience level.
6. Slow peeled DMEK grafts result in a wider scroll width but are associated with a greater reduction in ECD.
7. Early rebubbling of the graft has better visual outcomes and limits corneal scarring.

Publications

The PhD thesis is based on the following ten manuscripts:

1. **Borroni D.**, Romano V., Kaye S.B., Somerville T., Napoli L., Fasolo A., Gallon P., Ponzin D., Esposito A., Ferrari S. Metagenomics in ophthalmology: current findings and future prospectives. *BMJ Open Ophthalmol*. 2019 Jun 4; 4(1): e000248. doi: 10.1136/bmjophth-2018-000248.
2. **Borroni D.,** Parekh M., Romano V., Kaye S.B., Camposampiero D., Ponzin D., Ferrari S. Next-generation sequencing for the detection of microorganisms present in human donor corneal preservation medium. *BMJ Open Ophthalmol*. 2019 Apr 20; 4(1): e000246. doi: 10.1136/bmjophth-2018-000246.
3. Parekh M., **Borroni D.**, Ruzza A., Levis H.J., Ferrari S., Ponzin D., Romano V. A comparative study on different Descemet membrane endothelial keratoplasty graft preparation techniques. *Acta Ophthalmol*. 2018 Sep; 96(6): e718-e726. doi: 10.1111/aos.13746.
4. Parekh M., Baruzzo M., Favaro E., **Borroni D.**, Ferrari S., Ponzin D., Ruzza A. Standardizing Descemet Membrane Endothelial Keratoplasty Graft Preparation Method in the Eye Bank-Experience of 527 Descemet Membrane Endothelial Keratoplasty Tissues. *Cornea*. 2017 Dec; 36(12): 1458–1466. doi: 10.1097/ICO.0000000000001349.
5. Parekh M., Leon P., Ruzza A., **Borroni D.**, Ferrari S., Ponzin D., Romano V. Graft detachment and rebubbling rate in Descemet membrane endothelial keratoplasty. *Surv Ophthalmol*. 2018 Mar–Apr; 63(2): 245–250. doi: 10.1016/j.survophthal.2017.07.003. Epub 2017 Jul 22.
6. Tzamalis A., Vinciguerra R., Romano V., Arbabi E., **Borroni D.**, Wojcik G., Ferrari S., Ziakas N., Kaye S. The "Yogurt" Technique for Descemet Membrane Endothelial Keratoplasty Graft Preparation: A Novel Quick and Safe Method for Both Inexperienced and Senior Surgeons. *Cornea*. 2020 Sep; 39(9): 1190–1195. doi: 10.1097/ICO.0000000000002401.
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8. **Borroni D.**, Gadhvi K., Wojcik G., Pennisi F., Vallabh N.A., Galeone A., Ruzza A., Arbabi E., Menassa N., Kaye S., Ponzin D., Ferrari S., Romano V. The Influence of Speed During Stripping in Descemet Membrane Endothelial Keratoplasty Tissue Preparation. *Cornea*. 2020 Sep; 39(9): 1086–1090. doi: 10.1097/ICO.0000000000002338.
9. **Borroni D.**, Rocha de Lossada C., Parekh M., Gadhvi K., Bonzano C., Romano V., Levis H.J., Tzamalis A., Steger B., Rechichi M., Rodriguez-Calvo-de-Mora M. Tips, Tricks, and Guides in Descemet Membrane Endothelial Keratoplasty Learning Curve. *Journal of Ophthalmology*. 2021. Article ID 1819454. doi.org/10.1155/2021/1819454
10. Rocha-de-Lossada C., Rachwani-Anil R., **Borroni D.**, Sánchez-González J.M., Esteves-Marques R., Soler-Ferrández F.L., Gegúndez-Fernández J.A., Romano V., Livny E., Rodríguez Calvo-de-Mora M. New Horizons in the Treatment of Corneal Endothelial Dysfunction. *J Ophthalmol*. 2021 Jul 9; 2021:6644114. doi: 10.1155/2021/6644114

Background of the study

Descemet membrane endothelial keratoplasty (DMEK) has gained popularity for many surgeons to treat specific corneal endothelial failure cases. DMEK offers early rehabilitation with optimal visual acuity (VA). Its minimal invasive nature and low immunological rejection rate make this technique favourable for surgeons. Even with these advantages, the technique still requires refinement in terms of tissue preservation, graft preparation, delivery, unfolding and positioning in the recipient eye.

DMEK tissue preservation could be improved with Metagenomic analysis, a diagnostic approach that was originally associated with the studies of genetic material collected directly from environmental samples. Currently only pre-specified pathogens can be detected by conventional culture-based techniques or Polymerase Chain Reaction (PCR), but there are conditions to state whether metagenomics could revolutionize the diagnosis of ocular diseases.

New and improved donor preparation techniques like prestripped DMEK tissues are being taken up rapidly because of less manipulation that is required in the surgical theatre. Donor graft delivery in the recipient eye has also been improved because of new products like prestripped and preloaded membranes. As DMEK is at its budding stage, only early outcomes have been known so far. Early graft detachment, rebubbling rates, and primary failures are still being studied in DMEK. Graft detachment and rebubbling rates have been a huge challenge both during the surgery and also while reporting postoperative data. We highlight the

importance of defining graft detachment and rebubbling rates and their surgical relevance, which may also have an impact on graft preparation and insertion techniques.

The PhD thesis starts from the current concepts of DMEK surgery analyzing the potential improvements from the eye banking side to the surgical applications and innovations. It concludes with tips and tricks for new surgeons.

Pētījuma priekšvēsture

Descemeta membrānas endotēlija keratoplastika (DMEK) ir ieguvusi popularitāti daudzu ķirurgu vidū, īpaši radzenes endotēlija mazspējas gadījumu ārstēšanā. DMEK nodrošina agrīnu rehabilitāciju ar optimālu redzes asumu. Ķirurgi izvēlas šo metodi minimālās invazivitātes un zemā imunoloģiskā noraidīšanas līmeņa dēļ, taču, pat ņemot vērā šīs priekšrocības, tehnika joprojām prasa pilnveidošanu attiecībā uz audu un transplantāta sagatavošanu, piegādi, izvēršanu un ievietošanu recipienta acī.

DMEK audu saglabāšanu varētu uzlabot ar metagenomisko analīzi – diagnostisku pieeju, kas sākotnēji bija saistīta ar ģenētiskā materiāla pētījumiem, kas savākti tieši no vides paraugiem. Pašlaik var noteikt tikai iepriekš zināmus patogēnus, izmantojot parastās mikroorganismu kultūras metodes vai polimerāzes ķēdes reakciju (PĶR), taču ir iespējas noskaidrot, vai metagenomika varētu mainīt oftalmoloģisko slimību diagnostiku.

Jaunas un uzlabotas donoru sagatavošanas metodes, piemēram, iepriekš paņemti DMEK audi, tiek ātri izmantotas, tādējādi operāciju zālē ir jāveic mazāk manipulāciju. Donoru transplantāta piegāde saņēmēja acī ir uzlabojusies arī tādu jaunu produktu dēļ kā, piemēram, iepriekš noņemtas un ieliktas membrānas. Tā kā DMEK ir izpētes sākuma stadijā, līdz šim ir pieejami tikai agrīni rezultāti. Agrīna transplantāta atdalīšanās, atkārtotas gaisa pūslīšu ievades biežums un primārās neveiksmes joprojām tiek pētītas saistībā ar šo metodi. Transplantāta atdalīšanās un atkārtotas gaisa pūslīšu ievades biežums ir bijis milzīgs izaicinājums gan operāciju laikā, gan ziņojot par pēcoperāciju datiem. Mēs uzsveram transplantāta atdalīšanās un atkārtotas gaisa pūslīšu ievades ātruma noteikšanas nozīmi, tai skaitā, to ķirurģisko nozīmi, kas var ietekmēt arī transplantāta sagatavošanas un ievietošanas paņēmienus.

Promocijas darba pirmā puse ietver pašreizējos DMEK ķirurģijas priekšstatus, analizē potenciālos uzlabojumus, sākot no acu bankas līdz ķirurģiskam pielietojumam un novitātēm, savukārt darba otrajā daļā ir aprakstīti padomi jaunajiem ķirurgiem.

Novelties

1. Metagenomic deep sequencing has the potential to improve the microbiological analysis of samples starting from low concentrations. The current study is the first in the literature to apply Metagenomics analysis in corneal banking.
2. Corneal donor tissues have previously been prepared with 9.5 mm diameter. Considering pneumatic dissection or liquid separation the graft size can easily be obtained up to 10 mm, a novelty using our protocol. We prepare the tissues with 9.5 mm diameter in the eye bank and the final diameter can be decided by the surgeon based on the size of the descemetorhexis or the eye.
3. Stripping techniques with epithelial spatula and Sinskey hooks have served the best in terms of graft quality and economic feasibility. From this study, we have observed that M2, tissue preparation using Sinskey hook, could serve as the best graft preparation method considering all the parameters that include cell death, endothelial cell loss, time required to prepare the graft and costs.
4. The novel Yogurt DMEK technique by means of a guarded hinge punch, resembling
   the opening of a yogurt cup, seems to be an easy, quick, efficient, and safe method to prepare DMEK grafts independently of surgeon's experience level.
5. A slow stripped DMEK graft is associated with a wider graft scroll conformation.

Novitātes

1. Metagenomiskā dziļā sekvencēšana var uzlabot paraugu mikrobioloģisko analīzi, sākot no zemām koncentrācijām. Pašreizējais pētījums ir pirmais pieejamais literatūrā, kurā metagenomikas analīze tika pielietota radzenes bankās.
2. Iepriekš sagatavoti radzenes donoru audi 9,5 mm diametrā. Ņemot vērā pneimatisko sadalīšanu vai šķidruma atdalīšanu, var viegli iegūt transplantātu izmērā līdz 10 mm, – novitāte, kas ir iespējama, izmantojot mūsu protokolu. Acu bankā tiek sagatavoti audi 9,5 mm diametrā; nepieciešamo diametru izvēlas ķirurgs, pamatojoties uz *descemetorhexis* vai acs izmēru.
3. Noņemšanas paņēmieni ar epitēlija lāpstiņu un *Sinskey* āķiem ir kalpojuši vislabāk transplantātu kvalitātes un ekonomiskās pieejamības ziņā. Pētījumā tiek secināts, ka M2, audu sagatavošana, izmantojot *Sinskey* āķi, varētu kalpot kā labākā transplantāta sagatavošanas metode, ņemot vērā visus parametrus, kas ietver šūnu

bojāeju, endotēlija šūnu zudumu, transplantāta sagatavošanai nepieciešamo laiku un izmaksas.

4. Jaunā DMEK *Yogurt* tehnika, izmantojot transplantātam drošu viras perforatoru, kas līdzinās jogurta trauka atvērumam, šķiet viegla, ātra, efektīva un droša metode, lai sagatavotu DMEK transplantātus neatkarīgi no ķirurga pieredzes līmeņa.
5. Lēni ņemts DMEK transplantāts ir saistīts ar plašāku transplantāta konformāciju.

1 Study 1

The first study analyses the optimizations that metagenomics application can have on corneal tissue preservations.

Abstract in English

Title: Next-generation sequencing for the detection of microorganisms present in human donor corneal preservation medium.

Aim: To detect the presence of microorganisms in the storage media of human donor corneas using next-generation sequencing method.

Methods: Seven samples from organ culture (OC) group (Cornea Max, Eurobio, Les Ulis, France) with one control (sterile media without any cornea) and seven samples from hypothermic storage group (Cornea Cold, Eurobio) with one control were used for this study. The corneas were placed in the respective storage media for 14 days before collecting the samples. Storage media (2 mL) from each sample were collected in RNAase-free tubes and shipped for ribosomal RNA sequencing of 16S and 18S. Simultaneously, another 1 mL of media sample was used for conventional diagnostic method (CDM) using Bactec instruments.

Results: In both, OC and hypothermic storage and control samples, the most abundant genera were *Pseudomonas, Comamonas, Stenotrophomonas, Alcanivorax, Brevundimonas* and *Nitrobacter*. *Acidovorax, Acetobacter* and *Hydrogenophilus* were detected mostly in the hypothermic storage group. The most abundant fungal pathogen detected belonged to the genus *Malassezia*, which was found in both the storage conditions. CDM was negative for microorganisms in all the samples.

Conclusion: Metagenomics provides full taxonomic profiling of the detected genomic material of the organisms and thus has the potential to deliver a much wider microbiological diagnostic approach than CDM. The costs and turnaround time need to be reduced, and; the detection of viable organisms would help this technology to be introduced into routine clinical practice.

Keywords: 16S; 18S; NGS; bacteria; cornea; eye bank; fungus; media; microbiology; preservation; storage.

Abstract in Latvian

Pētījumā tika analizēta optimizācija, pielietojot metagenomiku radzenes audu saglabāšanā.

Nosaukums: Nākamās paaudzes sekvencēšana mikroorganismu noteikšanai cilvēka donora radzenes audu saglabāšanas vidē.

Mērķis: Ar nākamās paaudzes sekvencēšanas metodi noteikt mikroorganismu klātbūtni cilvēka donora radzenes audu uzglabāšanas vidē.

Materiāli un metodes: Kopā tika izmantoti septiņi paraugi no orgānu kultūras (no angļu val. – *organ culture* (OC)) grupas (*Cornea Max, Eurobio, Les Ulis*, Francija) ar vienu kontroli (sterila barotne bez radzenes audiem) un septiņi paraugi no hipotermiskās uzglabāšanas grupas (*Cornea Cold, Eurobio*) ar vienu kontroli. Pirms paraugu savākšanas radzenes audus ievietoja attiecīgajās uzglabāšanas vietās uz 14 dienām. Uzglabāšanas vide (2 ml) no katra parauga tika ievietota mēģenēs, kas nesatur RNS-āzi, un tika nosūtīta 16S un 18S ribosomu RNS sekvencēšanai. Vienlaikus 1 ml barotnes parauga tika izmantots konvencionālajai diagnostikas metodei (no angļu val. – *conventional diagnostic method* (CDM)), izmantojot *Bactec* instrumentus.

Rezultāti: Gan OC, gan hipotermiskās uzglabāšanas un kontroles paraugos visbiežāk novēroja *Pseudomonas, Comamonas, Stenotrophomonas, Alcanivorax, Brevundimonas* un *Nitrobacter* ģintis. *Acidovorax, Acetobacter* un *Hydrogenophilus* ģintis tika atklātas galvenokārt hipotermiskās uzglabāšanas grupā. Visbiežāk konstatētais sēņu patogēns piederēja *Malassezia* ģintij, kas tika atrasts abās uzglabāšanas vidēs. Ar CDM visi paraugi bija negatīvi attiecībā uz mikroorganismiem.

Secinājumi: Metagenomika nodrošina pilnīgu taksonomisko profilu noteikto organismu genomiskajam materiālam, tādējādi sniedzot daudz plašāku mikrobioloģiskās diagnostikas pieeju nekā CDM. Metodes izmaksu un apgrozījuma laika dzīvotspējīgu organismu noteikšanai samazināšana palīdzētu šo tehnoloģiju ieviest ikdienas klīniskajā praksē.

Atslēgas vārdi: 16S; 18S; NGS; baktērijas; radzene; acu banka; sēnes; barotnes; mikrobioloģija; audu saglabāšana un uzglabāšana.

1.1 Introduction

Descemet membrane endothelial keratoplasty (DMEK) is a selective replacement of dysfunctional corneal endothelium with healthy donor Descemet membrane and endothelium. Although the donor preparation and surgical methods still remain a challenge, DMEK is gaining popularity in terms of early rehabilitation and visual outcomes.

To be able to prepare a transplantable DMEK tissue, a healthy donor corneal tissue is a mandatory pre-requisite.

To improve the preparation and preservation of corneal tissue improvements in microbiological analysis can lead to better tissue.

1.1.1 Microbiological analysis of donor corneal tissue before transplantation: the rise of metagenomics and its differences with conventional culture methods

The human body is populated with an enormous variety of bacteria, archaea, fungi and viruses, which form a commensal, symbiotic and pathogenic community collectively known as human microbiome. The estimated amount of microorganisms is in the order of trillions, at least 10 times more than the number of human cells. This evidence has increased the interest of the scientific community to understand the role of these microorganisms in a day-to-day life. [1, 2]

The Human Microbiome Project (HMP) launched in 2008 by the United States National Institutes of Health had the aim to reveal and characterize the microbial populations of five main body areas, i.e. gut, mouth, nose, skin and urogenital tract. In the same year, the European Commission granted a project called MetaHIT, focused on understanding the correlations between human intestinal microbiota and some disorders, in particular inflammatory bowel disease and obesity (*http://www.metahit.eu*). [3, 4]

Metagenomics refers to the genomic analysis of microorganisms' populations, based on the development of Next generation sequencing (NGS) technology, which overcome the need to separate the genomes or to culture the microbes. [5]

Ideally, NGS can detect all the microorganisms present in a clinical sample, producing huge amounts of sequencing data that need to be decoded, and has the potential to improve diagnostic yield, as it is inherently unbiased and hypothesis-free.

Metagenomics has already shown its efficiency by providing a correlation between the changes in gut microbiota that has found to be associated with several diseases like cancer, obesity, asthma, atherosclerosis and diabetes. [6, 7]

Evolution of NGS technology, allowed its adoption by producing massive sequencing data. Indeed, the previous Sanger sequencing method was a low-throughput approach based on dideoxynucleotide chain termination. Although it was a suitable tool to sequence specific genes or fragments, but as was too laborious and expensive, it was difficult to investigate complex samples due to its sequencing speed, which was only a few thousand nucleotides per week. [8]

The application of NGS allows sequencing from thousands to millions of nucleic acid segments simultaneously in a single run. Thus, allowing to decode important large genomes, such as the human genome. This also allows full taxonomical profiling ("who is it?") and

compare the functions ("what is it doing?") of microbial communities from different areas, in a short span of time compared to the Sanger method. [9, 10]

After sample collection and nucleic acid extraction (DNA or RNA), the term NGS is generically used to indicate the two main sequencing methods: the marker gene sequencing approach (also called targeted-amplicon sequencing) and the shotgun approach.

In the former, amplicons from a single conserved gene are produced by Polymerase Chain Reaction (PCR) (library preparation and cluster generation). The conserved gene that is most commonly used is the 16S rRNA, because it is ubiquitous and formed from constant and variable regions that allow the definition of taxa. In addition, a universal target for eukaryotic organisms is the 18S rRNA gene. Moreover, internal transcribed spacer (ITS) regions of rRNA operons are frequently used to identify fungal species. Targeted-amplicon sequencing is used mainly in microbiome analysis with taxonomic purposes. [11]

In the shotgun approach, instead, the sequencing is performed across random fragments of all DNA in a given sample and can be used also in case of unknown microbial target. Both methods have advantages and disadvantages. Targeted-amplicon sequencing is usually used to characterize a particular microbial group in a sample, while shotgun is the only possible approach for the identification of previously uncharacterized microbes. Both can be used to detect pathogens, even if sometimes one type of technique is more appropriate than the other.

For example, if the etiological agent is suspected to be a virus, shotgun metagenomics is warranted, while in the case of a low biomass sample, marker gene metagenomics may be able to sequence the infectious agent more adequately.

However, metagenomics does not only sequence, but also interprets the retrieved data and perhaps this is the most critical step. Indeed, the high-throughput capabilities of NGS approach leads to an exponential accumulation of sequence data that need to be interpreted. Hence the requirement to develop increasingly appropriate bioinformatics tools, i.e. specific bioinformatics algorithms that transform raw sequence of NGS signal outputs in suitable and organized information. Complex and computationally expensive data analysis processes are therefore required. [12, 13]

Conventional culture-based techniques are still currently used for microbial identification but, due to their limitations, high performance diagnostic techniques are being developed and implemented (PCR and, mostly, NGS). Indeed, with culture-based methods, the incubation and inoculation of the clinical specimens (usually in high volumes) have to be performed on a range of appropriate media. The sensitivity is found to be relatively poor (yield of 40–70 %) and the probability of false-positive results could be significant. Results could also be biased due to the fast-growing microorganisms, which can be easily cultivated on a standard

media. Moreover, studies comparing conventional culture techniques and molecular analysis have shown very often, that results obtained by these methods are incomplete or biased by false-positive data, thus highlighting the limitations of traditional culture-based techniques in terms of sensitivity and reproducibility. [14, 15]

A comprehensive analysis of the microbial diversity is usually possible only through the support of innovative technologies like NGS. [16]

1.1.2 Metagenomics: selecting the test

The two methods (shotgun and marker-based metagenomics) can be used in different instances: the marker-based approach is used to get the taxonomic profiles of the community under study, whereas shotgun approach gives wider information on function and an extended phylogenetic breadth. For both methods, there are pros and cons: marker-based studies are well suited for analysis of large number of samples, that is, multiple patients, longitudinal studies, and so on, and are cheaper; however, there are well-known amplification biases and the amount of information is limited to the taxonomy. On the other hand, shotgun metagenomics is usually more expensive. It may miss low-abundant species and when host-associated metagenomes are studied, most of the reads derive from the host genome, especially when studying sites with low bacterial biomass. It offers, however, increased resolution, enabling the possibility to discover new microbial genes and genomes as well as a more specific taxonomic and functional classification of sequences (in some cases). Importantly, shotgun metagenomics allows the simultaneous study of viruses, bacteriophages, archaea and eukaryotes. Sample collection and storage methods are critical for most metagenomic studies: they are often arbitrary and rely on the common practices developed in single laboratories or even by single researchers.

However, in some cases, such as the study of the human faecal microbiome, there are well-established standard procedures. [6]

A standardized protocol for sample collection, handling and storage for metagenomic studies in ophthalmology is still under development (data not shown). In addition, as all low biomass samples, corneal surfaces are particularly vulnerable to external contaminations, which could also derive from the reagent kits, therefore, a proper experimental design should include a number of blank controls and the use of ultrapure reagents to minimise this risk. Several significant efforts to unravel bacterial identity with a resolution as high as the level of strain have already been published. The integration of the metaomics (collective name that stands for metagenomics, metatranscriptomics, metaproteomics, and so on) with information such as clinical history, dietary information and genetic background of the patient may be useful in the implementation of mechanistic models explaining the microbiome structure and

function. Biomarker discovery needs a high number of replicates; one pipeline developed for this task is LEfSe which relies on the linear discriminant analysis of effect size. It detects consistent abundance patterns among features (that can be either taxa or coding genes) in a multidimensional data set such as a species-per-sample metagenomic matrix. It is highly scalable and it has proved to achieve a discrete performance in reducing the false-positive detection, although as explicitly admitted by the developer, the false-negative rate is slightly higher. Other pipelines are also available for biomarker discoveries, however, a benchmark among them is beyond the scope of this review. Last, but not least, the complex tasks described above require high computational power and specific expertise in the field of biostatistics and informatics. [8, 9, 14]

1.1.3 Eye banking and Metagenomics: Why?

Infections of the eye such as endophthalmitis may occur following a corneal transplant. The incidence of endophthalmitis over a 7-year period in the UK following penetrating keratoplasty was 0.67 %. It can be challenging to identify and distinguish the source of the infection, which includes endogenous source such as the host and a variety of exogenous sources such as the donor cornea. It is well known that donor corneas may be the source of contamination as they contain viable cells and as such cannot undergo typical sterilization processes. Risk factors for the development of an infection following a corneal transplant include immunosuppressive treatment following surgery and cornea's avascular state. It has been suggested that discontinuation of the topical antimicrobials with concomitant use of steroids may allow growth of sequestered microorganisms. Transmission of the herpes simplex virus type 1 from the donor cornea has shown to increase the risk of rejection. Fungal infections have also been reported in the preservation media affecting graft survival after transplantation. [17, 18]

The specific diagnosis of infection remains a challenge as it still relies on conventional microbial culture techniques for the identification of the suspected pathogen. Most of the environmental microorganisms that are difficult to culture using conventional techniques can be detected using molecular methods. Such techniques use a hypothesis-free short-read approach that is suited for taxonomic and functional profiling applying the high-throughput DNA sequencing (DNA-seq) techniques. [19, 20, 21]

This helps to determine microbes with low sample volume increasing the diagnostic yield. PCR analysis has already been applied to identify pathogenic agents in ocular tissues, including the aqueous humour and vitreous, and has been used for the diagnosis of infections that would have been otherwise difficult to identify. RNA sequencing can also be performed to

detect fungus, parasites and viruses, however, this technique has limitations, as it requires proper specimen handling. [22]

For pathogens with DNA genomes, metagenomic DNA-seq can circumvent this challenge, as DNA is more stable at ambient temperatures.

In this first study, we investigate whether there are microorganisms present in the storage media that are undetected using the conventional microbiological assays and can potentially complicate a corneal transplant.

1.2 Materials and Methods

1.2.1 Ethical statement

Human donor corneas were obtained by the Veneto Eye Bank Foundation, Venice, Italy, with written consent from the donor's next-of-kin to be used for research purposes. The study followed the 2013 Tenets of Declaration of Helsinki. The tissues were used under the laws of Centro Nazionale Trapianti, Rome, Italy. The corneas were suitable for research and unsuitable for transplantation due to low endothelial cell counts (< 2200 cells/mm^2). No other complications or indications were recorded in the donor corneas such as diabetes, HIV or hepatitis B virus.

1.2.2 Corneal preservation

Human donor corneas (n = 7) were excised and preserved in Cornea Max (Eurobio, Les Ulis, France) for 14 days at 31° C, that is, the protocol currently used for organ culture (OC). Other (n = 7) samples were preserved in Cornea Cold (Eurobio) for 14 days at 4° C, as current hypothermic protocol. Both the media are commercially available and contain penicillin and streptomycin as antibacterial agents and amphotericin B as an antifungal agent. The control samples (n = 1) from OC and hypothermic media, without human donor corneal tissues, were used separately as controls.

1.2.3 Sample collection from corneal preservation media

Two millilitres of the storage media from each sample was extracted and preserved in 2 mL sterile Eppendorf tubes (Eppendorf Biopur safe-lock microtubes, Sigma-Aldrich, Italy) and shipped to IMGM laboratories, Germany, at room temperature (OC media) and in dry ice (hypothermic media), for metagenomic analysis, respectively. Microbiological analyses were also carried out in-house on the same samples using a Bactec Instrument (Becton Dickinson,

Franklin Lakes, NJ, USA), which is a colorimetric assay, in order to compare the difference between metagenomic analysis and conventional eye banking microbiological tests.

1.2.4 DNA isolation

Genomic DNA (gDNA) was isolated from both the preservation media using a NukEx Pure RNA / DNA kit (Gerbion, Kornwestheim, Germany) according to the manufacturer's instructions. gDNA concentrations were quantified using the highly sensitive fluorescent dye-based Qubit double-stranded (dsDNA) HS Assay Kit (Invitrogen, Waltham, MA, USA). In brief, 1 µL of each sample was used to determine dsDNA concentration (ng/µL) in comparison to a given standard provided with the kit.

1.2.5 Amplicon based sequencing analysis for 16S rRNA and 18S rRNA

The amplification strategy combined amplicon generation with library preparation for Illumina sequencing. The amplicon tagging scheme was based on a combination of an inner target-specific (TS) primer pair extended with a universal tag and an index primer pair comprising a complementary tag, indices and sequencing adapters. By incorporating sample-specific indices, all single-plex PCR products generated by single-plex PCR were pooled together to run in a single sequencing experiment. The study will report ribosomal RNA (rRNA; 16S rRNA and 18S rRNA) typing. Bacterial-specific primer pair Bakt_341F and Bakt_805Rwas chosen to cover variable regions 3 and 4 of the 16S rRNA gene. The primer pair 18 S-574f and 18 S-897r was chosen to cover variable region 4 of the eukaryotic 18S rRNA gene.

Subsequent to TS-PCR, the index PCR was performed on tagged 16S rRNA or 18S rRNA amplicons to barcode all samples with different indices for pooling, using 1 µL of each TS-PCR product as template. The quality check was performed using an aliquot of each final PCR product including the positive control. 2 % agarose gel (Midori Green-stained) was used to analyse the quality of the generated amplicons and to evaluate the expected amplicon size.

After amplicon purification using solid phase reversible immobilisation paramagnetic bead-based technology (AMPure XP beads, Beckman Coulter) and library normalisation and pooling, the quantification of the library pool was performed. Library denaturation and sequences were performed at a final concentration of 4 pM and with a 10 % PhiX control library spike-in (Illumina, San Diego, CA, USA). Cluster generation and sequencing was performed using primers hybridise to the adapter sequences at the end of the fragments. Bidirectional sequencing was performed on a MiSeq using its 500-cycle v2 chemistry, starting first at the end

of the sense strand (read 1) and subsequently at the end of the complementary strand (read 2). Both the reads have a length of 250 bases, finally producing 500 bases of sequence information in 2×250 bp paired-end reads.

1.2.6 Data processing

The data were processed using Illumina software MiSeq Reporter (MSR) V.2.5.1.3 on the MiSeq system and the Illumina Sequence Analysis Viewer (SAV) V.2.1.8 used for imaging, data processing and evaluation of the sequencing run performance. The image and signal processing was carried out by the Illumina MiSeq inherited MSR software packages applying the 'FastQ only' processing pipeline. This processing pipeline allows 3′-end trimming of adapter sequences, which is recommended by Illumina. De-multiplexing of all passed filter reads was performed using indices and corresponding sample IDs. Single read 1 and read 2 fastq files, containing quality values and sequence information, were generated. In-depth bioinformatics analysis was performed using the GLC Genomics Workbench version 9.5.3 and the implemented microbial genomics module version 1.6.1. Trimming of reads was performed according to TS primer sequences, base quality and read length, whereby a probability quality limit of 0.05 was applied to ensure high-quality data for subsequent analysis. To guarantee similarity and a sufficiently high level of sequence information for phylogenetic classification, sequences < 400 bp for 16S rRNA and < 300 bp for 18S rRNA, respectively, were discarded and longer sequences were trimmed to this length.

The remaining sequences were clustered at a 97 % identity threshold defining operational taxonomic units (OTU), according to the taxonomy of the SILVA 16S / 18S rRNA sequence database version 128. Chimeric sequences, representing PCR and sequencing artefacts, were filtered out and discarded during this step. Out of each cluster, one reference sequence of an OTU was defined. OTUs with less than 10 combined reads over all samples were discarded. A graphical overview on the taxonomy results per sample was generated as an interactive Krona Chart.

For Alpha diversity that describes the number of species (or similar metrics) in a single sample, multiple sequence alignment was performed including all sequences of the sample using the multiple sequence comparison by log-expectation algorithm and based on this alignment a phylogenetic tree was calculated using a maximum likelihood approach.

1.3 Results

1.3.1 Donor characteristics

The mean age of the donor corneas preserved in OC was 57.29 (± 10.78) years comprising of five male donors and two female donors with a mean post-mortem time of 3.42 (± 1.71) hours. Mean donor age of the corneas preserved in hypothermic storage was 73.43 (± 3.87) years comprising of six male donors and one female donor with a post-mortem time of 14.06 (± 8.67) hours.

1.3.2 Quality assurance

The purity and concentration of isolated gDNAs are listed in Table 1.1. Although DNA concentration of all samples was low with values below 1 ng/µL, it was still considered suitable for metagenomic analysis. All reads from low-quality clusters as well as mixed read clusters, which did not pass quality criteria, were discarded during the primary analysis pipeline. Read counts for all samples and amplicons are provided in Table 1.1. All 16S rRNA samples had enough reads for downstream analysis. For the 18S rRNA, 6 out of 16 samples generated less than 10 000 reads per sample. These samples corresponded to the low performing samples in 18S rRNA two-step PCR.

Table 1.1

Purity and concentration of isolated genomic DNA and distribution of sequenced reads per sample

	Organ culture storage	Hypothermic storage	16S for Organ culture storage	16S for Hypothermic storage	18S for Organ culture storage	18S for Hypothermic storage
	Concentration (ng/µL)	Concentration (ng/µL)	Absolute reads per sample	Absolute reads per sample	Absolute reads per sample	Absolute reads per sample
Sample 1	0.34	0.65	43 651	99 618	2969	122 045
Sample 2	0.48	0.64	27 772	58 045	36 165	52 231
Sample 3	0.39	0.76	48 668	51 075	9705	103 025
Sample 4	0.44	0.71	43 354	83 645	28 366	94 914
Sample 5	0.54	0.81	50 231	48 027	11 972	109 777
Sample 6	0.37	0.62	67 907	55 904	4642	132 767
Sample 7	0.62	0.67	29 398	76 440	7392	107 276
Control	0.51	0.84	33 477	25 475	4001	2501

1.3.3 Presence of bacterial genomic material in organ culture and hypothermic storage

Cumulative results of top 10 microorganisms with the highest reads per sample for 16S rRNA in OC and for hypothermic storage are shown in Figure 1.1 (A) and (B), respectively. The relative abundance of 16S rRNA pathogens in OC storage is shown in Figure 1.1 (C) and in hypothermic storage is shown in Figure 1.1 (D). Similar communities were profiled between OC and hypothermic storage. Only a small difference was observed while screening both the bacterial and fungal components of the samples. Evidence of *Pseudomonas, Comamonas, Stenotrophomonas* and *Alcanivorax spp*. were found commonly in OC at high abundance, with *Brevundimonas* and *Nitrobacter spp*. found frequently, but in low abundance. *Pseudomonas, Comamonas, Stenotrophomonas, Alcanivorax, Brevundimonas, Acidovorax and Hyrogenophilus spp*. were common and abundant in samples from hypothermic storage, with *Acetobacter* and *Nitrobacter spp*. being less abundant. The sequence reads per sample in hypothermic storage and OC storage showed the presence of genomic material in the samples as shown in Table 1.2.

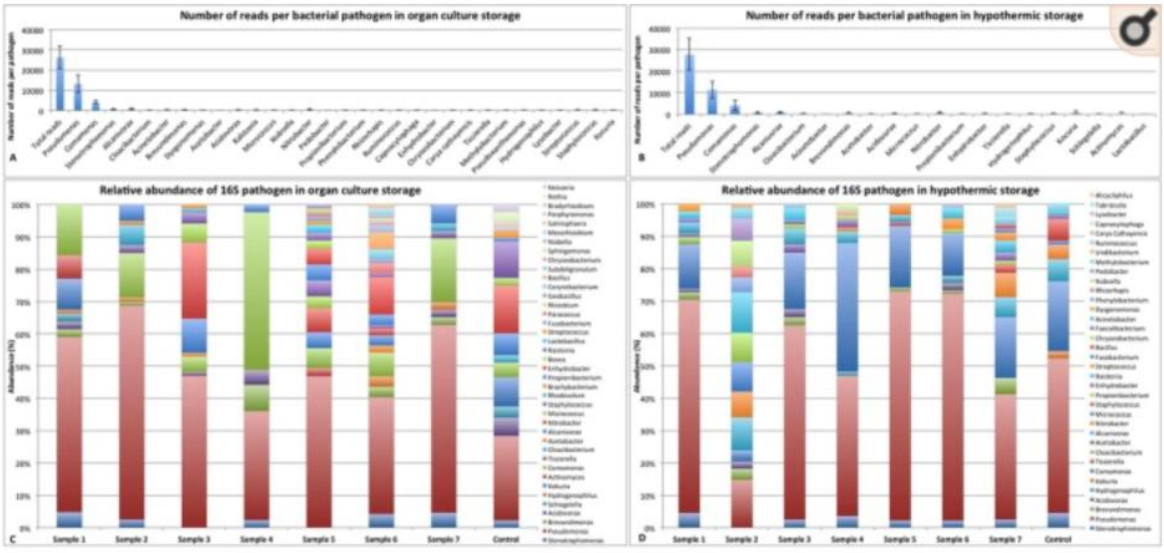

Figure 1.1 **Bacterial genomic material in organ culture and hypothermic storage**

(A) Cumulative number of reads per sample for 16S in organ culture and (B) in hypothermic storage. (C) Relative abundance of 16S pathogens in organ culture storage and (D) in hypothermic storage.

Table 1.2

Sequence reads of 16S per sample in organ culture and hypothermic storage showing the presence of genomic material in the samples

16S Organ culture								
Pathogen	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Control
Total reads	31 098	7308	29 264	19 971	33 246	45 341	20 212	24 222
Pseudomonas	18 094	986	13 919	7069	20 797	28 446	6136	10 133
Comamonas	3848	0	4034	6536	5553	5298	3022	4551
Stenotrophomonas	1200	0	445	652	581	991	486	1000
Alcanivorax	924	689	1177	661	503	0	986	1469
Brevundimonas	552	0	525	0	417	557	869	0
Nitrobacter	0	496	0	275	872	0	1219	941

16S Hypothermic								
Pathogen	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Control
Total reads	56 853	33 610	13 416	34 765	14 736	18 751	33 165	17 476
Pseudomonas	25 506	17 690	5443	9918	5582	6351	16 515	4067
Comamonas	7219	3560	477	14 519	780	1222	5563	680
Stenotrophomonas	2365	639	0	685	0	773	1396	387
Alcanivorax	676	1380	1144	768	651	539	1734	1057
Brevundimonas	908	388	0	2295	0	613	517	0
Acidovorax	619	322	193	1509	0	0	546	811
Hydrogenophilus	676	404	0	315	0	484	708	0
Acetobacter	0	464	136	0	0	439	280	0
Nitrobacter	0	0	2633	185	906	0	0	2221

1.3.4 Presence of fungal genomic material in organ culture and hypothermic storage

Classification of fungal organisms was assigned based on the last available taxonomy. As above, cumulative results of top 10 organisms with highest reads per sample for 18S rRNA in OC are shown in Figure 1.2 (A) and for hypothermic storage are shown in Figure 1.2 (B). Relative abundance of fungal pathogen in OC storage is shown in Figure 1.2 (C) and in hypothermic storage is shown in Figure 1.2 (D). Only *Pinales* and *Malassezia spp*. were found abundant both in OC and hypothermic storage. *Malassezia sp*. showed the highest number of reads in both the media. Most OTUs were classified from plants, mainly *Malassezia*, indicating that the other fungi (from plants) were absent due to the antifungal compounds present in the media. The number of reads and presence of genomic material in each sample are shown in Table 1.3.

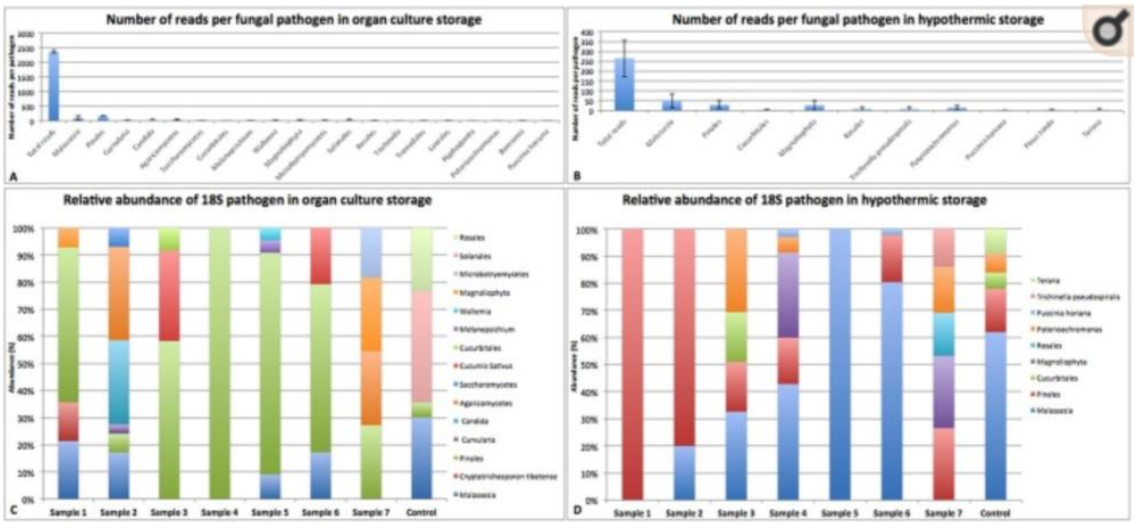

Figure 1.2 **Fungal genomic material in organ culture and hypothermic storage**

(A) Cumulative number of read per sample for 18S in organ culture and (B) in hypothermic storage.
(C) Relative abundance of fungal pathogen in organ culture storage and (D) in hypothermic storage.

Table 1.3

Sequence reads of 18S per sample in organ culture and hypothermic storage showing the presence of genomic material in the samples

18S Organ culture								
Pathogen	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Control
Total reads	3	512	118	393	184	110	461	341
Malassezia	0	10	19	61	8	81	81	211

18S Hypothermic storage								
Pathogen	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Control
Total reads	2866	2384	1464	5516	2500	1672	1828	722
Malassezia	86	131	0	0	48	83	0	356

1.3.5 Alpha diversity of 16S rRNA and 18S rRNA in organ culture and hypothermic medium

Refraction curves represent the species richness for a given number of individual samples. The number of different OTUs was plotted against the amount of reads per sample. The curves became flatter with increasing amount of reads in 16S rRNA samples indicating that only a few additional OTUs were likely to appear after deeper sequencing both, in OC (Figure 1.3 (A)) and in hypothermic media (Figure 1.3 (B)). The curves for 18S rRNA did not reach 10 000 reads both in OC (Figure 1.3 (C)) and in hypothermic media (Figure 1.3 (D)).

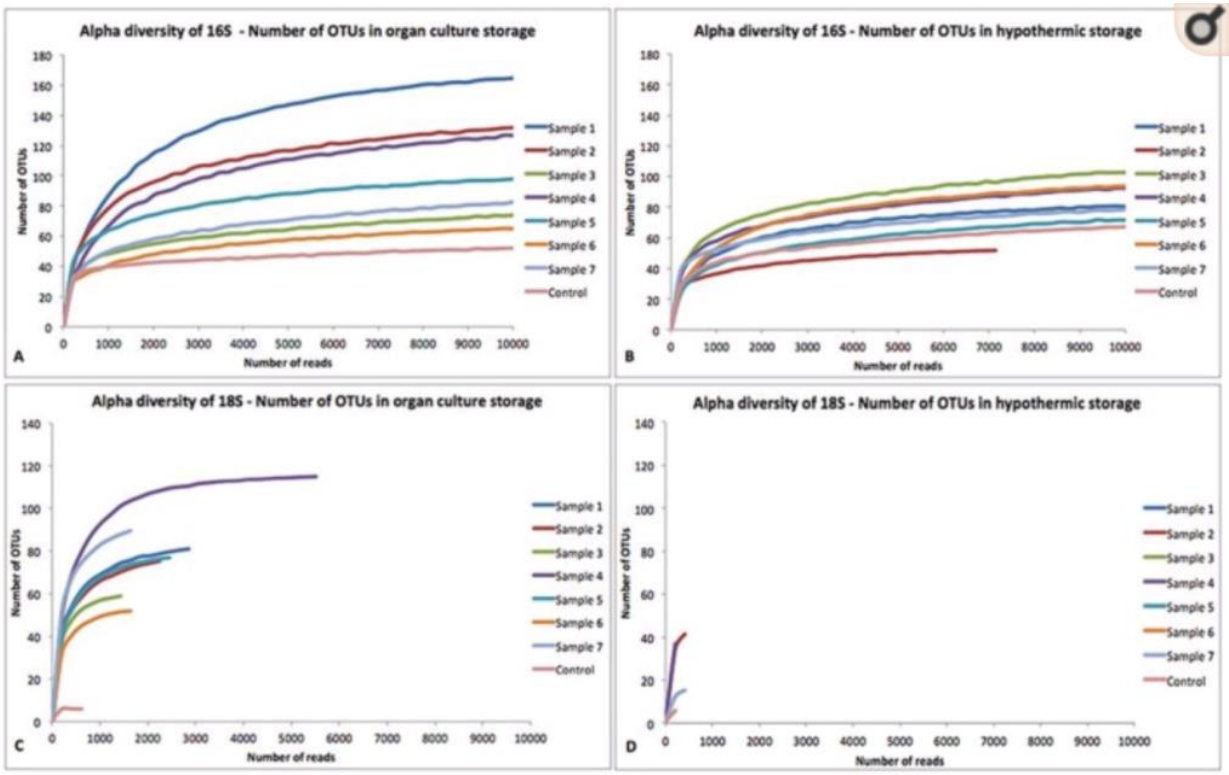

Figure 1.3 **Alpha diversity of 16S**

(A) 16S in organ culture, (B) 16S in hypothermic storage, (C) 18S in organ culture and (D) 18S in hypothermic storage. OTUs, operational taxonomic unit.

1.3.6 Conventional diagnostic method and control group analysis

All the samples, including controls, showed negative results from CDM analysis using Bactec instrument. However, even the control samples showed presence of genomic 16S rRNA (Figure 1.4 (A) for OC and 1.4 (B) for hypothermic media) and 18S rRNA (Figure 1.4 (C) for OC and 1.4 (D) for hypothermic media) material that was determined as sterile by conventional microbiological assays and industrial assays.

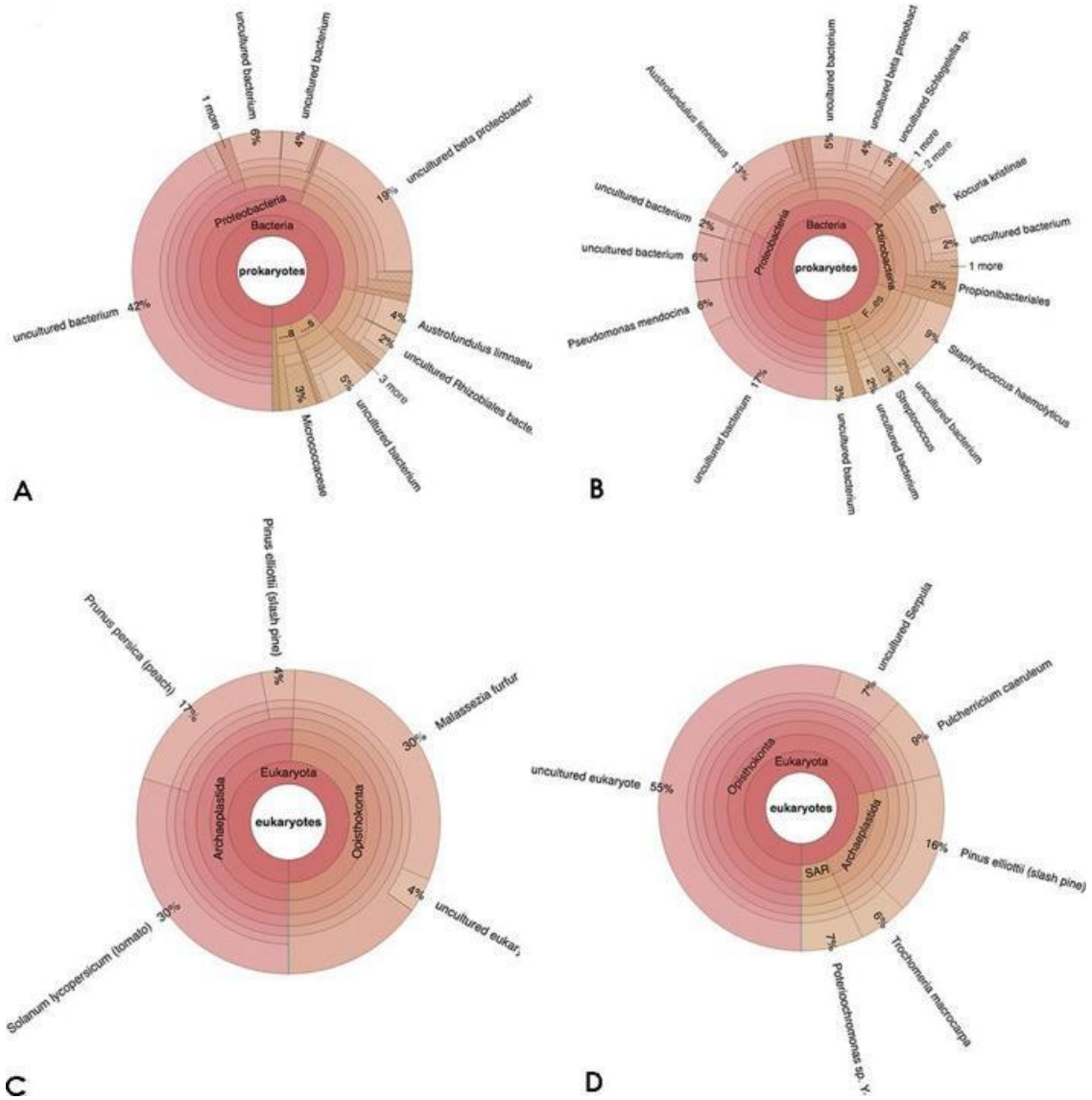

Figure 1.4 **Krona chart of control samples showing presence of genomic material**

In (A) 16S rRNA for organ culture and (B) for hypothermic media and
(C) 18S rRNA for organ culture and (D) for hypothermic media.

1.4 Discussion

Although corneal tissue and its preservation solution should be pathogen-free before a transplant can be carried out, there are reports of corneal infection and endophthalmitis after corneal transplantation due to contaminated donor tissue. It is well known that traditional culture methods only detect a fraction of the available microbiota. Studies on the ocular surface have shown the presence of high bacterial load per 1 ng of total DNA. Dong et al reported 59 distinct bacterial genera on the ocular surface microbiome using 16S rDNA gene deep sequencing. [5, 23]

As there are multiple diagnostic tools available for the detection of an infection, the choice of the diagnostic method becomes important. 16S rRNA and 18S rRNA approaches are used to detect prokaryotes and eukaryotes, respectively, whereas shotgun is used for deep

genome sequencing resulting into the identification and taxonomical classification of all microorganisms. [24, 25, 26]

Although 16S rRNA is useful for large number of laboratory or clinical samples, it offers limited taxonomical and functional resolution compared with shotgun sequencing. Shotgun sequencing can be expensive, but it has high-resolution data obtaining capacity, thereby enabling specific taxonomic and functional classification of sequences and identifying new microbial genes. [27, 28, 29]

Eye banks that collect, preserve, process and distribute donated human ocular tissues, store corneas using two different approaches. In Europe and New Zealand, corneas are predominantly stored in an OC medium, whereas in the USA, Asia and Australia, most donor corneas are stored in short-term hypothermic conditions between 2 °C and 8 °C.

The length of culture period (7–30 days) and the temperature (typically 31 °C–37 °C) of an OC medium facilitate the growth and detection of certain types of microorganisms. Endophthalmitis has been reported to occur more commonly if the donor had septicaemia. Septicaemia is a contraindication if the prospective donor cornea is stored in hypothermia. With OC, patients with bacterial septicaemia are not precluded as donors, as long as concomitant microbiological testing is performed.

Antibacterial agents such as penicillin and streptomycin, and antifungal agents such as amphotericin B are usually used as an empiric cocktail in OC corneal preservation media. Conventional microbiological controls are currently performed using standard bacteriological media in aerobic and anaerobic environments, whereas Sabouraud broth is a routine medium for detection of fungi. Other options include the use of Bactec blood bottles (Becton Dickinson) incubated in the Bactec instrument (based on the detection of CO_2 produced by microorganisms), which offer many advantages over the standard microbiological techniques. These techniques, however, only detect the presence of microorganisms but not their identity.

In this study, all the samples and controls showed evidence of the presence of microorganisms or its genomic content using 16S rRNA and 18S rRNA approaches. In particular, we also found same microorganisms in both, hypothermic and OC storage media. The presence of genomic material in the preservation media, however, does not necessarily relate to viable microorganisms in the storage solution. It is, therefore, not clear whether the difference between the 16S rRNA and 18S rRNA approaches and conventional culture reflects inhibition, but not eradication, of microorganisms by antimicrobials in the OC medium, differences in sensitivity and or the absence of living microorganisms or gDNA.

It is worth considering possible sources of the microbial DNA. It is possible that different genomic materials in our solutions could have come from either raw materials or packaging items when the media was manufactured. For example, genomic material of abundant microbes such as *Pseudomonas, Stenotrophomonas and Comamonas spp*. could have come from the industrial water. To produce highly purified water, microorganisms present in water are treated using ultrafiltration, followed by ultraviolet light that lyses the bacteria releasing genomic material into the media. The genomic material of *Alcanivorax sp*. could be related to the cap of the storage vial, as it is the only component that contains material derived from oil. The cap undergoes irradiation (beta or gamma), thus leading to release of genomic material. All the batches of the media were tested for 14 days in culture and the sterility in the industry is confirmed before releasing the batch. The presence of a low abundance of *Brevundimonas sp*. could be from the ocular surface when the corneas are cleaned with polyvinyl pyrrolidone before placing them in the storage solution. It is possible that the genomic material of non-viable microorganisms may have stuck to the epithelial cells and would have been released in the storage media during preservation. Fungal (18S rRNA) contamination was at a very low abundance rate. Interestingly, OC showed a higher number of bacterial and fungal OTUs compared with that in the hypothermic media. Indicating that larger number of species could be possibly available when the conditions are optimum for the growth of an organism.

Comparing the two majorly used protocols of corneal preservation, we expected that hypothermic storage media would have less genomic material compared with OC, as OC preservation system supplements the growth condition (temperature and supplements) of microorganisms much better than hypothermic condition. The concentration of fungal DNA was higher than bacterial DNA. The absolute reads were higher in hypothermic samples compared with OC samples. As the medium is an industrial product, most of the organisms identified in our study are from the industrial raw material or water that may contain more organisms of fungal rather than of bacterial origin and therefore less bacterial DNA was observed in the samples. There are chances that such a variation could also have been due to technical issues but as all the samples were processed at the same time, this possibility could be ruled out. However, the raw materials and the final vials used for hypothermic media sampling are different than those of OC media. Some constituents or the materials could have been a possible source of more DNA concentration found from the hypothermic group compared with those from the OC group.

Multiple factors such as different concentrations of antibiotics, media formulation, raw materials, downstream processing, temperature differences, etc could have also led to the presence or release of more DNA from organisms before, during or after preservation. Industrial

procedures to detect live microorganisms is sufficient, but could be improved with more specific and sensitive assays like next-generation sequencing (NGS), whereas sequestered microbes in the tissue will not be detected and they have been considered to be the risk for infections such as endophthalmitis. Most of the DNA (regardless its provenience) came from taxa usually found in industrial water. Some of those taxa contain species that could be pathogenic.

However, the amount of reads detected suggests that the actual contamination is negligible (if not just the background noise). All our samples showed negative results using Bactec colorimetric analysis, which would suggest that the samples were unlikely to contain sufficient viable microorganisms for the samples to be found positive, thus indicating that the currently used antibacterial and antifungal cocktails used in the respective media are also reliable for corneal preservation.

Aldave *et al* observed an insignificant increasing trend in the rate of fungal infection; they determined that it is not sufficiently compelling to pursue antifungal supplementation for donor storage media. In this study, we also report that fungal contaminants were found at a very low abundance rate. The other microorganisms detected that might have arisen from the cornea or the media may have been below the detection limit of CDM or were killed by the antimicrobials present inside the media. The 10 most abundant genera found on the ocular surface include *Pseudomonas, Propionibacterium, Bradyrhizobium, Corynebacterium, Acinetobacter, Brevundimonas, Staphylococcus, Aquabacterium, Sphingomonas and Streptococcus*, which were also observed in our samples.

16S rRNA and 18S rRNA data were acquired and analysed, which only provides data on the detection of genes and not necessarily viable microorganisms, which could be considered as a potential limitation of this study. This could, however, be supplemented with proteomics to detect live organisms. The other limitation is that the method measures only rRNA and therefore other genomic information is missing and specificity of identification is reduced. By law, if the storage media is contaminated, the corneal samples must be discarded. With further improvements, NGS could be advantageous by detecting the presence of genomic material in a short span of time and with reduced costs. A controlled comparative in vitro study of NGS and CDM with enrichment culture and removal of antibiotics in the medium is needed.

Current study showed the presence of gDNA in the negative control samples. A positive control of a known organism and concentration would have been beneficial for understanding the efficiency and sensitivity of metagenomics. Because of the high sensitivity of this technique, technicians must strictly follow a total sterility protocol avoiding contamination during sample processing. The cornea sheds epithelial cells during the preservation phase. Regeneration of

these cells in OC particular, if co-infected by intracellular microorganisms, highlights the need for their detection by NGS especially as it has been observed that ocular surface contains a small number of bacterial cells.

To conclude, Metagenomic deep sequencing has the potential to improve the microbiological analysis of samples starting from low concentrations.

The costs, presence of live organisms, turnover time, downstream processing and data analysis could be considered as limitations when it comes to routine eye banking procedures especially when the empiric solutions already seem to be relatively safe. Given the current trends in genomic technology development, the costs are likely to be reduced significantly and more narrowed and standardized results will be obtained in the near future. With adequate staffing, the final protocol could be completed in less than 48 hours. NGS could therefore be of significant value for checking the microbiological load in industrial production to ensure the safety of healthcare products. Metagenomics has a role for detecting organisms with high specificity and sensitivity, which may also be important at the centres where Good Manufacturing Practices (GMP) rules are stringent. [30–41]

2 Study 2

The first study showed how metagenomics can improve the preservation of corneal donor tissues. Starting from preserved corneal tissues, the second study aims to standardize DMEK tissue preparation.

Abstract in English

Title: Standardizing Descemet membrane endothelial keratoplasty graft preparation method in the eye bank-experience of 527 Descemet membrane endothelial keratoplasty tissues

Aim: To provide a standardized protocol for Descemet membrane endothelial keratoplasty (DMEK) graft preparation.

Material and methods: 527 prestripped DMEK tissues were prepared between 2014 and 2017. The experience of using different instruments and techniques has been described, and a standardized technique for preparing DMEK grafts has been identified. The tissues in general were prepared by superficially tapping the endothelial side with a Moria trephine (9.5 mm diameter). The plane of cleavage was identified using a cleavage hook, and the DMEK graft was deadhered from the trephined site throughout the circumference for ease of excising the graft. The DMEK graft was peeled using either one or multiple quadrant methods depending on the challenges faced during excision. The graft was finally marked with the letter "F" to identify the orientation during surgery. Data on endothelial cell loss (ECL) and challenging cases were observed, monitored, and recorded during this period.

Results: Less than 1 percent trypan blue-positive cells with tissue wastage of < 6 % was observed during the study period. Our standardized stripping technique has resulted in an overall ECL of 4.6 %. Marking Descemet membrane showed 0.5 % cell mortality.

Conclusions: Standardizing DMEK technique using specific tools and simple techniques would help new surgeons to decide the instruments and improve their tissue preparation skills also in challenging cases such as previous cataract incisions or horseshoe-shaped tears, further reducing ECL or tissue wastage.

Keywords: DMEK, tissue preparation, tips, tricks, Metagenomics, preservation; storage

Abstract in Latvian

Pirmais pētījums pierāda, kā metagenomika spēj uzlabot radzenes audu saglabāšanu. Otrā pētījuma mērķis ir standartizēt DMEK audu sagatavošanu.

Tēzes

Nosaukums: Descemeta membrānas endotēlija keratoplastikas transplantāta sagatavošanas metodes standartizēšana 527 DMEK audu paraugos.

Mērķis: Nodrošināt standartizētu protokolu DMEK transplantāta pagatavošanai.

Materiāli un metodes: Laikā no 2014. līdz 2017. gadam tika sagatavoti 527 iepriekš noņemti DMEK audi. Ir aprakstīta dažādu instrumentu un tehniku lietošanas pieredze, un ir identificēta standartizēta tehnika DMEK transplantātu sagatavošanai. Audi tika sagatavoti, virspusēji, noklājot endotēlija pusi ar Morija trefīnu (diametrs 9,5 mm). Sadalīšanas plakne tika identificēta, izmantojot sadalīšanas āķi. Lai atvieglotu tā izgriešanu, DMEK transplantāts tika atdalīts no trefinētās vietas visā apkārtmērā. Tā atdalīšanai tika izmantotas atšķirīgas kvadranta metodes, kuras tika individuāli pielāgotas ekscīzijai. Visbeidzot transplantāts tika apzīmēts ar burtu "F", lai operācijas laikā atvieglotu atrašanās vietas noteikšanu. Šajā posmā tika novēroti, uzraudzīti un reģistrēti dati par endotēlija šūnu zudumu (no angļu val. – *endothelial cell loss* (ECL)) un par sarežģītākie klīniskiem gadījumiem.

Rezultāti: Tika novērots mazāk nekā 1 % tripānzilā-pozitīvu šūnu ar audu zudumu < 6 %. mūsu standartizētās materiāla noņemšanas tehnikas ECL ir 4,6 %. Descemeta membrānas iezīmēšana uzrādīja 0,5 % šūnu mirstību.

Secinājumi: Standartizēta DMEK tehnika, izmantojot īpašus rīkus un vienkāršas metodes, palīdzētu jaunajiem speciālistiem izvēlēties atbilstošu tehniku un uzlabotu viņu audu sagatavošanas iemaņas arī sarežģītākos klīniskos gadījumos, piemēram, iepriekšējas kataraktas incīzijas vai pakavveida plīsumi, tādējādi vēl vairāk samazinot ECL vai kopējo audu zudumu.

Atslēgas vārdi: DMEK; audu sagatavošana; padomi; metagenomika; audu saglabāšana.

2.1 Introduction

Endothelial keratoplasty has become a gold standard for the treatment of endothelial failure over a decade. Characteristics of Descemet membrane endothelial keratoplasty (DMEK) such as its minimally invasive nature, fast optimal visual recovery and extremely low occurrence of post-operative immunologic rejection have made DMEK a choice for many surgeons. The technique still requires overcoming major challenges, mainly related to delivery, unfolding, and positioning of the graft in the recipient eye. Very recently, eye banks have started preparing and transporting precut donor tissues with a desired diameter for Descemet stripping automated endothelial keratoplasty (DSAEK), ultrathin DSAEK, and pre-stripped tissues for DMEK. Precut DSAEK and pre-stripped DMEK grafts have been shown to reduce surgical

effort, time, and cost in the theatre. The popularity of pre-cut / pre-stripped tissues is therefore seen to be increasing rapidly in our setting at the Veneto Eye Bank Foundation, Venice, Italy. [42, 43, 44]

Different surgeons have described various DMEK graft excision techniques. Busin et al introduced pneumatic dissection that uses an air bubble formed with full pressure introduced in the stroma–Descemet membrane interface. In a study by Parekh et al a liquid bubble was used to create full-length graft separation by forcing the liquid into the stroma–Descemet membrane interface. Liquid pressure has also been used as a cutting tool by Muraine et al to separate Descemet membrane (DM) and endothelium with stroma. [45, 46, 47]

A study by Studeny showed a preparation technique using the stromal rim. The no-touch method was introduced by Dapena in which the peripheral endothelium was scored at the trabecular meshwork and peeled as an entire graft with full diameter.

Although many techniques have been introduced for preparing a DMEK graft, we believe that there are several hidden tricks to achieve optimum results. We therefore intend to share our experience that we have built using 527 pre-stripped DMEK tissues that also include difficult cases and describe the challenges and methods to overcome them while preparing a DMEK graft. The article also lists the standardized procedure that we use for preparing a pre-stripped DMEK lenticule. [48, 49]

2.2 Materials and methods

The corneal tissues (n = 527) were collected by the Veneto Eye Bank Foundation, Venice, Italy, with written consent from the donor's next-of-kin to be used for trans plantation. DMEK grafts were prepared after determining the endothelial cell density (ECD), which was between 2500 and 3000 cells/mm^2 in all tissues. Tissues were stained using trypan blue and placed in sucrose solution after briefly washing in phosphate-buffered saline. The ECD and trypan blue-positive cells (TBPCs) were recorded by manually counting the cells using a $10_{\times}10$ reticule fixed inside the eyepiece of an inverted microscope (Zeiss, Milan, Italy) at $100_{\times}$ magnification. An average of 3 readings was recorded to avoid false-positive measurements centrally and paracentrally.

2.2.1 Stripping

Although there are many techniques that show excision of DM from the cornea, we have modified the stripping technique that was initially introduced by Melles as described further. The basic tools that are required for stripping a DMEK graft usually include 1) vacuum base, 2) trephine, 3) cleavage hook, and 4) forceps for stripping.

2.2.2 Vacuum base and trephine

There are multiple vacuum bases that are available along with trephines from different companies; however, our choice varies depending on the purpose. We have used the Moria trephine base (Moria, Antony, France) (Fig. 2.1 (A)) for regular DMEK superficial cuts. The base of the Moria trephine is shallow, and therefore it usually provides a fin vertical cut at the trephine site. This helps a lot in terms of tissue recognition and stripping as the tissue can be held easily using cleavage hooks and forceps. The Moria trephine is required to be tapped on the endothelial side gently. This can be very subjective and therefore may sometimes create deep cuts that are further difficult to manage while stripping.

However, if the tissues stay in a deep base like the Barron trephine (Katena Products, Inc, Denville, NJ) (Fig. 2.1 (B)), irregular trephines may make stripping more difficult Because of curvature, we usually prefer the Barron trephine when the tissue needs a full trephination and not just a superficial cut like DMEK; therefore, it can be used in the surgical theatre while performing a second cut. Standardized superficial trephines from e.janach (Como, Italy) (Fig. 2.1 (C)) are available in larger diameters up to 10 mm; therefore, depending on the size of the graft that is required, although not used commonly, the e.janach trephine can be used for full-size grafts. Moreover, these trephines are calibrated, and therefore subjective tapping can be reduced over a single full forced trephination that is currently applied for penetrating keratoplasty. The calibrated trephines come with a full force depth of 200 mm. A normal trephine when tapped gently can still cut as deep as 300 mm, whereas the calibrated trephine, even after applying full force to trephinate the cornea, can cut a maximum of 150 mm. Although difficult but achievable, DMEK is possible after full-thickness trephination, but shallow trephination can reduce the effort during identification of the cleavage plane, therefore reducing graft wastage that occurs because of tears and inappropriate graft detachment. An “appropriate” cut is necessary to reduce tears and inappropriate “graft detachment. Different Descemet incision techniques using trephines are shown in Supplemental Video 1 (see Supple mental Digital Content, *http://links.lww.com/ICO/A551*).

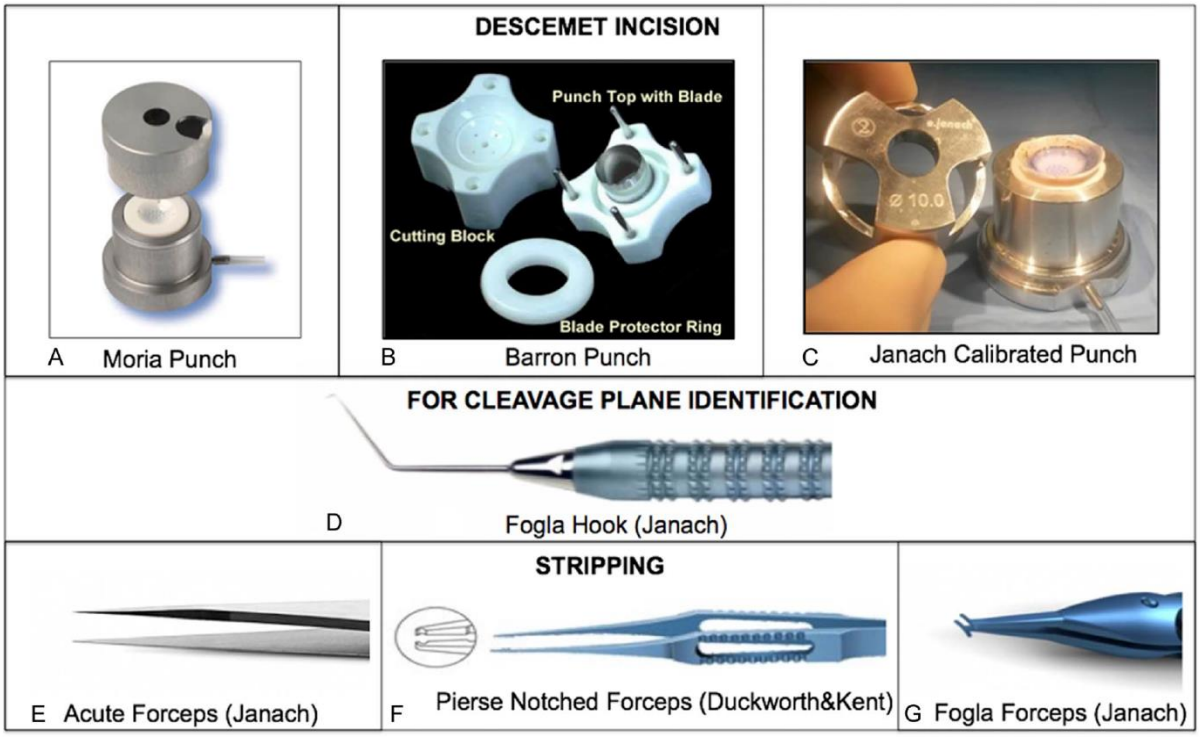

Figure 2.1 **Instruments required for DMEK excision**

Descemet incision can be performed using (A) Moria trephine, (B) Barron trephine, or (C) janach trephine. The cleavage plane can be identified using (D) the Fogla hook, whereas stripping can be performed using forceps, such as (E) acute forceps, (F) Pierse notched forceps, or (G) Fogla forceps depending on the technique to be used. Instruments required for DMEK excision.

2.2.3 Cleavage hook

It is important to use a cleavage hook when stripping is considered a choice for DMEK excision. The cleavage hook scores the periphery of the cut and ensures detachment of Descemet membrane with stroma. We use the cleavage hook to mainly de-adhere the DMEK graft at the trephined site. If de-adherence is not performed correctly, then the tissue can be left adhered at several points resulting in tears while peeling. It has been observed several times that with forced trephination of donor tissue, DM falls and sticks inside the stroma at the trephined site. It therefore becomes important to remove the tissue from the cut depth and lift it out for peeling.

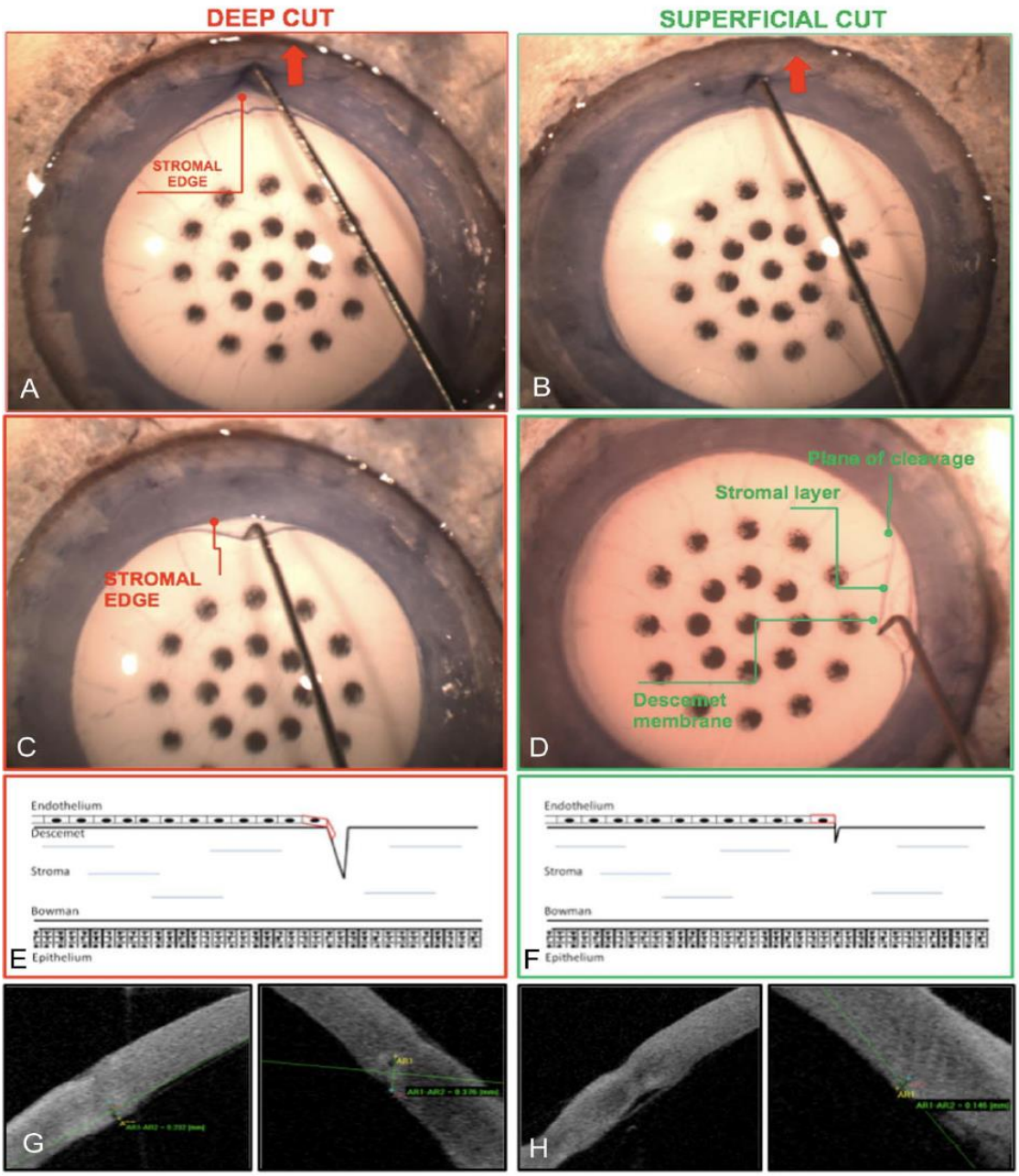

Figure 2.2 **Incision cut**

(A) deep versus (B) superficial that can be identified using a cleavage hook by pushing the edge further away. (C) Deep cut showing the stroma that can be identified using a cleavage hook; (D) superficial cut showing Descemet membrane, posterior stroma, and stromal layer. (E) in deep cut, the endothelial cells can enter the stromal layer that can be difficult to excise without obtaining tears against (F) the superficial cut where the endothelial cells stay on the surface. This can be observed using optical coherence tomography (OCT) scans with (G) deep and (H) superficial cuts.

However, if the cleavage plane is difficult to identify, a small part of the tissue from the peripheral end can be cut using a blade to identify DM and separate it from the stroma. Usually, in our setting, we de-adhere DM at the periphery radially in the clockwise direction making de-adherence easy with limited-to-no tears in the periphery. We prefer a Fogla Hook (e.janach), which is the only cleavage hook currently available that is specific for DMEK grafts for cleavage plane identification as shown in Figure 2.1 (D).

2.2.4 Stripping the tissue with defined forceps

The tissue can be stripped using specific forceps. We have used Acute forceps (0.12 mm straight pointed) (e.janach) (Fig. 2.1 (E)), Pierse notched forceps (Duckworth and Kent, Baldock, United Kingdom) (Fig. 2.1 (F)), and Fogla forceps (e.janach) (Fig. 2.1 (G)). The use of forceps usually depends on the technique that is used to peel the DMEK graft. If the DMEK graft is planned to be peeled radially, then acute forceps can be used. Although possible, using double forceps for peeling has been difficult in our experience. It takes great skill as the tissue must be held at 2 points and peeled simultaneously along the radial axis. If there is a mismatch in the hand movements, then the tissue can be torn easily. It was also noted that Pierse notched forceps and Fogla forceps serve as good candidates for peeling. However, we did not use them because these forceps touch a larger area of the endothelium and hence damage a higher number of cells. These forceps hold a larger area of DM, making it slightly difficult to grab the tissue and peel it radially. It is very subjective to explain such an event. We use acute forceps as they have served the best for us so far. Different stripping techniques are shown in Supplemental Video 2 (Supplemental Digital Content, *http://links.lww.com/ICO/A552*).

2.2.5 Understanding the cut planes

It is not difficult to misjudge a posterior stromal layer with that of DM, especially when there is no visually significant difference between a deep and superficial cut. It is important to analyse the cut before the stripping procedure, and trypan blue staining therefore becomes mandatory in this phase. We have found that when the cut is “deep,” the stromal edge is well defined compared with the “superficial” cut. It can be identified by pushing the trabecular meshwork superiorly to recognize the presence of the stromal layer as shown in Figure 2.2 (A) and (B). A thick layer of stroma can be easily observed in Figure 2.2 (C) along the stromal edge. Figure 2.2 (D) shows 2 different layers when the cut is performed superficially, indicating clear DM that is adhered to the posterior stroma.

Identification of DM makes it easier to detach the periphery and reduce any possible tear, further helping to initiate the main stripping phase. It has been observed that with deep cuts, the endothelium tends to enter the stromal layer because of the force of cutting (Fig. 2.2 (E)) compared with when the cut is superficially applied (Fig. 2.2 (F)). Deep cuts make it difficult to de-adhere DM and remove it from the stroma, which is tightly packed. Therefore, it is highly recommended to stay as superficial as possible, which can be achieved by a gentle tapping method or using a calibrated trephine. Optical coherence tomography images show the

amount of cut created by deep (Fig. 2.2 (G)) and superficial trephines (Fig. 2.2 (H)). The plane of cleavage identification is shown in Supplemental Video 3 (Supplemental Digital Content, *http://links.lww.com/ICO/A553*).

2.2.6 Stripping the tissue using the single-touch and peel method

A technique described by Tenkman et al shows that DMEK tissue can be peeled in sectors or quadrants. For example, for the right eye tissue, primary peeling starts from the superior end following the radial axis up to one-third of the tissue. The second part starts from the temporal side toward nasal, and the last one-third is opposite to the second part. The tissue is then peeled from the superior position to the inferior in total or leaving a hinge behind. This ensures minimal tearing and excises a complete DMEK graft easily. We adopted the technique from Tenkman et al with a slight modification whereby we excise the graft from the superior to inferior position without undergoing quadrant method or using multiple quadrant methods or oscillating movements in challenging cases. Although minimal touching of the endothelium results in minimal cell loss, it is insignificant considering that the periphery of the first peel will be removed before the second trephine. As shown in Figure 2.3 (A), we superficially trephine the tissue and remove the excess peripheral membrane. [It should be noted that the second trephine (8.00–8.50 mm diameter) is performed by the surgeon in the operating room. The peripheral 1 mm diameter is removed along with the hinge in this phase and the surgeon gets a DMEK graft equivalent to an optical zone]. As shown earlier, using a cleavage hook, the endothelium was scored circumferentially in the clockwise direction (Fig. 2.3 (B)), which has been shown to de-adhere the graft easily without any tear. DM (9.00–9.50 mm diameter) was further stripped leaving a hinge behind (Fig. 2.3 (C)) and restored on the cornea for maintenance of the stripped DMEK tissue and shipping purposes.

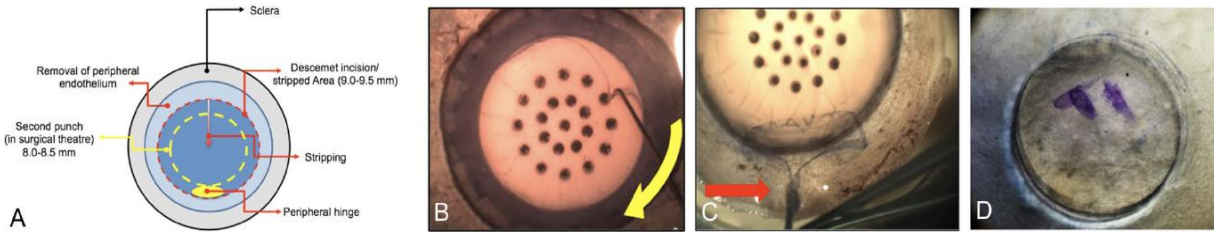

Figure 2.3 **Stripping technique showing**

(A) schematic representation of different cuts (peripheral and central) with a hinge. (B) Cleavage identification and scoring the periphery of DMEK using the cleavage hook in the clockwise direction (yellow arrow). (C) Stripping the DMEK tissue from superior to inferior leaving a hinge behind (red arrow) and (D) marking the tissue with letter "F" for tissue orientation identification.

Stripping by the quadrant method, challenging cases, and ways to overcome them are shown in Supplemental Video 4 (Supplemental Digital Content, *http://links.lww.com/ICO/A554*).

2.2.7 Marking the tissue

It has been observed that inverse grafting is one of the main causes of primary graft failure, especially when a DMEK is considered. Veldman et al have previously reported an "S" stamp on DM to reduce this issue and graft the tissue in the most precise and appropriate way with correct orientation. Therefore, to reduce the phenomenon of inverse grafting, we modified the "S" stamp into "F" as we mark with the help of a cleavage hook. Once the DMEK tissue is peeled, a skin biopsy punch (2 mm diameter) is used to make a trephine hole in the stroma. DM is restored on the cornea, and the cornea is inverted with the epithelial side facing the air. The trephined stromal part is removed, and using the Fogla hook and skin marker as dye, the letter "F" is marked on the Descemet side and the stromal piece is restored (Fig. 2.3 (D)). The pre-stripped and marked tissue is then ready for evaluation.

Step-By-Step Guide of Our Standardized Technique for DMEK Graft Preparation Using the Stripping Method

1. Donor corneal tissues were fixed on a vacuum block with the endothelial side facing the air after checking the ECD and mortality before stripping.
2. A donor trephine (9.5 mm, Moria) was gently tapped on the endothelial surface to obtain a superficial cut.
3. The cut margins were visualized using trypan blue.
4. Peripheral endothelium was removed and a small drop of tissue culture medium was added topically on the endothelium to keep it moist while stripping.
5. Cleavage hook (Fogla hook, e.janach) was used to identify the plane of cleavage (moved radially), slightly de-adhering the DMEK tissue at the periphery.
6. Acute forceps (0.12 mm straight pointed, e.janach) was used to peel the tissue from the superior side toward the inferior side. This step was performed either as a single-peel method or by quadrant method depending on the tissue difficulty. If the tissue was tightly adhered, then it was peeled using a quadrant method or oscillating fashion.
7. The DMEK tissue was peeled (80–90 %) leaving a small hinge behind, and it was left in the same position without restoring back on the cornea.

8. Small (2.0 mm) skin biopsy punch was used to trephine the stroma. The peeled DMEK tissue was replaced back on the cornea after stromal trephination using sterile sponges to reverse the flow of liquid and open the DMEK tissue on the cornea.
9. The vacuum was released and the entire corneal tissue was reversed with the epithelial side facing the air. The punched stromal part was removed exposing bare DM resting on the rest of the stroma.
10. The cleavage hook tip was coloured with a skin marker and the letter “F” was marked on DM.
11. The punched stroma was replaced back on the cornea and the tissue was evaluated for the ECD and mortality. Trypan blue stain (0.25 %) was applied on the top of the endothelial cells for 20 seconds and washed briefly with phosphate-buffered saline. The corneal tissue was placed in sucrose (hypotonic solution) for evaluation and measurement of ECD and TBPCs.
12. The tissue was fixed using a corneal claw and placed in the transport medium for shipment.

2.2.8 Evaluation of the pre-stripped tissues

Once the tissue is restored on the cornea, the entire tissue can be placed in a hypotonic sucrose solution with endothelium facing the bottom of the Petri plate that is in direct contact with sucrose (1.8 %). Hypotonic solution (sucrose) allows the intercellular borders to swell and makes it easier to count the endothelial cells. Trypan blue is a dye that can be used to stain the endothelial cells for checking the amount of TBPCs that indirectly refers to the number of necrotic or dead cells. It is used by the eye bank to check the endothelium and decide the suitability of the tissue for transplantation. The periphery of the cut can also be viewed after the trephine (Fig. 2.4 (A)), and as the cells can be viewed, they can also be counted before further preservation (Fig. 2.4 (B)) and the morphology checked in terms of polymegathism or pleomorphism at higher magnifications (Fig. 2.4 (C)). Sucrose and TBPCs is an evaluation technique that our eye bank has been using for a decade along with many other eye banks in Europe. In the United States, the evaluation method differs from the one that is used by our eye bank as they do not use TBPCs as a criterion of mortality and only check the number of cells using specular microscopy.

2.2.9 Stripping the tissues with tight adherence

Tight adherence has been reported in tissues from young donors or from the ones that have peg-like interlockings [19]. The difference in thickness and structure of the interfacial matrix zone or high amounts of adhesive glycoproteins has also contributed to tight adherence of Descemet membrane with stroma. A study by Greiner et al [20] showed that diabetes mellitus increases the risk of unsuccessful graft preparation. DM graft adherence to stroma can be handled by stripping DM slowly and in an oscillating fashion. This loosens the grip of DM to stroma and makes peeling slightly easier, avoiding large tears. In such tissues, the central optical zone is usually the tightest. Therefore, the central zone should be stripped gently with simple oscillating movements as described above, which can also be observed in Supplemental Video 4 (Supplemental Digital Content, *http://links.lww.com/ICO/A554*).

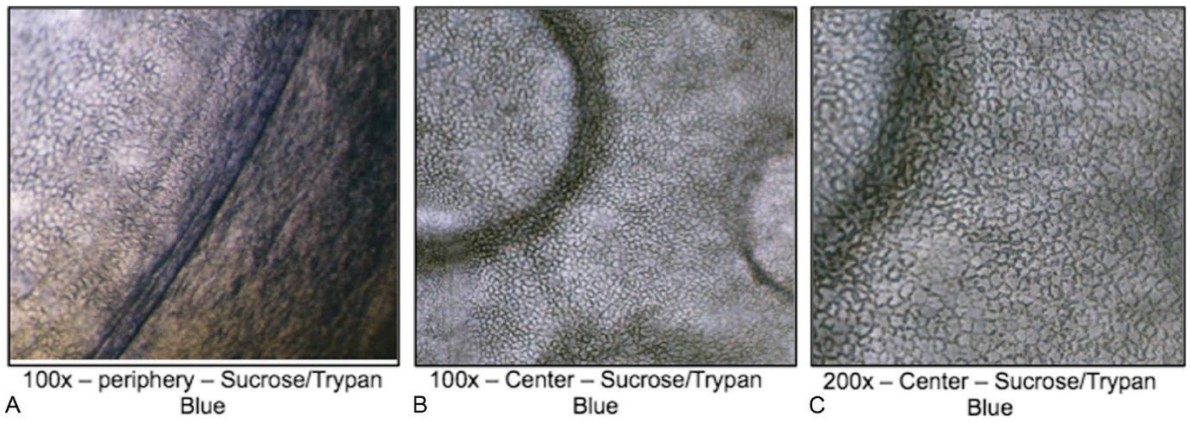

Figure 2.4 **Endothelial cell evaluation after trypan blue staining and sucrose to swell the endothelial cell borders**

(A) cut site, (B) 0·central endothelium after stripping at 100 magnification, and (C) 20 magnification.

2.2.10 Managing cut tears

As previously described by Tenkman et al, [17] a successful DMEK tissue can be prepared even after multiple horseshoe shaped tears (HSTs) that can form in DM. We have observed similar conditions in which we obtained an HST (Fig. 2.5 (A)). The horseshoe or other peripheral tear condition can be managed by either cutting a part or completely removing the cut area depending on the size of the HST. If there are multiple HSTs (0.5 mm), then it could also be managed by lifting peripheral DMEK upward followed by gentle stripping. This will produce a DMEK graft with peripheral cuts; however, the surgeon can still use the graft considering that the peripheral endothelium will be discarded after the second cut. The peripheral tears can be managed; however, if the tissue is torn in the optical zone, then it cannot be further used and the tissue is usually discarded. One of the contraindications of tissue

selection for DMEK is the donor tissue with previous cataract surgeries. Performing DMEK excision in a cornea having an incision from previous cataract can be dangerous in terms of tissue tears (Fig. 2.5 (B)). The incision areas are usually outside the optical zone, and therefore centralized DMEK should not be considered for such an excision. Rather, a decentralized DMEK can be a good option to ensure removal of the incision site from the preparation area (Fig. 2.5 (C)). This can lead to better graft excision and lower tissue wastage and further involve more corneas that are rejected for DMEK because of previous cataract interventions. This technique does not involve any major endothelial cell damage in the periphery (Fig. 2.5 (D)) or centrally (Fig. 2.5 (E)).

2.2.11 DMEK failure

Excision of DM can fail in many cases. Peripheral tears are the most common complications, and if not taken care of, they can lead to partial tear leading to complete tearing. For the surgeons who require DMEK rolls, the tissue can be stripped completely; however, for the surgeons requesting a DMEK tissue with an attached hinge, DMEK preparation failure usually is seen when the tissue is completely excised and there is no hinge left. This results in a free-floating DMEK tissue as a roll and can still be used as a roll for transplantation purpose. This condition usually occurs either when the tissue is being stripped to the end or while the tissue is being restored on the cornea but with more force than usual. Therefore, it is advised to replace the tissue gently until the tissue is completely restored on the cornea. DMEK can also fail if the tissue is tightly adhered. If the tissue is still stripped with radial movements or using different sites, it may face a lot of tearing leading to a total tear and cannot be further used as a DMEK tissue. If the tissue is peeled successfully using multiple sites and locations, the tissue will show greater endothelial damage. It is advised to use gentle oscillation movements while stripping such highly adhered tissues. DMEK failure cases and techniques to overcome them are shown in Supplemental Video 5 (Supplemental Digital Content, *http://links.lww.com/ICO/A555*).

2.3 Results

So far, at the Veneto Eye Bank Foundation, we have pre-stripped 527 DMEK tissues since May 2014. Ages of donors for DMEK grafts have been older than 65 years with ECD > 2500 cells/mm^2 and time in TCM + dextran of 24 to 48 hours with major contraindications being diabetes mellitus and previous cataract surgery. TBPCs on average of 1 % have been observed so far with tissue wastage of 6 % that included central or peripheral

tears, total DM detachment, and strong adherence. Our first validation using the stripping method resulted in 2 % ± 2.7 % TBPCs after the preparation phase (n = 10). Endothelial cells before stripping (Fig. 2.6 (A)) did not show any TBPC, minimal cell loss was found after stripping (Fig. 2.6 (B)) and after 7 days of preservation (Fig. 2.6 (C)) showed no TBPCs when the tissues were preserved in transport media. However, an overall endothelial cell loss (ECL) noted was 4.6 % because of morphological changes that occur during these phases followed by loss of cells. It was also observed that marking the tissue with an "F" leads to around 0.5 % TBPCs overall with maximum mortality seen at areas where the "F" is marked using gentian violet

(Figs. 2.6 (D) and (E) compared with the central endothelium (Fig. 2.6 (F)) where there is no marking.

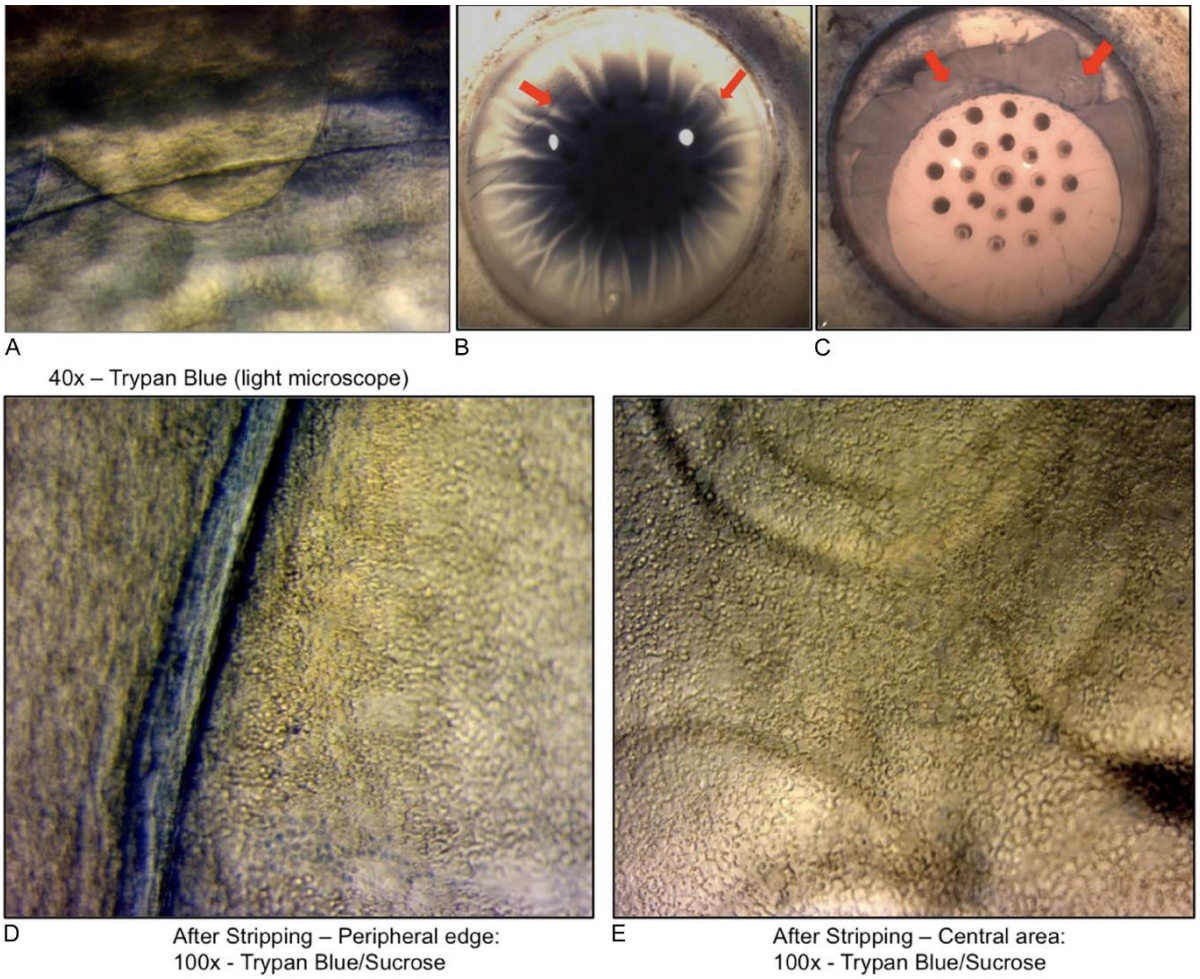

Figure 2.5 **Challenging cases with**

(A) horseshoe-shaped tear, (B) cataract incision that can be left in the periphery initiating the stripping away from the incision site, (C) DMEK tissue stripped away from the incision site previously formed because of cataract. Proper DMEK can be excised without any tear or endothelial cell damage. (D) Peripheral edge showing minor mortality after stripping at 10 magnification and (E) central endothelium showing no mortality and maintenance of endothelial cells after performing DMEK excision in challenging cases such as previous cataract incision.

2.4 Discussion

Donor tissues have previously been prepared with 9.5 mm diameter. Considering pneumatic dissection or liquid separation where the graft size can easily be obtained up to 10 mm, using our standard protocol, we prepare the tissues with 9.5 mm diameter in the eye bank, and the final diameter can be decided by the surgeon based on the size of the descemetorhexis or the eye.

Although a 9.5 mm graft can be transplanted, many surgeons prefer a graft of 8.25 to 8.50 mm.

We believe that preparing a larger diameter graft would allow not only transplantation of more cells but also so-called putative stem cells that are assumed to be at the periphery of the cornea, and therefore, although speculative, such a procedure may result in higher graft survival, as also discussed by Anshu for DSAEK and Romano et al for ultrathin DSAEK.

There is noted endothelial cell mortality during the stripping procedure as the endothelium is touched by the forceps at the periphery. During the second trephine at surgery, the dead cells in the periphery are eliminated, and therefore the surgeon gets a viable tissue, which is the central zone.

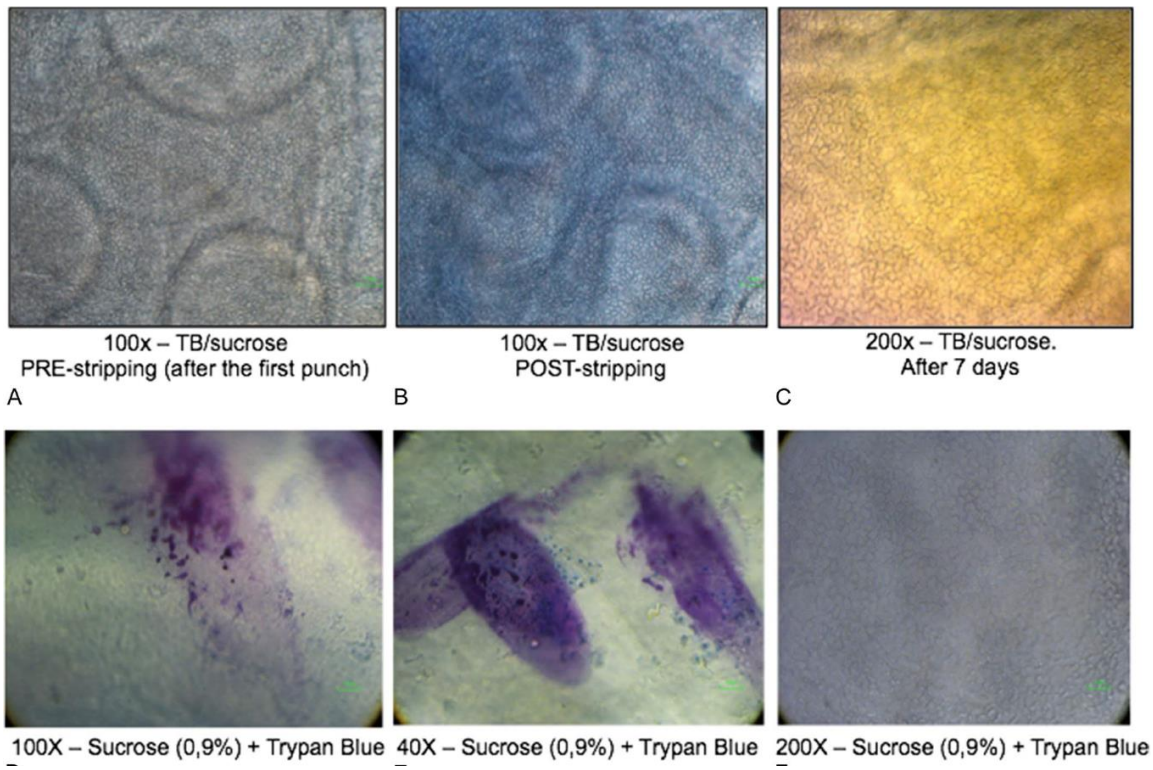

Figure 2.6 **Endothelial cells during the validation study showing**

(A) endothelium before stripping (after the first trephine), (B) after stripping – 100 magnification, and (C) stripped DMEK tissue left with a hinge after 7 days of preservation in the transport at medium at 200 magnification. Endothelial cell damage after "F" mark on different tissues (D and E) at 4 magnification and (F) 20 magnification showing central endothelium without any trypan blue-positive cell. Mortality was usually found near the F mark (0.5 %).

Although this ECL is not huge at the periphery and the number of cells that are transplanted with the larger diameter graft is more, it could also be recommended to graft a larger diameter DMEK tissue to increase the chances of more endothelial cells and possibly longer graft survival. Moreover, preparing a larger diameter graft was noted to be more feasible compared with a smaller diameter graft considering that the tissue is tightly adhered to the centre and initiating stripping from the tightly adhered part was found to be risky. We also note that inter- and intra-personnel counting of endothelial cells and mortality per square millimetre could be the limitation of this study.

However, although only experienced eye bankers have been preparing the DMEK grafts in our eye bank (A.R., M.B., and E.F.), we strictly assume that the calculations for ECD would be appropriate, considering that we practice a manual counting technique. Apart from DMEK excision, it is important to find an appropriate graft loading technique and instrument for implantation. There are a few techniques that have been introduced for graft loading, of which only 2 methods have been used so far, endothelium rolled outward and endothelium flapped inward. Currently, most DMEK grafts are aspirated inside a modified glass tube with the endothelium on the outside of the rolled surface. This is due to the spontaneous rolling of DM when in fluid, which increases the risk of endothelial damage during injection and during manipulation inside the eye, the latter being technically difficult.

However, recently Busin and our group, have reported a technique of flapping the endothelium inward, which is not natural, but it opens up spontaneously inside the recipient eye and therefore serves an additional advantage to the surgeon without any significant ECL that has been noted so far. Therefore, apart from identifying the best suitable technique for excision of a DMEK graft, it is also important to understand the most appropriate method to load a graft and further implant it, be it the endothelium-in or endothelium-out techniques for loading; or injection or bimanual pull-through techniques for implantation. We believe that the techniques described above will help new surgeons to decide the best suitable instruments and improve their tissue preparation and surgical skills even while handling challenging cases. The stripping technique has resulted in less endothelial damage and tissue wastage, and therefore it is highly recommended. [50, 51, 52]

If the corneas from previous cataract incision cases and HST along with those DMEKs that are tightly adhered to the stroma due to diabetes can be used using simple tricks, then the donor pool will increase automatically. Therefore, it is recommended to plan the excision technique and tools before starting peeling if the contraindications are previously known because it will help reducing the effort while peeling.

3 Study 3

Following a standardization of DMEK surgery technique showed in Study 2, Study 3 compares different DMK graft preparation techniques.

Abstract in English

Title: A comparative study on different Descemet membrane endothelial keratoplasty graft preparation techniques.

Purpose: To compare different Descemet membrane endothelial keratoplasty (DMEK) graft preparation methods.

Methods: Stripping from the trabecular meshwork (M1) using epithelial spatula; stripping by scoring the peripheral endothelium (M2) using Sinskey hook; stripping by punch method (M3) using donor trephine; Submerged hydro-separation (M4); and pneumatic dissection method (M5) were evaluated. Preparation time, costs, endothelial cell loss (ECL) post-preparation, cell death and morphology were compared. Hoechst / Ethidium / Calcien AM (HEC) staining and Zonula Occludens-1 (ZO-1) expression were analyzed. Statistical analysis was performed using one-way ANOVA and; Tukey as post hoc test.

Results: A total of 35 corneas (seven per group) were used. Endothelial cell loss (ECL) represented as Mean (SD), in M1, M2, M3, M4 and M5 was 2.7 (5.0), 3.0 (7.4), 1.2 (7.4), 3.3 (7.3) and 4.1 (7.1) %, respectively not showing any difference between the groups ($p = 0.96$). A significantly higher cell death ($p < 0.05$) was observed in M4 and M5 compared with M1, M2 and M3. Graft preparation time was significantly shorter in M4 and M5 and longest in M3 ($p < 0.05$). M3 was the most expensive preparation technique. Minimum pleomorphic cells were observed in M1, M2 and M3, whereas moderate pleomorphism was seen in M4 and M5. Hoechst, Ethidium homodimer and Calcein AM (HEC) staining showed high Ethidium positivity (dead cells) in M4 and M5 with minimum positivity in M1, M2 and M3. Zonula Occludens-1 (ZO-1) was expressed in all the conditions except the denuded areas.

Conclusion: Graft preparation using Sinskey hook (M2) and donor punch (M3) are reliable methods in terms of efficiency and quality with acceptable range of ECL. The preparation time and associated costs could be a limitation for M3.

Keywords: DMEK; comparison; graft preparation.

Abstract in Latvian

Ņemot vērā otrajā pētījumā aprakstīto standartizēto DMEK ķirurģijas tehniku, trešais pētījums savstarpēji salīdzina dažādas DMEK transplantāta sagatavošanas tehnikas.

Nosaukums: Salīdzinošs pētījums par dažādām descemeta membrānas endoteliālās keratoplastikas transplantāta sagatavošanas metodēm.

Mērķis: Salīdzināt dažādas DMEK transplantāta sagatavošanas metodes.

Materiāli un metodes: Materiāla ņemšana no trabekulārā tīkla (M1) ar epiteliālo lāpstiņu; materiāla ņemšana, izvērtējot perifēro endotēliju (M2), lietojot *Sinskey* āķus; materiāla ņemšana ar perforēšanas metodi (M3), izmantojot donortrefīnu; iegremdēta materiāla hidroatdalīšana (M4); pneimatiska disekcijas metode (M5). Tika salīdzināts metodes ilgums, izmaksas, ECL pēc sagatavošanas, šūnu bojāeja un morfoloģija. Tika analizēta HEC krāsošanas metode un ZO-1 ekspresija. Statistikas datu analīze tika veikta, izmantojot vienfaktora dispersiju analīzi (ANOVA) un Tukey Post Hoc testu.

Rezultāti: Kopā tika izmantotas 35 radzenes (7 katrā grupā). ECL, kas izteikts kā vidējais (SD), M1, M2, M3, M4 un M5 bija 2,7 (5,0), 3,0 (7,4), 1,2 (7,4), 3,3 (7,3) un 4,1 (7,1) %, attiecīgi nepierādot statistiski nozīmīgu atšķirību staro grupām ($p = 0,96$). Ievērojami augstāka šūnu bojāeja ($p < 0,05$) novērojama M4 un M5, salīdzinot ar M1, M2 un M3. Transplantāta sagatavošanas laiks bija ievērojami īsāks M4 un M5, visilgākais – M3 ($p < 0,05$). Visdārgākā sagatavošanas tehnika bija M3. Minimālais pleomorfo šūnu skaits tika novērots M1, M2 un M3, savukārt mērens pleomorfisms tika novērot M4 un M5. HEC krāsošana uzrādīja augstu etīdija pozitivitāti – bojā gājušās šūnas – M4 un M5, minimālu pozitivitāti – M1, M2 un M3. ZO-1 tika ekspresēta visos apstākļos, izņemot bojātos apgabalus.

Secinājumi: Transplanta sagatavošana, izmantojot *Sinskey* āķi (M2) un donora perforatoru (M3), ir uzticamas metodes efektivitātes un kvalitātes ziņā ar pieņemamu ECL diapazonu. Sagatavošanas laiks un saistītās izmaksas varētu būt ierobežojums M3 izmantošanai.

Atslēgas vārdi: DMEK; salīdzinājums; transplantātu sagatavošana.

3.1 Introduction

DMEK offers early rehabilitation with optimal visual acuity (VA). Its minimal invasive nature and low immunological rejection rate make this technique favourable for surgeons.

Even with these advantages, the technique still requires refinement in terms of graft preparation, delivery, unfolding and positioning in the recipient eye. Recently, the eye banks have been providing pre-stripped DMEK tissues that aim to reduce the surgical efforts, time

and costs in the theatre. With such requests, the eye banks are challenged to find a standardized method in terms of feasibility of preparation, good graft quality, availability of the instruments and associated costs. Earlier, air bubble and liquid bubble techniques to separate the stroma and Descemet membrane (DM) have been described. Groeneveld-van Beek et al. also suggested no-touch method for DMEK stripping. It was noted from the literature that the bubble and stripping techniques have been used for DMEK graft preparation. [53,54,55,56]

The following study therefore describes five methods that are slight modifications of the conventional bubble and stripping techniques.

We further determine the effects of each technique on endothelial morphology and cell loss with an intention to highlight the most feasible option in terms of costs, quality and efficacy allowing eye bankers and surgeons to optimize the best choice for DMEK graft preparation.

3.2 Materials and methods

3.2.1 Ethical statement

Human donor corneas [n = 35] were collected from the Veneto Eye Bank Foundation, Mestre, Italy (FBOV) with written consent from the donors' next-of-kin for use in research. The study followed the tenets of 2013 Declaration of Helsinki.

3.2.2 Donor characteristics and tissue preservation

Donor characteristics were obtained from the FBOV database. All the corneas were preserved at 31 °C in tissue culture medium (TCM) that was composed of 2 % newborn calf serum with MEM-Earle as a base medium along with 25 mm HEPES Buffer, 26 mm sodium bicarbonate, 1 mm pyruvate, 2 mm glutamine, 250 ng/ml amphotericin B, 100 IU/ml penicillin G and 100 mg/ml streptomycin. TCM was prepared at FBOV with full regulatory compliance.

3.2.3 Endothelial evaluation

The endothelium of all the samples was stained using trypan blue (0.25 %) for 20 seconds and washed with 1X phosphate-buffered saline (PBS). The corneas were placed in a sterile petri dish containing a hypotonic sucrose solution (to increase definition of cell borders) with the epithelium uppermost. To estimate endothelial cell density (ECD), the cells in five 1-mm^2 squares of a 10 9 10 mm reticule inserted in the eyepiece of an inverted microscope (Primovert; Zeiss, Jena, Germany) were counted manually at 1009 magnification. The number of blue-stained (trypan blue positive cells – TBPCs) cells allowed percentage cell

death to be determined. The ECD and cell death were counted both, before and after DM stripping.

3.2.4 Alizarin red staining

After DMEK preparation, the endothelium was stained (immersed) with alizarin red S (Sigma Aldrich – A3757, St. Louis, MO, USA) for approximately 5 min, washed with PBS and viewed at 1009 magnification using an inverted microscope to observe the morphology (pleomorphism, polymegethism and hexagonality) of the cells.

3.2.5 Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live / dead cells

The tissues after trypan blue staining were washed with PBS prior to Hoechst, Ethidium homodimer and Calcein AM (HEC) staining. 4 ll of Hoechst 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), 4 ll of Ethidium Homodimer EthD-1 (E) and 2 ll of Calcein AM (C) (Live / Dead viability/ cytotoxicity kit; Thermo Fisher Scientific) were mixed in 1 ml of PBS. About 300 ll of the final solution was directly added on the endothelium of the DMEK tissue resting on the cornea and incubated at room temperature (RT) in the dark for 30 min. The DMEK lenticule was excised and placed on a slide with the endothelium uppermost. Four radial cuts were made to obtain a flat mount of the tissue on the slide and covered with coverslips without the mounting medium. The endothelium was viewed at 1009 magnification of Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam, the Netherland) using NIS Elements software (Nikon).

3.2.6 Expression of tight junctions using Zonula Occludens-1 (ZO-1) marker

The tissues were washed with PBS and fixed in 4 % paraformaldehyde (PFA) at RT for 10 min. The cells were permeabilized with 0.1 % Triton X-100 in PBS for 10 min. After blocking with 1 % bovine serum albumin for 1 hr at RT, the tissues were incubated with 0.1 % bovine serum albumin and primary antibody [anti-ZO-1, 1:200 (ZO1-1A12 Alexa Fluor 488 conjugate; Invitrogen, Carlsbad, CA, USA)] for 3 hr at RT. After each step, the cells were washed thrice with 19 PBS. The tissue was flat mounted on the slide and fixed with a mounting medium containing 40, 6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Cells were examined under Nikon Eclipse Ti-E (Nikon) microscope at 1009 magnification and the images were captured with NIS Elements software (Nikon). The study was divided into the following five groups, each with seven corneas. M1 was performed by Davide Borroni, M2 by Vito

Romano, M3 by AR, M4 and M5 by Mohit Parekh. Each technician has prepared more than 50 tissues with the method they were assigned and therefore their level of experience was deemed suitable for this study.

3.2.7 Stripping from the trabecular meshwork

A cornea was centred on a punch base using suction; however, it is not mandatory to use a suction base for this method. The tissue was stained with trypan blue for ease of visualization for 1 min. The point of initiation (PoI; first point of contact on cornea by instrument for making incision) was 1 mm away from the trabecular meshwork (TM) on the sclera. An epithelial spatula (Blink Medical Ltd., Birmingham, UK) was used to swipe the TM towards the cornea detaching the peripheral endothelium as observed in Fig. 3.1 (A) and (B). The TM was excised completely exposing the periphery of the DMEK graft (Fig. 3.1 (C)). The DMEK was grasped at its periphery using a 120 mm straight pointed acute forceps (e.Janach, Como, Italy) and peeled using different peeling sites to release the DMEK tissue avoiding peripheral tears (Fig. 3.1 (D)). Note that trypan blue stain remains on the tissue keeping the tissue moist and there is no liquid (TCM) on the endothelium. The preparation method is shown in Video S1.

3.2.8 Stripping by scoring the peripheral endothelium

A cornea was centred on a punch base using suction; however, it is not necessary to use the suction base in this method. The PoI of this technique is at the periphery of the DMEK graft, just before the TM as seen in Fig. 3.1 (E) - (F). An intraocular lens (IOL) manipulator [Sinskey] hook with blunt tip (Beaver-Visitec International Ltd., Warwickshire, UK) was used to score the peripheral circumference on the endothelial side of the cornea (Fig. 3.1 (G)) detaching the periphery of the DMEK for further peeling. About 120 mm straight pointed acute forceps (e.Janach) was used to peel the DMEK graft using a single peel (superior to inferior) method (Fig. 3.1 (H)). The cells were kept moist with a single drop of PBS and were not totally submerged in the liquid. In challenging cases where the tissue was tightly attached to the stroma, multiple quadrant method (where the edge of the DMEK tissue is grasped at different sites and peeled in parts obtaining a single graft) was used. Note that unlike method 1, the tissue can be stained to see the scored area if necessary.

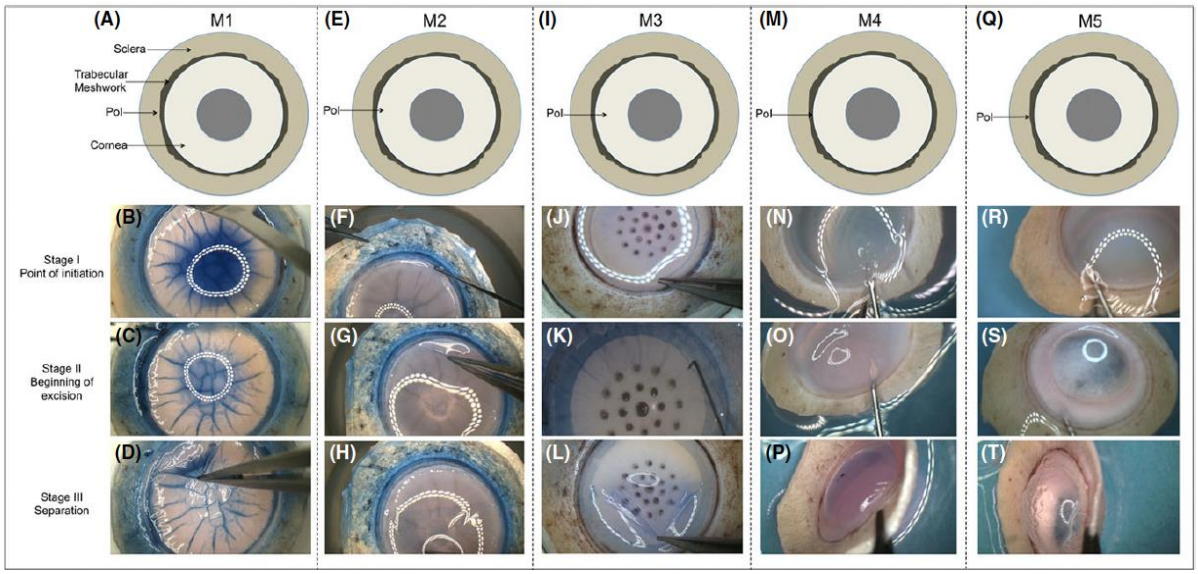

Figure 3.1 **Schematic representation, stages I, II and III of different Descemet membrane endothelial keratoplasty (DMEK) graft preparation methods**

M1 represents the technique that uses an epithelial spatula to swipe the DMEK graft all the way from the trabecular meshwork (TM). (A) Point of Initiation (PoI) is shown just before the TM, (B) PoI on a donor cornea, (C) Excision begins after removing the TM at the periphery of the DMEK graft and (D) Separation of a DMEK graft from the underlying stroma is carried out using acute forceps using stripping method. M2 represents the technique that uses a Sinskey hook to score the peripheral endothelium. (E) Point of initiation (PoI) is shown at the periphery of the endothelium just before the TM on the corneal side. (F) Point of initiation (PoI) near the TM, the donor endothelium is scored using a Sinskey hook along the circumference of the cornea and (G) Separated from the stroma followed by (H) excision of DMEK graft using acute forceps using stripping method. M3 represents the technique that uses a donor punch. (I) Point of initiation (PoI) 9.5 mm from the central cornea, (J) Donor punch that is used for superficial tapping on the endothelial side, (K) Cleavage hook used to separate the DMEK graft from the trephined site and (L) Acute forceps used to strip and excise the DMEK graft from the cornea. M4 represents the submerged hydro-separation technique. (M) Point of initiation (PoI) is just beneath the TM, (N) A needle of the syringe is advanced in the stroma-DM plane until the bevel is completely inside, (O) with moderate to high pressure, the liquid is forced in the plane to create a liquid bubble, (P) a full-length liquid bubble separating the stroma with DM. M5 represents the pneumatic dissection technique. (Q) Point of initiation (PoI) is from the sclera just outside the TM, (R) a 25G needle of the syringe is advanced tangentially, (S) an air bubble is initiated with full force, if there is a central air bubble with stromal lamellae filled with air, the PoI can be changed to another site and the bubble is generated, (T) A full length air bubble is thus created. The circle with white dots is an impression originating from the LED light fixed on the microscope.

3.2.9 Stripping technique after creating a superficial trephine cut

A cornea was centred on a punch base using suction. Vacuum was created using a syringe and the cornea was secured on the base. The PoI of this technique can vary depending on the donor punch (8.25–10.00 mm diameter) that is selected. For this study, we used our standard protocol with a 9.5 mm donor trephine making it the PoI (Fig. 3.1 (I)). The endothelium was gently tapped with the trephine (Moria, Antony, France) to make a superficial cut (Fig. 3.1 (J)). Trypan blue was applied on the endothelium for about 20 seconds to determine the trephined zone. Excess peripheral endothelium was removed using 120 mm straight pointed acute forceps (e.Janach) and the tissue restained to help see the nonendothelial part. The endothelium was kept moist during the entire procedure using TCM to create a film

of fluid on the top of the endothelium. The membrane was slightly lifted at the periphery using a cleavage hook, and the procedure was continued along the entire circumference of the trephined area to ensure limited peripheral tearing while peeling (Fig. 3.1 (K)). The DMEK tissue was stripped with a longitudinal movement using a 3-quadrant method, ensuring no stress lines were generated, thus reducing cell death. Once the DM was stripped, leaving a peripheral hinge, it was laid back onto the stroma (Fig. 3.1 (L)) as previously described. The method is shown in Video S3. For Methods 1, 2 and 3, the DM was not completely stripped but left attached by a peripheral hinge. The DM was laid back onto the stroma for morphological and molecular analysis as described above.

3.2.10 Submerged hydro-separation (SubHyS)

In this technique, a cornea was submerged in a sterile petri dish half-filled with the TCM to keep the endothelium moist throughout the procedure. The tissue was held at the sclera with sterile forceps. A needle (25 gauge) connected to a 1 ml syringe was inserted beneath the TM (PoI; Fig. 3.1 (M) and (N)) and advanced radially in the posterior stroma or stroma–DM interface until the bevel of the needle was completely inserted. Tissue culture medium (TCM) from the syringe was injected into the stroma or the DM-stroma interface with pressure enough to separate the DM and the stroma. A small, clear bubble was visible (Fig. 3.1 (O)) with the initiation of the process. The bubble was enlarged with an increased pressure to achieve an approximately
10–11 mm diameter bubble (Fig. 3.1 (P)). The liquid was removed by sucking the liquid back in the syringe (Parekh et al. 2014), and the tissue was used for further analysis.

3.2.11 Pneumatic dissection

In this technique, a cornea was placed in a sterile petri dish with endothelial side upwards. Using a 25G needle attached to a 1 ml syringe, the needle was advanced in a tangential direction from the limbus (Fig. 3.1 (Q)) at the stroma-DM interface up to 2 mm (Fig. 3.1 (R)). Air was injected to obtain a detachment of DM and stroma (Fig. 3.1 (S)), and the bubble was enlarged up to the corneal periphery (Fig. 3.1 (T)). If the bubble was not achieved, then another PoI was considered and the bubble was enlarged from that site until a complete bubble was obtained. The air was removed by sucking it back in the syringe and the tissue was used for further analysis.

3.2.12 Time and cost analysis

The time for the preparation of graft was monitored by stopwatch, and the costs were analysed based on the most expensive instruments used in the technique.

3.2.13 Statistical analysis

Comparisons between these groups were performed by one-way ANOVA followed by Tukey test as a post hoc analysis test. Data were deemed significantly different when $p < 0.05$.

3.3 Results

3.3.1 Donor characteristics

Average age of the donors, gender, post-mortem hours, ECD, number of days the tissue was stored in TCM at 31°C and cause of death from each group are listed in Table 3.1.

3.3.2 Preparation time

The time required to prepare the grafts in all the groups was statistically significant ($p < 0.05$; Table 3.2). It was observed that M3 required longer graft preparation time compared with any other methods; M4 and M5 required the least time (Fig. 3.2, Table 3.2). M1 and M2 required almost same time to prepare the graft.

3.3.3 Tissue wastage

There was no total tissue wastage from M1, M2 and M3. Two tissues were partially peeled (60 %) from M1 (although not lost completely) due to tight attachment of the DM to stroma compared with others. M2 and M3 did not show any tissue wastage. One tissue was lost due to overfilling of stroma with liquid and not forming a liquid bubble in M4. Two tissues were lost due to stromal swelling caused by air, which did not generate a bubble in M5. There were three sites used for pneumatic dissection to generate air bubbles but all three failed in M5. The wasted tissues were not considered in this study, and other tissues were obtained for evaluation purposes using the same technique.

3.3.4 Endothelial cell density, cell loss, cell death and morphology

Minimal cell death (assessed by trypan blue, Fig. 3.3) was observed in M1 (Fig. 3.3 (A)), M2 (Fig. 3.3 (B)) and M3 (Fig. 3.3 (C)). Both M4 (Fig. 3.3 (D)) and M5 (Fig. 3.3 (E)) resulted in higher cell death than the other methods with M5 showing the highest

percentage of dead cells (Fig. 3.4, Table 3.2). Cell death was statistically significant ($p < 0.05$) between the groups (Table 3.2). Endothelial cell loss (ECL) was found to be highest in M5, however, there was no significant difference between the groups (Table 3.2). The morphology in all the conditions did not differ pre- and post-preparation; however, there were some pleomorphic cells observed after Alizarin red staining (Fig. 3.5 (A)–(E). Denuded areas with no endothelial cells were noted after Alizarin red staining especially in M4 (Fig. 3.5 (D)) and M5 (Fig. 3.5 (E)). As the DM was laid back onto the stroma and analysis was carried out without further preservation just after stripping, no cellular overgrowth on the DM side could have possibly occurred in this study.

3.3.5 Cost

Bubbling techniques (M4 and M5) were found to be least expensive with < an approximate cost of $1 (majorly being the syringe) compared with the stripping methods that used an epithelial spatula ($8) or Sinskey hook ($10). The most expensive technique was M3 that used donor punches costing up to $150 also mentioned in Table 3.3.

3.3.6 Hoechst, Ethidium Homodimer and Calcein AM staining

Triple endothelial cell labelling with Hoechst 33342 (H), Ethidium Homod imer (E) and Calcein AM (C) showed the presence of ‘E’ in red representing the dead cells, blue representing the nuclei ‘H’ and green indicating the viable cells ‘C’. Higher numbers of dead cells were observed in M4 (Fig. 3.6 (D) and M5 (Fig. 3.6 (E) compared with M1 (Fig. 3.6 (A)), M2 (Fig. 3.6 (B)) and M3 (Fig. 3.6 (C)). The number of apoptotic cells was very low and as the cells were compact it was difficult to evaluate the exact number of apoptotic cells in different conditions.

3.3.7 ZO-1 staining

Expression of ZO-1 was observed in all the conditions M1 – M5 (Fig. 3.7 (A) – (E) respectively) and the hexagonality was partially maintained in all the cases wherever the cells were present. The presence of ZO-1, a component essential for tight junction integrity, was confirmed at cell borders and the morphology correlated with Alizarin red staining. There were small, denuded areas due to the detachment of endothelial cells where ZO-1 was not expressed.

Table 3.1

Donor characteristics in terms of age, gender, post-mortem hours, endothelial cell density, graft storage time and cause of death from corneas used in each methods M1–M5

	Age (years)	Male/Female	Post-mortem (hours)	ECD (cells/mm^2)	Graft storage time (days)	Cause of death
M1	75.0 (3.3)	6/1	16.8 (5.3)	2042 (113.3)	26 (3.1)	1 Prostatic tumour 2 haemorrhagic shock 3 Lung tumour 4 Lung tumour 5 Ischaemic heart disease 6 Ovarian cancer 7 Liver Metastasis
M2	73.3 (2.5)	4/3	12.5 (4.1)	1971 (48.8)	26 (3.6)	1 Hepatocellular carcinoma 2 Cardiac arrest 3 Gastric cancer 4 Hepatocellular carcinoma 5 Lung tumour 6 Hepatocellular carcinoma 7 Ovarian cancer
M3	73.0 (4.1)	5/2	10.7 (6.9)	1975 (95.7)	27 (3.2)	1 Cerebral haemorrhage 2 Sepsis and hypertensive 3 Acute respiratory failure 4 Chronic cardiovasculopathy 5 Lung tumour 6 Cardiac arrest 7 Cerebral haemorrhage
M4	65.8 (10.4)	5/2	13.7 (6.3)	1960 (54.8)	28 (4.0)	1 Liver metastasis 2 Cardiac arrest 3 Advanced oesophageal cancer 4 Prostate neoplasia 5 Cerebral haemorrhage 6 Pulmonary carcinoma 7 Ovarian cancer
M5	72.1 (3.9)	4/3	10.1 (3.6)	2042 (78.7)	28 (2.5)	1 Chronic cardiovasculopathy 2 Sepsis and hypertensive 3 Malignant melanoma 4 Suicide 5 Ovarian cancer 6 Cardiac arrest 7 Prostatic tumour

All the data are presented as Mean (SD) except the data on gender. ECD = endothelial cell density.

Table 3.2

Comparison between different graft preparation methods:
1st column cell death evaluated with trypan blue, 2nd column ECD evaluated with light microscope, 3rd column overall endothelial cell loss, 4th column time evaluated with stopwatch for each method

	Dead cells (%) Mean (SD) [Range]	ECD (cells/mm^2) Mean (SD) [Range]	ECL (%) Mean (SD) [Range]	Time (min) Mean (SD) [Range]
M1	1.29 (0.96) [0.3–3]	1928 (75) [1800–2000]	2.71 (4.99) [0–10]	9.43 (3.91) [4.5–15]
M2	0.21 (0.17) [0–0.5]	1921 (90) [1800–2000]	2.96 (7.42) [0–14.2]	7.64 (3.37) [4.5–14]
M3	0.13 (0.14) [0–0.4]	1957 (97) [1800–2100]	1.18 (7.37) [0–4.76]	19.41 (2.77) [15–23.5]
M4	8.71 (2.14) [8–12]	1914 (89) [1800–2000]	3.34 (7.25) [0–10]	0.27 (0.14) [0.1–0.4]
M5	11.00 (3.11) [8–16]	1900 (81) [1800–2000]	4.06 (7.06) [5–10]	0.17 (0.16) [0.7–0.2]
p Value	<0.05*	0.96	0.96	<0.05*

ECD = endothelial cell density, ECL = endothelial cell loss.
* Statistically significant values.

3.4 Discussion

Descemet membrane endothelial keratoplasty (DMEK) has shown advantages such as early visual rehabilitation rates and outcomes. Endothelial keratoplasty using precut for Descemet stripping automated endothelial keratoplasty (DSAEK) or prestripped for DMEK grafts have shown increased interest recently, and the eye banks are increasingly requested to produce these challenging grafts. [57, 58, 59, 60]

The most suitable method for DMEK graft preparation has become an area of debate. Although protocols to standardize graft excision and transplantation have been introduced earlier; however, as there are many procedures followed for DMEK, an affordable and easy to prepare a good quality standard graft must be identified, as this is one of the reasons why uptake of DMEK by surgeons is growing at a slower rate than DSAEK or penetrating keratoplasty (PK). Eye bank technicians have experience preparing a significant volume of corneas for selective transplantation on a daily basis, and therefore a pre-prepared graft from the eye bank could be a valuable option for the surgeons to reduce the graft preparation time, tissue wastage and overall costs in the surgery. [61, 62, 63, 64]

Using pre-prepared tissue may, therefore, facilitate smoother surgery. As there are several possible techniques for DMEK graft preparation, we studied few methods that are routinely used and compared them to find out the most suitable technique for DMEK graft preparation. Hence, we selected three different ways of stripping and two bubble techniques and compared multiple parameters.

We observed that both, M4 and M5, are the techniques that use pressure between stroma and DM to create a bubble. Pneumatic dissection (M5) is carried out with full single force of air compared with submerged hydroseparation (SubHyS; M4) that utilized medium to high force of liquid depending on the primary small bubble. The pressure to obtain a complete bubble could have caused high cell death in M4 and M5 as it stretches the tissue. It was also observed that if the liquid is injected in the stroma, the force of the liquid cleaves the stroma and enters the stroma-DM plane to create a bubble. However, this phenomenon is not seen with air. The air is blocked in the stromal lamellae and as the density of air is less than liquid, it does not further cleave the posterior stroma and hence higher tissue wastage was noted in M5.

Tissue wastage is significantly low in stripping techniques compared with bubble methods. Time required to produce a bubble from M5 was least if the bubble is generated from the first PoI. M4 is a controlled method hence requires more time than M5. However, it depends on the tissue and technique, if the DM to the stroma is tightly attached, the liquid can leak from the PoI. In such cases, the tissue should be immediately switched to a stripping technique. M3 requires longer time for graft preparation due to an extensive technique that requires punching, separating the graft and peeling compared with relatively easy methods such as M1 and M2. [65, 66]

Stripping methods have shown reduced cell death rate. All three stripping methods with different PoIs were considered for this study. However, as M3 technique provides less endothelial damage, it could be worth investing the time but due to the expenses of this technique it may not be economically advantageous by many eye banks or surgeons with low

volume grafts. Other techniques such as M1 and M2 have also shown an acceptable range of cell death with both the methods having an economical advantage.

The epithelial spatula or the Sinskey hook is not expensive and, therefore, if this technique is mastered, it can provide affordable and better quality DMEK grafts with minimal cell death. The loss of tissue owing to technical failure could also be seen as part of the overall cost of a particular method that was observed in M4 and M5 in this study. For tightly attached tissues, a gentle oscillating stripping method can be used to strip the DMEK graft. However, for cases such as previous cataract incision and horse shoe shaped tears, it is recommended that the PoI should be from the opposite ends of the incision or rejecting the area of incision and initiating the stripping just after the incision.

A paracentral DMEK graft can be achieved this way (Parekh et al. 2017). However, these kinds of tissues can only be stripped and cannot be bubbled as the bubble will pop out and the tissue will be torn or the liquid will leak from the incision site never allowing a bubble generation. There are several graft excision techniques that have been published in literature with advantages and disadvantages.

The limitation of this study was that we were only able to get data on five techniques that are routinely prepared by our eye bank and collaborators. As stripping techniques have provided better graft quality compared with bubble techniques in this study and also from our previous experience, it is recommended to use a stripping method.

M3 has shown to have the best graft quality, however, as the overall expenses and time of preparation for this method is significantly high for any low volume eye bank, it may have strict limitations.

Stripping techniques with epithelial spatula and Sinskey hooks have served the best in terms of graft quality and economic feasibility. From this study, we have observed that M2, tissue preparation using Sinskey hook, could serve as the best graft preparation method considering all the parameters that include cell death, endothelial cell loss, time required to prepare the graft and costs. We believe that the techniques described in this article would help the surgeons and eye bankers to select the best option for DMEK graft preparation.

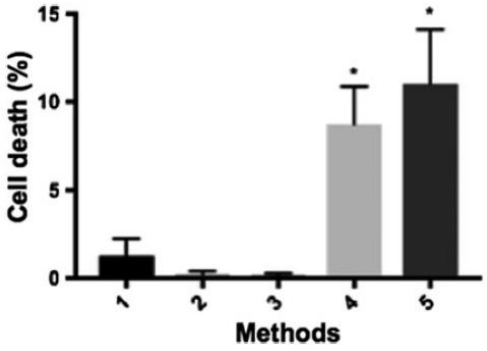

Figure 3.2 **Time required to prepare the graft in each method was statistically significant with M3 taking highest time to prepare a graft**

* Methods take more time than others.
** Method is the slowest of all.

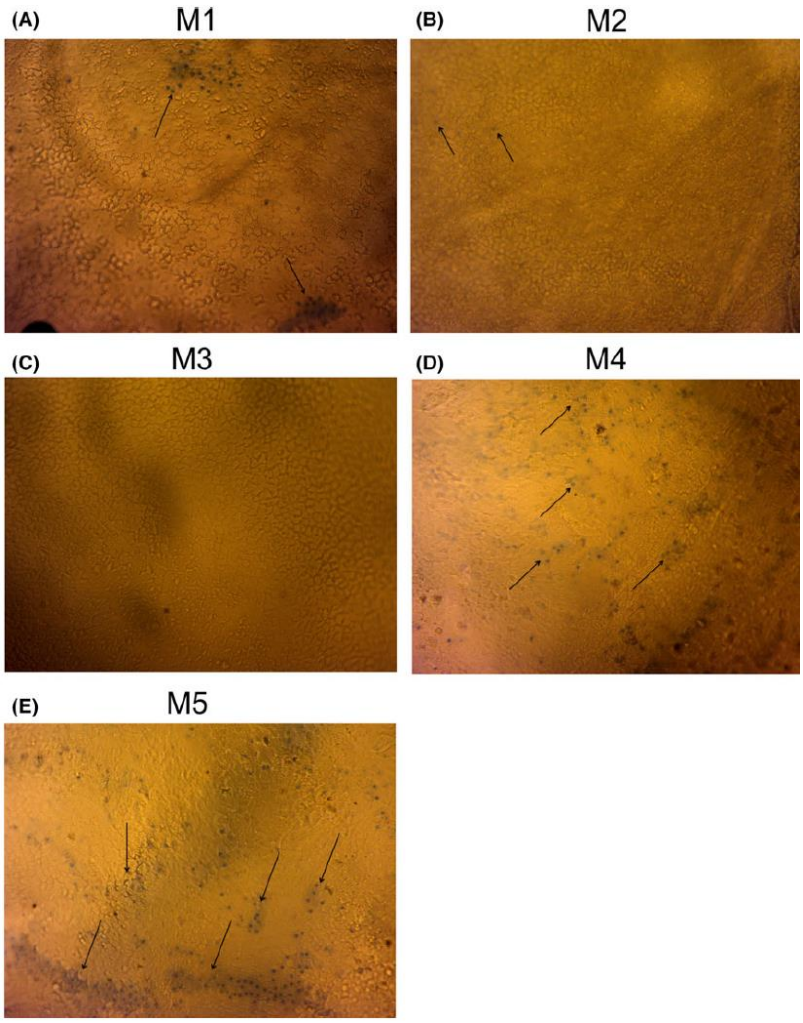

Figure 3.3 **Endothelial cell density and cell death observed using trypan blue positive cells (marked with arrow)**

(A) M1 showed acceptable range of cell death, (B) M2 showed minimal amount of cell death, (C) M3 did not show any cell death, (D) M4 and € M5 showed critical levels of cell death.

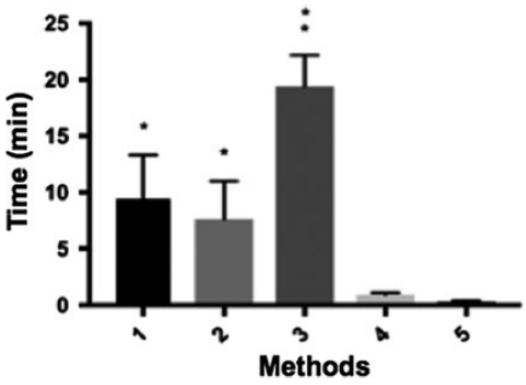

Figure 3.4 **Endothelial cell death was statistically significant ($p < 0.05$) between all the methods**

* Methods cause significant cell death compared with others.

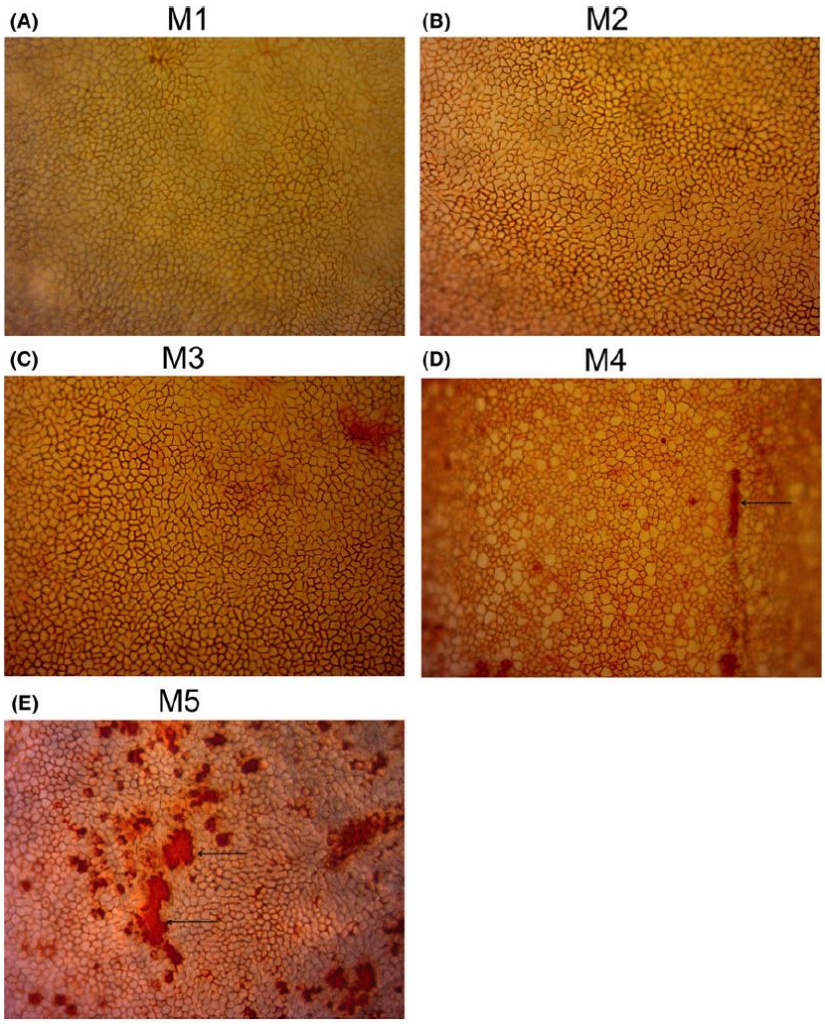

Figure 3.5 **Alizarin red staining for morphological analysis showed**

(A) M1, (B) M2 and (C) M3 that did not differ in morphology, however, (D) M4 showed pleomorphic cells and areas without cells (marked with arrow) and (E) M5 showed pleomorphic cells and serve amount of denuded areas (marked with arrows). Scale: 100× magnification.

Table 3.3

Parameters considered: instruments used, diameter of the graph, risk of peripheral tears or bubble burst, inclusion of suction base, forceps and overall coasts

	Instrument	Diameter of graft obtained (mm)	Risk of peripheral tears	Risk of bubble burst	Suction base	Forceps*	Costs† approx (USD)
M1	Epithelial spatula	11	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	8
M2	Sinskey hook	10.5	High	Not applicable	Not mandatory	Medium forceps (J3683, e.Janach) size 3, straight, pointed tips, 120 mm	10
M3	Donor trephine	8.25–10 (Wide range)	Moderate	Not applicable	Mandatory	Medium forceps (J3683, e. Janach) size 3, straight, pointed tips, 120 mm	150.0
M4	25G needle with 1 ml syringe	11	Not applicable	Moderate	Not required	Not applicable	<1
M5	25G needle with 1 ml syringe	11	Not applicable	Moderate-High	Not required	Not applicable	<1

* Forceps – depends on the surgeon. We prefer acute forceps as they grasp sufficient tissue required for peeling and to reduce overall endothelial cell damage.

† Costs – the overall costs are mainly for the instruments that are used. As these costs are the primary costs that an eye bank or the surgeon will have to pay, we considered this as our point of reference. This mainly includes the price of the instrument (Column one).

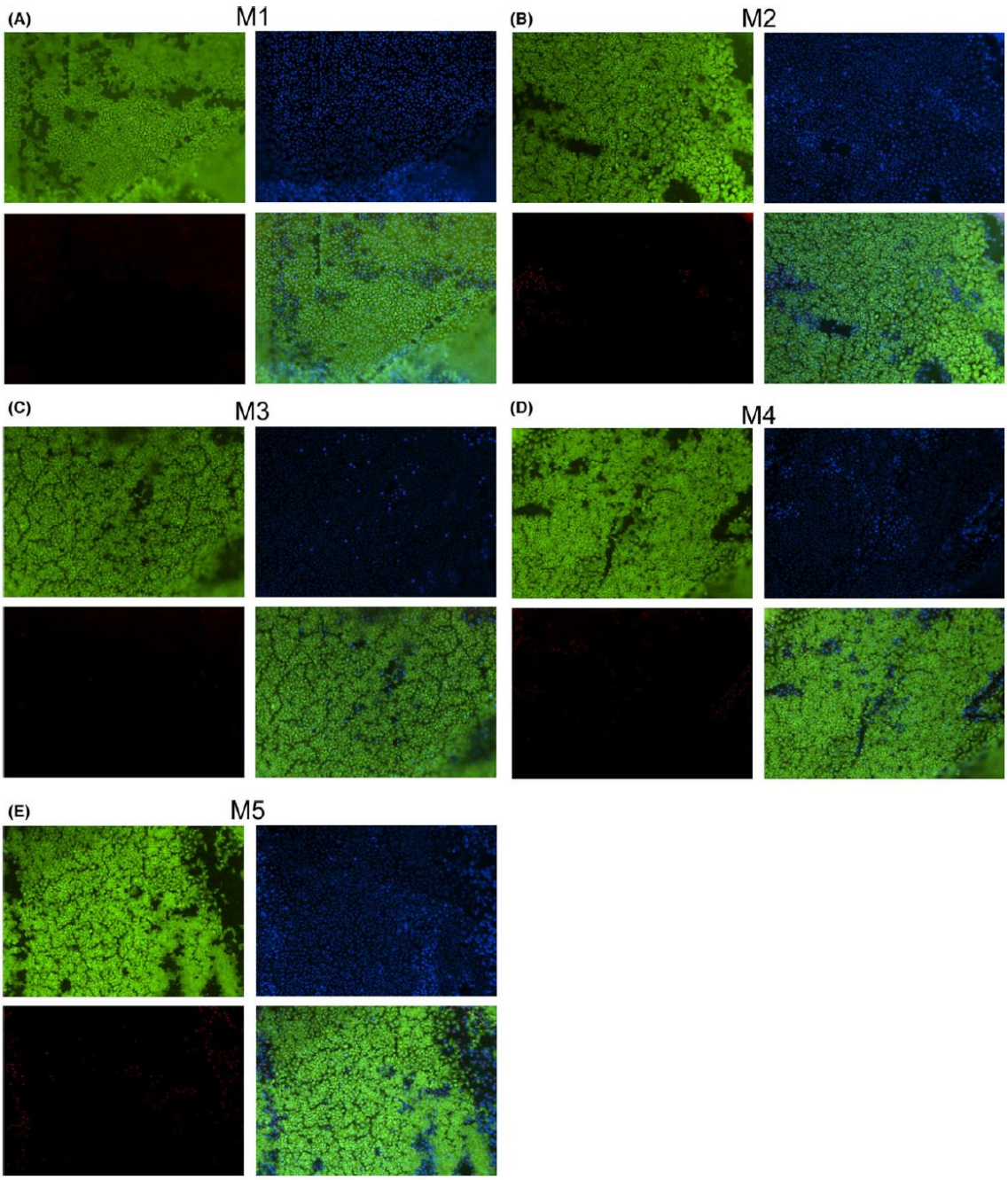

Figure 3.6 **Hoechst, Ethidium homodimer and Calcein AM (HEC) staining to determine live / dead cells**

Live cells were determined by green (Calcein AM staining), nuclei of the cells were determined by blue (Hoechst staining) and the dead cells were determined with red (Ethidium homodimer staining) colours. All the results were correlated with trypan blue staining. It was observed that (A) M1 and (B) M2 showed acceptable cell death but (C) M3 did not show any cell death, whereas (D) M4 and (E) M5 showed critical levels of dead cells. There were some denuded areas also found in M4 and M5.

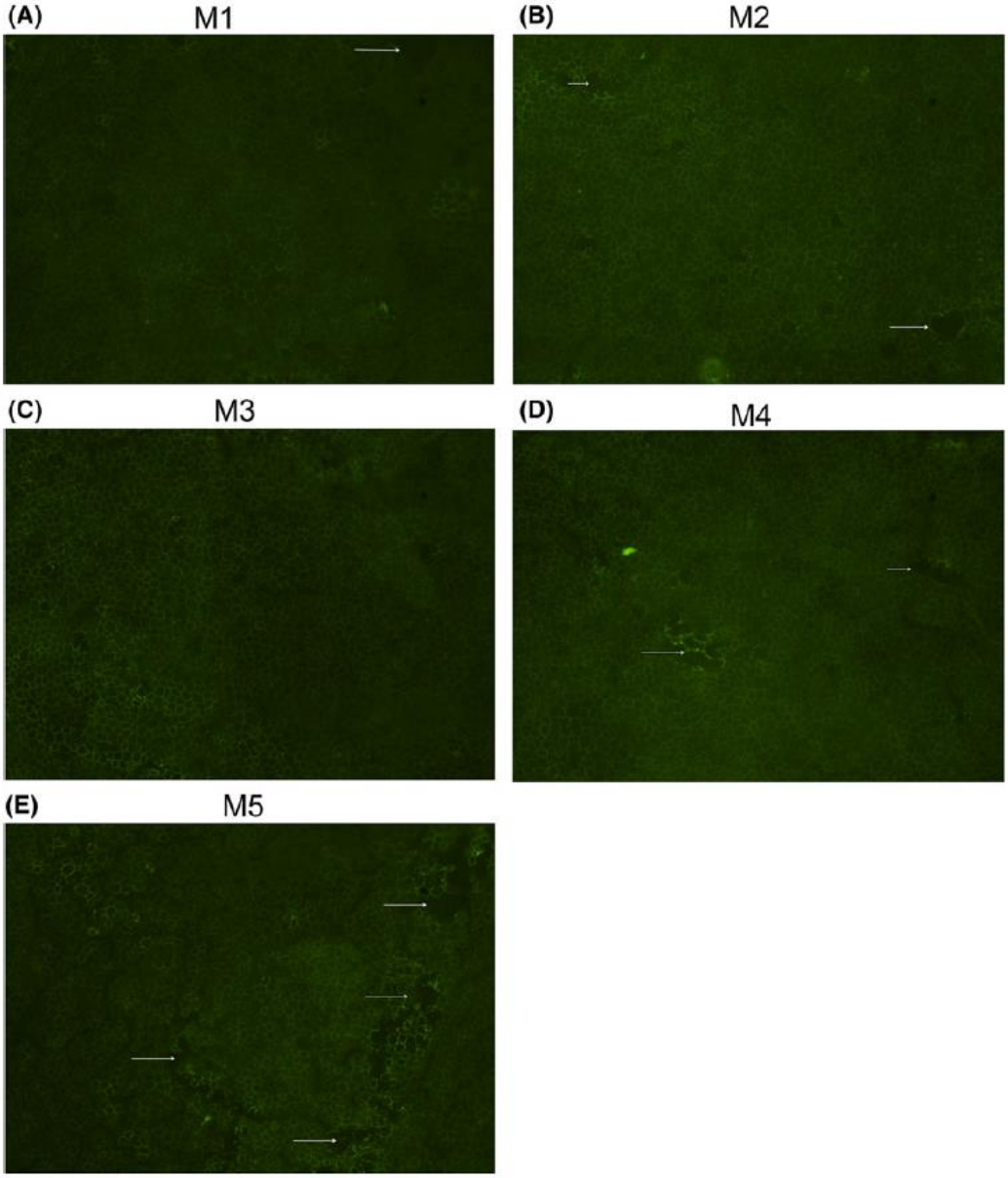

Figure 3.7 **Zonula Occludens-1 (ZO-1) expression was seen in all the methods wherever the cells were present**

(A) – (E) there were denuded areas as observed earlier and ZO-1 was not expressed in those areas. The morphology and hexagonality were observed and normal to pleomorphic cells in all the cases were found. Scale: 100× magnification.

4 Study 4

Study 4 shows a novel technique for DMEK tissue preparation.

Abstract in English

Title: The “Yogurt” technique for Descemet membrane endothelial keratoplasty graft preparation: a novel quick and safe method for both inexperienced and senior surgeons.

Purpose: To describe and evaluate the efficacy and safety of a novel technique to prepare Descemet membrane endothelial keratoplasty (DMEK) donor grafts using a newly designed partial-thickness hinge punch.

Methods: The novel punch has a circular guarded blade missing 1 clock hour, creating an uncut hinge on the donor cornea. In addition, 2 straight cuts are made by the punch perpendicular to the edge of trephination toward the trabecular meshwork in the hinge area. After the donor corneoscleral rim is positioned endothelial side up, a partial-thickness trephination is performed avoiding any rotational movements. Descemet membrane is lifted from Schwalbe line in the hinge area, and DMEK graft is peeled after desired marking without further preparation.

Results: Three surgeons of different experience levels on DMEK (senior / independent / fellow) initially applied the new technique in 18 research corneas, divided into equal groups. Two failures in graft preparation were noted, defined as radial tears extending $\geq$ 0.5 mm. The mean preparation time was 6.21 $\pm$ 1.45 minutes. No statistically significant differences were noted in success rate, duration, and endothelial cell loss (ECL) between surgeons ($P > 0.05$). ECL was evaluated as an average of 5 readings on randomly selected graft areas, not including graft periphery. Fifteen additional research corneas were stripped by 1 single user in an eye bank setting. No tissue loss was recorded, whereas ECL and mortality rate remained unaffected after preparation ($P = 0.64$ and $P = 0.72$, respectively).

Conclusions: This new DMEK graft preparation technique, simulating the opening of a yogurt cup, seems to be a safe and an efficient method, providing shorter preparation time and low failure rates independent of surgeon's experience level.

Key Words: Descemet membrane endothelial keratoplasty, DMEK graft preparation, preparation technique

Abstract in Latvian

Ceturtais pētījums apskata jaunākās tehnikas DMEK audu sagatavošanā.

Nosaukums: Descemeta membrānas endoteliālās keratoplastikas transplantāta sagatavošana ar Yogurt tehniku: jauna, ātra un droša metode gan nepieredzējušiem, gan pieredzes bagātiem ķirurgiem.

Mērķis: Aprakstīt un izvērtēt tehnikas efektivitāti un drošību, lai sagatavotu descemeta membrānas endotēlija keratoplastikas (DMEK) donoru transplantātus, izmantojot nesen izstrādātu parciāla biezuma viru perforatoru.

Materiāli un metodes: Jaunajam perforatoram ir apļveida forma, kas perforēšanas rezultātā neskar eņģes apvidu. Papildus tas veic 2 taisnus griezienus perpendikulāri trefinācijas malai pret trabekulāro tīklu eņģu apvidū. Pēc tam, kad korneosklerālā mala ir pozicionēta ar endotēlija pusi augšup, tiek veikta parciāla biezuma trefinācija, izvairoties no jebkādām rotācijas kustībām. Descemeta membrāna tiek pacelta no Švalbes līnijas uz eņģu apvidu, un DMEK transplantāts tiek noņemts pēc vēlamā marķējuma bez nepieciešamības tālākai sagatavošanai. Pētījumā piedalījās trīs ķirurgi, kuri sākotnēji izmantoja jauno tehniku 18 pētījuma radzenēs, sadalot vienlīdzīgās grupās. Ķirurgi tika sadalīti dažādās DMEK pieredzes grupās – vecākais, neatkarīgais un nepieredzējušais.

Rezultāti: Tika konstatētas divas nepilnības, sagatavojot transplantātu, kas definētas kā radiāli plīsumi, kas stiepjas ≥ 0,5 mm. Vidējais sagatavošanas laiks bija 6,21 ± 1,45 minūtes; netika novērotas statistiski nozīmīgas atšķirības starp ķirurgu panākumu līmeņa, ilguma un endotēlija šūnu zuduma ($p > 0,05$). Endotēlija šūnu zudums tika novērtēts kā vidēji pieci rādījumi nejauši izvēlētās transplantāta zonās, neieskaitot transplantāta perifēriju. Pieejamas acu bankas apstākļos viens lietotājs noņēma 15 papildu radzenes. Audu zudums netika konstatēts, savukārt endotēlija šūnu zudums un mirstības līmenis palika nemainīgs (attiecīgi $p = 0,64$ un $p = 0,72$).

Secinājumi: Jaunā DMEK transplantāta sagatavošanas tehnika, kas simulē jogurta iepakojuma atveri, šķiet droša un efektīva metode, kas nodrošina īsāku sagatavošanas laiku un zemu neizdošanās līmeni neatkarīgi no ķirurga pieredzes līmeņa.

Atslēgas vārdi: descemeta membrānas endotēlija keratoplastika, DMEK transplantāta sagatavošana, sagatavošanas tehnika.

4.1 Introduction

Fuchs endothelial corneal dystrophy and pseudophakic corneal edema have lately become the main indicators for corneal transplantation, especially after the establishment of phacoemulsification as the gold standard in cataract surgery. The management of corneal endothelial failure has changed substantially in the past years, moving from penetrating keratoplasty (PK) to more elective lamellar procedures. Endothelial keratoplasty (EK) is now the standard of care because it offers better outcomes and faster visual recovery than PK. According to the Eye Bank Association of America, the number of PK procedures performed in the United States has significantly decreased from 2005 to 2015, whereas EK has been the most commonly performed corneal transplantation and continues to increase. [67,68,69,70]

Since its introduction by Melles et al in 2006, Descemet membrane endothelial keratoplasty (DMEK), although technically more challenging than other EK procedures, has gained in popularity becoming the first-line treatment of many corneal surgeons in the management of corneal endothelial dysfunction. According to recent surveys, one of the most common barriers among corneal surgeons to uptake EK is anxiety related to tissue preparation. The first step for successful surgery is to master a donor tissue–harvesting technique that can consistently provide an intact graft to transplant. [71, 72, 73, 74]

It has lately been established that endothelial graft preparation techniques are diverse and feature different strengths and weaknesses. Although many techniques have been proposed so far, there is no consensus on a so-called standardized method. The most commonly used techniques include pneumatic dissection, no-touch technique, sub merged hydro-separation technique, liquid separation technique, and few more that have been thoroughly evaluated earlier.

Although the stripping method has been the most widely adopted, it can sometimes be difficult, particularly when Descemet membrane (DM) is strongly adherent to the underlying stroma, making it occasionally challenging to identify a cleavage plane to start DM stripping. This phenomenon, more commonly seen in younger donors, could be attributed to variation in the intermediary “Bowman-like” zone of randomly arranged collagen fibers at DM–stroma interface, which have been shown to serve as an anchoring function between DM and posterior stroma. [75, 76, 77]

To our knowledge, although most methods applied worldwide have fair to excellent success rates, there is currently no corneal trephine system that overcomes all difficulties. Therefore, novel techniques and modalities might be of great value to further increase safety and efficacy in DMEK graft preparation.

The aim of this study was to present a novel method for DMEK graft preparation, evaluating its safety and efficacy when used by surgeons of different experience levels.

The new method is called the "Yogurt technique" because it resembles the opening of a yogurt cup using a newly designed corneal punch.

4.2 Materials and methods

4.2.1 Description of the technique (Yogurt Technique) and the Hinge-Guarded Punch

- The donor corneoscleral disc is grasped carefully with toothed forceps from the scleral rim, and it is positioned endothelial side up on the cutting block of the device.
- It is important that the donor disc is properly centred on the cutting block, ensuring that the limbus is equally distanced from the peripheral markings of the cutting block 360 degrees (Fig. 4.1 (A)).
- Vacuum is applied by means of a spring-loaded syringe attached to the cutting block (applying negative pressure) to secure position and stabilization of the corneoscleral disc.
- Trypan blue solution 0.4 % (Sigma-Aldrich, Deisenhofen, Germany) is applied on the endothelial side and left for 20 seconds in place to stain the endothelium / DM and facilitate better visualization of the procedure.
- Trypan blue solution is rinsed off with a balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX) and using a triangle ophthalmic sponge along the periphery avoiding any contact to the endothelium.
- A partial-thickness trephination with the 100 mm guarded punch blade is performed avoiding any rotational movements (Fig. 4.1 (B)).
- The above-described DMEK punch has a circular guarded blade missing 1 clock hour, creating an uncut hinge on the donor cornea (Fig. 4.1 (C)).
- After partial-thickness trephination, the donor disc is stained again for 20 seconds with trypan blue solution 0.4 % and then rinsed off with BSS.
- The uncut hinge of approximately 40 degrees arc is being identified and brought opposite to the surgeon's field at the 12-clock hour.
- In addition, during punching, 2 straight cuts are made almost perpendicular to the edge of the circular cross section toward the trabecular meshwork in the hinge area. The perpendicular cuts are also of partial thickness by means of a guarded 100 mm blade.

- A non-sharp, pointed instrument (e.g., Sinskey hook) is used to identify the end of DM at the level of Schwalbe line in the uncut hinge area (Fig. 4.1 (D)).
- DM with overlying endothelium is peeled off from the underlying corneal stroma using a curved spatula or a crescent blade (Fig. 4.2 (A)).
- DM peeling is performed carefully beyond both angles of the hinge (the 2 ends of the circular cross-section), taking care to avoid inducing any tears to the graft (Fig. 4.2 (B)).
- The peeled edge is placed back using BSS, and thereafter, the graft is stained again with trypan blue and rinsed off.
- The detached hinge is being cut with a blade to leave only an orthogonal triangle part that will act as marking when the graft is placed in the recipient's eye, allowing identification of correct graft orientation. The hypotenuse of the orthogonal triangle created lies clockwise to the right (90 degrees) angle, so that when inserted in the anterior chamber and unfolded, it should appear anticlockwise as the endothelial side should be facing downward (Fig. 4.2 (C)).
- The DMEK graft is grasped with forceps (tying, jewellers, or other DMEK forceps) from the triangle marking and further stripped in a single-peel technique (Fig. 4.2 (D)).

See Video 1 (Supplemental Digital Content 1, *http://links.lww.com/ICO/B46*).

4.2.2 Experimental evaluation of the novel technique

The new technique was initially evaluated on corneas provided by Manchester Eye Bank (Manchester Eye Tissue Repository; NHS, Manchester, United Kingdom) not suitable for transplantation because of low endothelial cell density (ECD 2200 cells/mm^2). Three surgeons with different levels of experience applied the new technique using 18 donor corneas, divided into equal groups (6 each). A senior surgeon (200 DMEK procedures performed), an independent surgeon (50 but 200 DMEK procedures performed), and a corneal fellow (50 DMEK procedures performed) participated in the study. The donor tissues were randomly assigned to surgeons. Fifteen additional research corneas were stripped by 1 single user (A.T.) in the setting of an eye bank, and all tissues were evaluated before and after preparation by a masked cell biologist to assess endothelial cell mortality and ECD. To calculate mortality rate and ECD after preparation, all grafts were stripped until almost 90 % from the donor underlying stroma, and then, they were placed back with

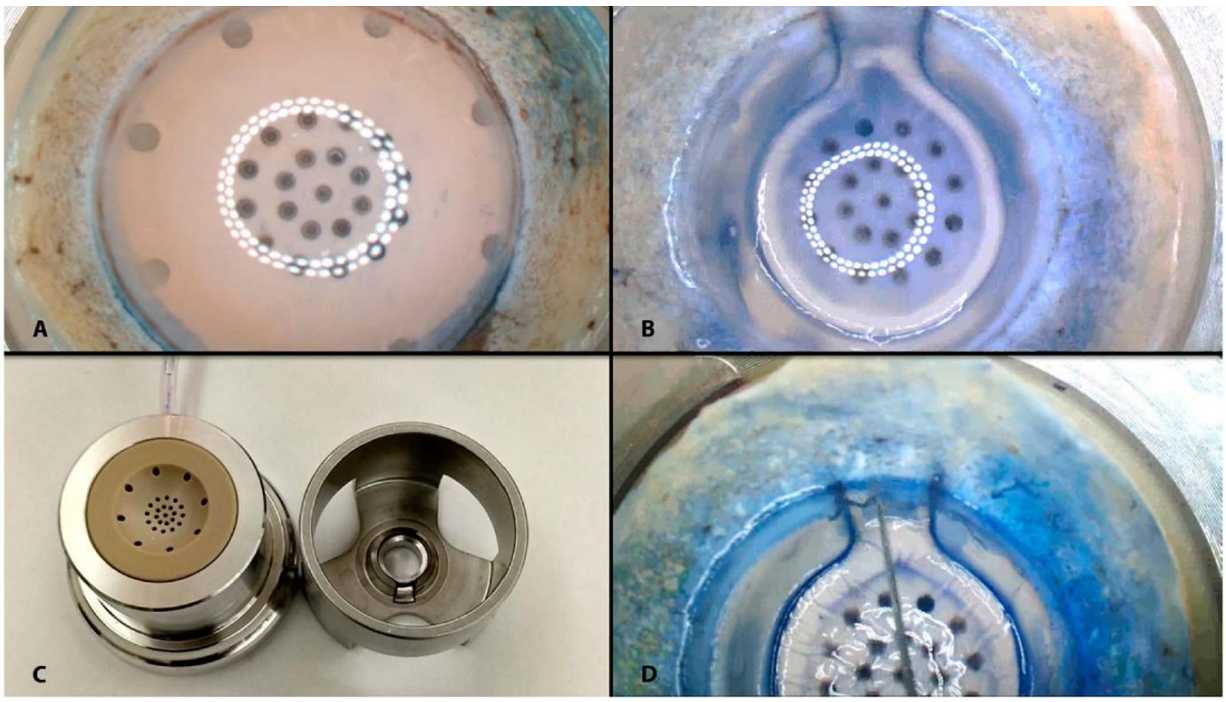

Figure 4.1 **(A) Corneal graft centred on the cutting block endothelial side up. (B) Configuration of cutting pattern after partial-thickness trephination with the guarded hinge punch. (C) Novel DMEK hinge punch featuring a circular guarded blade missing 1 clock hour, creating an uncut hinge on the donor cornea and perpendicular cuts to the periphery. (D) Identification of DM anatomical and at the level of Schwalbe line in the uncut hinge area with a Sinskey hook.**

The full colour version of this figure is available online at *www.corneajrnl.com*.

BSS to facilitate examination under the optical microscope by the masked cell biologist. The diameter of the circular blades used in the study was 8 mm in all cases.

All graft preparations were recorded and further analysed. The following measures were used to evaluate the safety and efficacy of the new technique:

- Tissue loss: Absolute success was defined as preparation without any radial tears, relative success as preparation with 1 radial tear less than 0.5 mm in length, and failure as multiple peripheral tears or a single large one extending more than 0.5 mm.
- Preparation time: The time of each preparation (in minutes and seconds) was recorded by an independent rater, and, thereafter, it was confirmed by viewing the video recordings. The timer was started on placement of the donor tissue on the cutting block, and the end point of the preparation was defined as the full reattachment of the 90 % stripped DMEK graft to the underlying donor stroma.
- Endothelial cell loss (ECL): Corneal grafts were evaluated by a masked cell biologist before and after preparation. All tissues were initially stained using trypan blue (0.4 %) for 20 seconds and washed with phosphate-buffered saline (PBS; Sigma-Aldrich). The corneas were placed in a sterile Petri dish containing a hypotonic sucrose solution (to increase definition of cell borders) with the epithelium

uppermost and examined at 100 magnification of an inverted microscope (Primovert; Zeiss, Switzerland). The tissues after trypan blue staining were washed with PBS before Hoechst, ethidium homodimer, and calcein AM staining.

Approximately 4 mL of Hoechst 33342 (Thermo Fisher Scientific, Rochester, NY), 4 mL of ethidium homodimer EthD-1, and 2 mL of calcein AM (Live / Dead viability / cytotoxicity kit; Thermo Fisher Scientific) were mixed in 1 mL of PBS. From the final solution, 300 mL was directly added on the endothelium of the DMEK tissue resting on the cornea and incubated at room temperature in the dark for 30 minutes. Both the ECD and cell death were counted before and after DM stripping.

Mortality rate and ECD were measured by manually counting the cells using a 10×10 reticule fixed inside the eyepiece of an (inverted optical microscope (100 magnification, Primovert; Zeiss).

Five readings were made on randomly selected graft areas, and the average was recorded. The damage induced by trephination to the graft edges was not evaluated.

4.3 Results

The novel DMEK graft preparation technique was initially applied in 18 research corneas by 3 surgeons of different experience levels (6 cases each).

Figure 4.2. **(A) Peeling of DM with overlying endothelium in the hinge area using a crescent blade**

(B) DM peeled beyond the angles of the hinge. (C) Orthogonal triangle marking. (D) DMEK graft stripped using tying forceps in a single-peel technique.
The full colour version of this figure is available online at *www.corneajrnl.com*.

The average age of donors was 69.7 ± 9.2 years (range = 58–79 years), and the mean endothelial cell count was 1892.4 ± 156.3 cells/ mm^2. Five donors (27.8 %) had a recorded history of diabetes mellitus, whereas 11 (61.1 %) suffered from arterial hypertension. No differences were noted in any of the donor tissue characteristics between surgeons (P = 0.05). Further details on donor tissues are provided in Table 4.1. All surgeons had no previous exposure to the above-described technique and were asked to perform it according to the above-mentioned instructions. Tissue loss was recorded in 1 case (independent surgeon), whereby a radial tear measuring half of the diameter of the DMEK graft was noticed during stripping.

One more case was considered as failure showing a 1.5 mm radial tear (corneal fellow). Success rate of graft preparation was subsequently 88.9 % (16 / 18 cases). Absolute success was noted in 15 cases (83.3 %), whereas in 1 case (5.6 %), a small peripheral radial tear (0.4 mm) was induced, and preparation was finished without further complications. No statistically significant difference was found between surgeons regarding tissue loss ($\times 2$ test, P = 0.56, contingency coefficient = 0.378). The time needed for graft preparation ranged between 3.2 and 9.1 minutes, yielding an average of 6.21 ± 1.45 minutes, not differing significantly between surgeons of different experience levels (analysis of variance, P = 0.39). Further details on graft preparation by each surgeon are provided in Table 4.2.

Table 4.1

Donor tissue characteristics and comparison between surgeons

	Total	Senior Surgeon	Independent Surgeon	Corneal Fellow	*P*
Age (yrs)	69.7 ± 9.2	68.3 ± 8.6	70.7 ± 9.6	70.2 ± 9.7	0.89*
Sex	10 M/8 F	3 M/3 F	4 M/2 F	3 M/3 F	0.79†
Death to preservation time (h)	12.1 ± 2.3	11.8 ± 2.4	12.1 ± 1.9	12.4 ± 2.7	0.94*
Death to preparation time (d)	12.9 ± 4.6	13.1 ± 5.4	13.2 ± 4.9	12.4 ± 4.3	0.61*
ECD (cells/mm^2)	1892.4 ± 156.3	1912.6 ± 141.2	1881.2 ± 167.5	1883.4 ± 149.7	0.93*
Diabetes mellitus	5/18	2/6	1/6	2/6	0.76†
Arterial hypertension	11/18	4/6	4/6	3/6	0.79†

*Assessed with analysis of variance test.
†Assessed with χ^2 test.
F, female; M, male.

Table 4.2

Success rates (with percentages in parentheses), preparation time (in minutes), and ECL (%) after DMEK graft preparation

	Total	Senior Surgeon	Independent Surgeon	Corneal Fellow	*P*
Absolute success	15/18 (83.3)	5/6 (83.3)	5/6 (83.3)	5/6 (83.3)	0.56*
Relative success	1/18 (5.6)	1/6 (16.7)	0/6 (0)	0/6 (0)	
Failure	2/18 (11.1)	0/6 (0)	1/6 (16.7)	1/6 (16.7)	
Preparation time	6.21 ± 1.45	5.53 ± 1.45	6.58 ± 1.24	6.51 ± 1.62	0.39†
ECL	2.67 ± 1.98	2.49 ± 1.81	2.74 ± 2.31	2.78 ± 2.19	0.92†

*Assessed with χ^2 test.
†Assessed with analysis of variance test.

The mean preparation time using the novel technique was plotted against the preparation time that the same surgeons needed to perform 12 successful DMEK graft preparations (4 each) using the scoring method. The analysis showed that the Yogurt technique resulted in a quicker preparation not only in cases of absolute success but also in cases with small radial tears ($P = 0.001$ and $P = 0.02$, respectively). The novel method was additionally evaluated in another eye bank setting (Veneto Eye Bank, Mestre, Italy). DMEK graft preparation was performed by a single user (A.T.) in 15 research corneas.

The donor mean age and ECD was 71.6 - 5.4 years and 1723.3 ± 182.3 cells/mm^2, respectively. No tissue loss or failure was noticed because absolute success was recorded in 14 of 15 cases (93.3 %) and a minor radial tear (,0.5 mm, relative success) in 1 case (6.7 %). Graft preparation time varied between 4.17 and 11.09 minutes with a mean of 5.86 ± 2.28 minutes. Cell mortality did not show any statistically significant difference before and after preparation (3.81 % ± 3.8 % vs. 4.57 % ± 5.2 %, respectively, $P = 0.72$, Student t test). ECD did not show any significant decrease after preparation (ECD = 1684.4 ± 260.7, $P = 0.64$), whereas cell loss yielded an average of 2.31 % ± 4.3 %.

4.4 Discussion

DMEK has been a breakthrough in the management of corneal endothelial pathologies in the past decade, offering a quick rehabilitation of vision and a superior final visual outcome in comparison with previous surgical techniques. A safe and efficient preparation method of donor grafts is a strong prerequisite for successful surgery, and in a recent survey, half of corneal surgeons participating in a DMEK wet laboratory expressed that anxiety related to tissue preparation is one of the major perceived barriers to uptake DMEK. Many techniques for DMEK graft preparation and modifications of them have been proposed so far, yielding varying results for safety and efficacy. [78, 79, 80, 81]

In a recent updated review, Birbal et al reviewed 25 techniques described in the literature from 2006 to 2018. In most techniques, the time needed for graft preparation was not reported, failure rates varied between 0 % and 17 %, and ECL (only reported in a few studies) was mostly less than 10 %. [82, 83, 84]

Although several authors have already tried to propose a standardized technique for DMEK graft preparation, there is still a great variation in techniques used by corneal surgeons and eye bank technicians all over the world. The main reason for this disparity is probably the fact that every user tends to adopt the technique that is most convenient and doable in their setting. Therefore, ease of use, a short learning curve, and a reasonable cost of instruments / disposables are important factors in the uptake of a new technique. One of the most popular

standardized protocols uses the application of a partial-thickness trephination and a cleavage hook to identify the cleavage plane and to de-adhere the periphery of the graft from the underlying stroma before peeling off DM. This might occasionally be challenging due to strong adherence of DM to the underlying stroma after punching. [85, 86, 87]

In contrary, DM with the overlying endothelium can be very easily peeled off from Schwalbe line because this is the anatomical end point of it. This has already been proposed as a no-touch technique to peel DM from Schwalbe line or the trabecular meshwork at 360 degrees.

However, this preparation method requires time and increases the risk of unintentional damage to the graft because it is grasped at many points with forceps. The novel technique described in this study has been designed to overcome some of the aforementioned main difficulties and, importantly, is reproducible. We have named the new method Yogurt technique because DMEK graft preparation resembles the opening of a yogurt cup. The technique depends on a specially designed punch that generates a circular graft but leaves a hinge at the limbus. Thus, a graft can be punched from the donor cornea, and Descemet–endothelium complex can be peeled by grabbing the tab generated by the uncut hinge that connects to the limbus. In that way, the problem of identifying a cleavage plane to peel the graft after punching is overcome because DM is stripped easily from its natural end that lies toward Schwalbe line. Furthermore, this technique is much faster in comparison with previously applied methods because it does not demand circumferential (360 degrees) detachment or peeling of DM edge.

Although all radial tears recorded in our cohort were not associated with leaving the graft edges unpeeled before striping the graft, one could always try to do so if they feel it provides extra safety to the preparation process. Moreover, it has a very low learning curve because it does not require special skills and can be easily performed by both experienced and inexperienced surgeons / users. Marking of the graft is helpful for corneal surgeons performing DMEK because graft orientation is often difficult to identify and a graft placed upside down can definitely lead to surgical failure and the need for reoperation. [88]

Various methods have been suggested for marking of the graft.

Most use direct marking such as cutting of an inner triangle or other asymmetric marking removing part of the graft periphery within the graft leading to ECL. This problem is overcome with the new technique because marking and manipulation of the graft is only performed through an orthogonal triangle tissue that is outside the circular DMEK graft. The technique has already been applied to real surgery conditions, leading to no further complications or increase of graft detachment rate. Finally, cost-efficacy is crucial for a new method to be established.

One further advantage of DMEK over previous lamellar keratoplasties such as Descemet stripping automated EK is the fact that it does not require expensive equipment, such as a microkeratome, to prepare the endothelial graft. The newly described technique might be less expensive compared with some other methods that use 2 corneal punches instead of 1.

However, one should always keep in mind that a designated punch might have additional costs. Furthermore, when only 1 punch is used, this minimizes the option for the operator to start peeling from another point if a radial tear occurs and re-punch in an area devoid of tears or damage. The new DMEK punch does not limit the location of the graft to the central cornea, and grafts could also be offset to avoid specific areas. One just needs to make sure there is enough space for the hinge formation toward the periphery and the trabecular meshwork. The results of our study show that this technique has a good safety and efficacy profile and might provide another option for surgeons and eye bank technicians when choosing a suitable method for DMEK graft preparation. However, it has several limitations that should be kept in mind.

Above all, ECL and mortality rate in this study have been evaluated in each case only as an average of 5 measurements on randomly selected graft areas, instead of examining the whole graft, as it has already been described in other cohorts.

This analysis might be prone to bias, and it probably underestimates the amount of ECL because we did not consider damage induced to the periphery of the graft. In fact, cell death caused by the punch alone at the edge of the trephination likely contributes to a significant proportion of the overall damage to the total graft. Therefore, the rates of ECL and mortality reported in this study should be considered carefully and only as a measure to compare between different type of users in a clinically oriented study and would not be appropriate to conclude on the real extent of endothelial cell damage in a laboratory study. Consequently, DMEK graft preparation time should also be taken into consideration thoughtfully, when ECL is not properly measured. Furthermore, variations in donor tissue characteristics, and especially the proportion of diabetic tissues, might affect success rates in DMEK preparation.

However, in our cohort, no significant differences were noted between surgeons in any of these features, strengthening our results.

In conclusions, the novel Yogurt technique by means of a guarded hinge punch, resembling the opening of a yogurt cup, seems to be an easy, quick, efficient, and safe method to prepare DMEK grafts independently of surgeon’s experience level. Further prospective studies are needed to evaluate its efficacy in real-time surgical conditions comparing it with other preparation techniques.

5 Study 5

Study 5 shows how the speed of stripping influences the graft's shape.

Abstract in English

Title: The influence of speed during stripping in Descemet membrane endothelial keratoplasty tissue preparation.

Purpose: To evaluate if the speed of stripping a DMEK graft influences the graft scroll width.

Methods: Human corneas suitable for research were selected for the study. Pairs of corneas were randomly divided into 2 groups. 1 cornea was stripped with a slow speed (Group 1) and the contralateral with a fast speed (Group 2). Slow speed was defined as total time greater than 150 seconds or speed <0.057mm/sec. Fast peeling was defined less than 75 seconds or speed > 0.11mm/sec. The grafts acquired were evaluated by microscopy for the graft scroll width and endothelial cell density (ECD) change pre- and post-preparation.

Results: 10 corneas of 5 donors were included in the analysis. The mean donor age was 70.6 ± 6.4 years. The mean total time of the tissue preparation in group 1 was 274.2 ± 37 seconds, and in group 2 was 153 ± 53 seconds (p-value = 0.0038). The mean speed of stripping in group 1 was 0.048 ± 0.006 mm/sec, in group 2 was 0.188 ± 0.063 mm/sec (p-value = 0.0055). The graft width in group 1 was 6.28 ± 0.92 mm and in group 2 was 2.92 ± 0.23 mm (p-value = 0.00066). The mean endothelial cell loss in group 1 was 292 ± 109 cells/mm^2 and in group 2 was 160 ± 23.66 cells/mm^2 (p-value = 0.039).

Conclusion: We found a correlation between the speed of stripping, scroll width and endothelial cell loss. Slow peeled DMEK grafts result in a wider scroll width but were associated with a greater reduction in ECD.

Key Words: Cornea, peeling, DMEK, speed

Abstract in Latvian

Piektais pētījums parāda, kā noņemšanas ātrums ietekmē transplantāta formu.

Nosaukums: Noņemšanas ātruma ietekme uz Descemeta membrānas endotēlija keratoplastikas audu sagatavošanu.

Mērķis: Novērtēt, vai DMEK transplantāta noņemšanas ātrums ietekmē transplantāta ritināšanas platumu.

Materiāli un metodes: Pētījumam tika atlasītas atbilstošas cilvēka radzenes. Radzeņu pāri pēc nejaušības principa tika sadalīti divās grupās. 1 radzene tika noņemta lēni (1. grupa), kontralaterālā – ātri (2. grupa). Lēns ātrums tika definēts kā kopējais laiks, kas pārsniedz 150 sekundes, vai ātrums < 0,057 mm/s. Ātra noņemšana tika noteikta par laiku, kas mazāks nekā 75 sekundes, vai ātrums > 0,11 mm/s. Iegūtie transplantāti tika novērtēti ar mikroskopijas palīdzību, lai noteiktu tā ritināšanas platumu un endotēlija šūnu blīvuma izmaiņas pirms un pēc sagatavošanas.

Rezultāti: Analīzē tika iekļautas 10 radzenes no 5 donoriem. Vidējais donora vecums bija 70,6 ± 6,4 gadi. Vidējais kopējais audu sagatavošanas laiks 1. grupā bija 274,2 ± 37 sekundes, bet 2. grupā – 153 ± 53 sekundes (p = 0.0038). Vidējais noņemšanas ātrums 1. grupā bija 0,048 ± 0,006 mm/sekundē, 2. grupā – 0,188 ± 0,063 mm/sekundē (p = 0,0055). Transplantāta platums 1. grupā bija 6,28 ± 0,92 mm, 2. grupā – 2,92 ± 0,23 mm (p = 0,00066). Vidējais endotēlija šūnu zudums 1. grupā bija 292 ± 109 šūnas/mm^2, 2. grupā – 160 ± 23,66 šūnas/mm^2 (p = 0,039).

Secinājumi: Tika atrasta saistība starp noņemšanas ātrumu, izritināšanas platumu un endotēlija šūnu zudumu. Lēni "nomizoti" DMEK transplantāti rada lielāku ritināšanas platumu, taču ir saistīti ar lielāku ECD samazināšanos.

Atslēgas vārdi: radzene, nomizošana, DMEK, ātrums

5.1 Introduction

Descemet membrane endothelial keratoplasty (DMEK), when compared to the other techniques of endothelial keratoplasty, provides a faster visual rehabilitation and a lower risk of graft rejection. DMEK however has a steep learning curve particularly when compared to Descemet stripping automated endothelial keratoplasty (DSAEK) with higher complication rates reported during graft preparation and during transplantation into the recipient eye.

During DMEK surgery, the graft conformation and particularly scroll width has a crucial role in its unfolding within the anterior chamber. From our experience, and that of others, the more tightly scrolled the graft and narrower the scroll width, the more difficult it is to unfold the tissue once in the recipient eye. This is particularly the case with younger donors. This variability in the donor tissue behaviour is influenced heavily by its conformation after stripping and frequently results in DMEK surgery being avoided in more complex cases such as eyes which are aphakic, vitrectomized or with coexisting glaucoma tubes. To aid tissue unfolding various adaptations have been proposed, for example higher temperature has been shown to increase the width of DMEK graft potentially aiding unfolding.

We note that Descemet membrane contains elastin and its elastic properties may contribute to the scrolling characteristics of the graft after stripping. [89, 90, 91, 92]

We postulate that this may be influenced by the speed of stripping of the Descemet's from the stroma during DMEK graft preparation secondary to the effect and duration of tension on the elastic recoil of the tissue. To our knowledge, no studies have been performed to investigate the effect of the stripping speed on the scrolling behaviour and final graft scroll width resulting in preparation for subsequent DMEK.

The aim of our study was to evaluate if the speed of stripping of the Descemets and endothelium from the stroma affects the scrolling pattern of the graft in a laboratory setting and final graft scroll width acquired. The secondary aim of the study was to assess if the speed of corneal stripping has an influence on safety and endothelial cell loss during preparation.

5.2 Material and methods

5.2.1 Human cornea

Human corneas suitable for research with endothelial cell density (ECD) between 2000–2200 cells/mm^2 were selected for the study. Corneas were provided by Venice Eye Bank. All the procedures conformed to the tenets of the Declaration of Helsinki. Immediately after initial evaluation in the eye bank, corneal tissues were preserved in organ culture medium (CorneaMax, Eurobio, Les Ulis, France). A dry incubator was used to store the vials at 31 °C. ECD and mortality [trypan blue positive cells (TBPCs)] was checked using trypan blue (TB) staining (0.06 %) ((Vision Blue®; DORC, Zuidland, The Netherlands) for 20 seconds followed by washing with phosphate buffered saline (PBS). TBPCs and endothelial cell density were recorded using an in-built eyepiece reticule (10×10) for inverted microscope (Axiovert, Zeiss, Oberkochen, Germany). An average of 5 manual readings were taken from different sites to lower the risk of manual error.

5.2.2 Tissue preparation and stripping

Each pair of corneas had 1 cornea stripped with slow speed (Group 1) and the contralateral with a fast speed (Group 2). Fast peeling was defined as the maximum constant speed at which the surgeon could strip the donor without regrasping the tissue, interruption to motion and < 75 seconds (> 0.11mm/second). Slow speed was defined as stripping at the lowest speed possible without regrasping tissue or interruptions to motion and > 150 seconds (< 0.057 mm/second). Corneas were stripped as previously described. Briefly, a single trephine technique was used and preparation was performed in 2 parts by a single experienced DMEK

surgeon (DB). The time for each part of preparation was recorded by an independent observer from the Venice Eye Bank (AG).

Part 1: The endothelium was stained with trypan blue 0.06 % (Vision Blue®; DORC, Zuidland, The Netherlands) for 10 seconds. Separation of the Descemet membrane (DM) was initiated from the periphery of the corneal tissue at the junction of the corneal and the Trabecular Meshwork ™ by gently swiping the DM layer from towards the centre using a pediatric crescent knife 2.3 mm (Alcon Laboratories, Inc., Fort Worth, Texas) angled with the bevel up. 2 mm of the peripheral DM was disengaged from its adhesion to the stroma using this method to create a flap to grasp for subsequent stripping during part 2 of preparation. The endothelium was then replaced on the corneal stroma and a Moria 8.5mm punch was used to trephine with care taken to include 1mm of free DM tissue within the area trephined to grasp in Part 2.

Part 2: The previously free edge of DM was then grasped with a non-toothed tying forceps and the DM totally peeled until free from stroma.

The graft was subsequently immediately submerged in a petri dish filled with balanced salt solution (BSS) and the graft's scroll width measured with a vertical ruler reticle (KR-235 Microscope World, Innovation Way Carlsbad, CA). The BSS was then removed from the petri dish, the tighter graft opened with flush of BSS and the ECD measured. The speed of stripping was calculated using the formula $s = d / t$ (speed = distance / time) considering that the standard linear distance to strip as that of the diameter of the punched tissue (8.5mm) and the time taken to strip measured in seconds.

5.2.3 Statistical analysis

The differences in the parameters between the group 1 and group 2 were analysed and the differences in cell counts from baseline to post stripping were analysed. Statistical analysis was performed using the one-tailed Student's *t*-test. Significance was determined as $p < 0.05$.

5.3 Results

10 corneas of 5 patients were included in the analysis. The mean donor age was 70.6 ± 6.4 years. Of the donors, 3 were females and 2 males. The mean total time of the tissue preparation in group 1 was 274.2 ± 37.33 seconds, and in group 2 (fast group) was 153 ± 53.99seconds (p-value = 0.0038). The mean total time of the part 1 of the tissue preparation was 93.4 ± 25.6 sec in group 1 and 103 ± 42.8 sec in group 2 (p value = 0.35). The mean total time of part 2 of the graft preparation was 180.8 ± 22.29 seconds in group 1 and 50 ± 15.43 seconds in group 2 (p-value = 0.00001). The mean speed of stripping in group 1 was

0.048 ± 0.006 mm/sec, in group 2 was 0.188 ± 0.063 mm/sec (p-value = 0.0055). The graft width in group 1 was 6.28 ± 0.92 mm and in group 2 was 2.92 ± 0.23 mm (p-value = 0.00066). The mean ECD in group 1 and group 2 pre-tissue preparation was 2174 ± 129.55 cells/mm^2 and 2180 ± 136.38 cells/mm^2 respectively (p-value = 0.475). Post tissue preparation the ECD reduced to 1882 ± 224.80 cells/mm^2 in group 1 and 2020 ± 134.01 cells/mm^2 in group 2 (p-value = 0.165) (Table 5.1). The mean endothelial cell loss in group 1 was 292 ± 109.069 cells/mm^2 and in group 2 was 160 ± 23.66 cells/mm^2 (p-value = 0.039).

5.4 Discussion

As DMEK becomes increasingly popular, there is a greater emphasis on optimizing the surgical steps of the procedure has developed. One of the challenges in mastering DMEK surgery is the unfolding of a tightly rolled graft within the eye.

In this study we demonstrate that by changing the speed of the graft stripping we can influence the tightness of the graft scroll that results in BSS and may potentially control an important variable in the surgical procedure of DMEK. In the present study we evaluated the effect of slow and fast stripping of DMEK graft tissue during preparation on scroll width and ECD loss. The marked difference in the speed of stripping (p-value = 0.0055) and the subsequent graft width (p-value = 0.00066) strongly suggests an influence of the speed of stripping on the final conformation of the graft (Figure 5.1).

The positivity of the coefficient of part 2 in DMEK preparation demonstrates that the slower speed of the tissue stripping, the wider the graft scroll width obtained in the final conformation of the graft in BSS (Figure 5.2). This finding may be due to the longer duration of stress that the tissue sustained during the peeling process and its effect on subsequent elastic recoil of the graft.

It has been suggested that a tightly scrolled graft is associated with a longer intraocular surgical duration and more manipulation within the anterior chamber during the unfolding steps of DMEK surgery.

Modabber demonstrated that a tightly scrolled graft prolongs the time to complete the surgical procedure potentially reducing the ECD. Thus, efficient unfolding and minimal manoeuvring of the graft within the eye may theoretically result in a shorter surgical time, graft trauma and reduction in the loss of endothelial cells. However, Sale CS et al did not find a correlation between unfolding time and tighter scrolls, ECD loss or graft failure rate.

Factors other than speed have been demonstrated to influence DMEK scroll width. It has previously been described that when certain shapes were punched out of the graft or outlines created the tightness of scrolling of the DMEK could be reduced and graft scroll width increased.

Unfortunately, these modifications were only obtained with additional loss of tissue volume which would have effects on subsequent total endothelial counts and potentially graft survival. Between the two speeds of stripping, we did not observe an increase of tears, complications or loss of tissue and full 8.5mm diameter grafts were obtained in all cases.

Although our findings support slow stripping of the DM in order to acquire wider DMEK graft scroll, we did find an association between a slow peeling speed and higher loss of endothelial cells during preparation (p-value = 0.039).

We postulate this may result from the extra stress, higher creases and folds or trauma sustained by the tissue during part 2 (peeling) of the preparation of the graft. Interestingly, although the number of endothelial cells is widely used to determine the health of the corneal endothelium and graft viability, recent studies suggest that cell function of the cells, plays an important role in maintaining a clear cornea.

The limitations of the study are the low numbers of samples and that this was a laboratory-based study only, unaccompanied by evaluation of the graft conformation achieved within an anterior chamber and subsequent ease of transplantation into the recipient eye. Additional limitations include the unaccounted-for variability in the DMEK preparation procedure and the needs to open tighter grafts to count ECD.

To reduce bias, we used pairs of corneas from a single donor to reduce donor tissue variability and a single expert DMEK surgeon to prepare all graft using the same technique.

From our findings, a slow stripped DMEK graft is associated with a wider graft scroll conformation with potential for easier unfolding within the eye. However, a slower stripping graft was associated with a greater drop in ECD. Our study suggests a fast peeling technique could be favourable when preparing tissues with a low or borderline ECD, while in tissue with a higher cell count and younger donors, a slow peeling speed will benefit the surgeon by reducing the scroll width and tightness. To further evaluate our findings, a study with a larger sample size would be needed to assess for other possible variables / confounders in DMEK graft preparation and evaluation of the impact of the scroll width in vivo.

Table 5.1

Group 1 and 2 parameters pre- and post- stripping

Donor	Age	Speed of Stripping	OD/OS	Scroll Width	ECD Prestripping	ECD Poststripping	ECL (%)
Donor 1	63	Slow	OD	6.0	2140	1850	13.55
	63	Fast	OS	3.0	2350	2220	5.53
Donor 2	73	Slow	OS	7.4	2000	1500	25
	73	Fast	OD	3.3	2000	1850	7.5
Donor 3	63	Slow	OD	4.9	2140	1930	9.81
	63	Fast	OS	3.0	2350	2220	5.53
Donor 4	77	Slow	OS	7.2	2400	2200	8.3
	77	Fast	OD	2.6	2200	2030	7.73
Donor 5	77	Slow	OD	5.9	2190	1930	11.87
	77	Fast	OS	2.9	2300	2100	8.7
Donor 6	70	Slow	OS	5.2	2100	1450	30.95
	70	Fast	OD	2.8	2050	1830	10.73
Donor 7	70	Slow	OS	7.1	2000	1600	20
	70	Fast	OD	2.9	2100	1820	13.33
Donor 8	78	Slow	OD	6.6	2100	1750	16.67
	78	Fast	OS	3.1	2000	1850	7.5
Donor 9	74	Slow	OD	5.9	2150	1550	27.91
	74	Fast	OS	2.1	2000	1900	5
Donor 10	60	Slow	OD	7.8	2000	1570	21.5
	60	Fast	OS	3.2	2050	1740	15.12

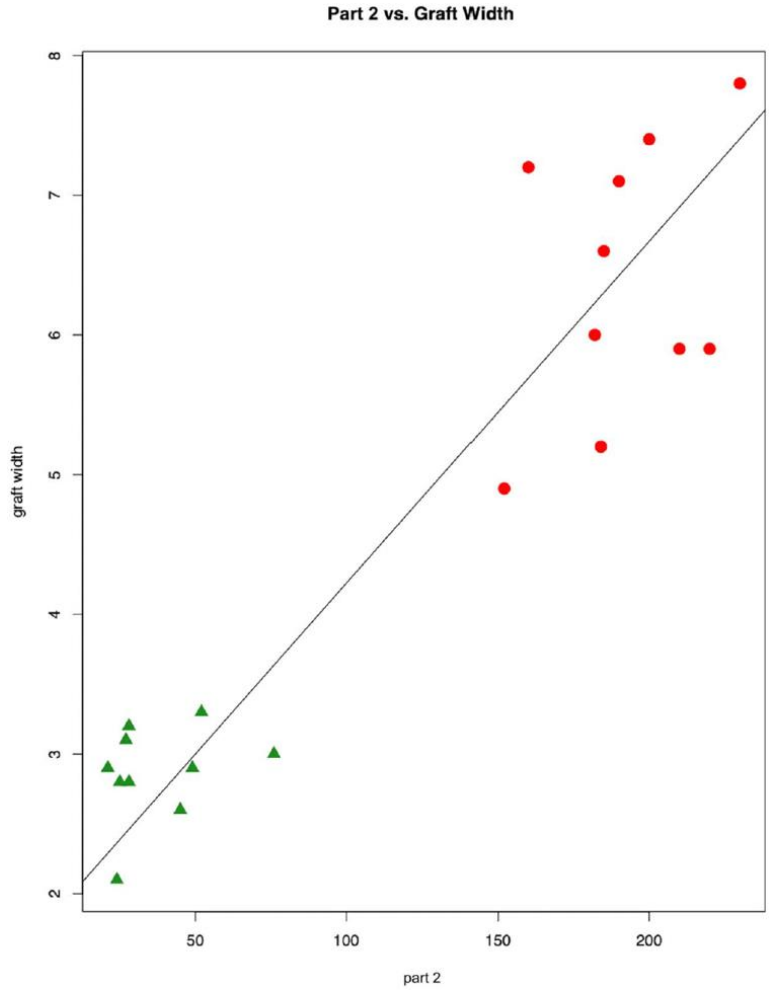

Figure 5.1 **Plot of graft width versus stripping speed obtained for each graft prepared**

Group 1 (circles) and group 2 (triangles). The full colour version of this article is available at *www.cornealjrnl.com*.

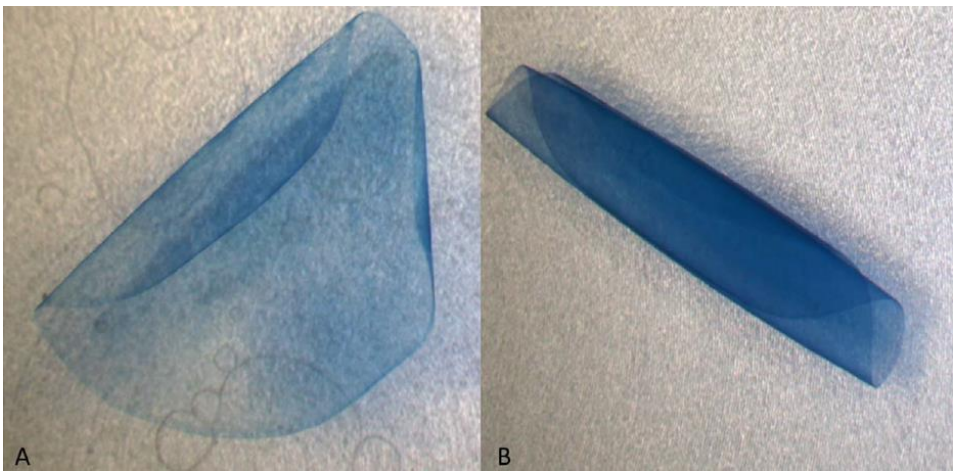

Figure 5.2 **DMEK graft tissues prepared from same donor**

(A) show-peeled graft (group 1) and (B) fast-peeled graft (group 2). The full color version of this article is available at *www.cornealjrnl.com*.

6 Study 6

Following improvements in tissue preparation presented in the previous studies, Study 6 aims to analyse Graft detachment and rebubbling rates.

Abstract in English

Title: Graft detachment and re-bubbling rate in Descemet membrane endothelial keratoplasty

Although the donor preparation and surgical methods still remain a challenge, DMEK is gaining popularity in terms of early rehabilitation and visual outcomes. New and improved donor preparation techniques like prestripped DMEK tissues are being taken up rapidly because of less manipulation that is required in the surgical theatre. Donor graft delivery in the recipient eye has also been improved because of new products like pre-stripped and preloaded membranes. As DMEK is at its budding stage, only early outcomes have been known so far. Early graft detachment, rebubbling rates, and primary failures are still being studied in DMEK. As there are different techniques that are currently used for preparation and injection of the graft, it becomes difficult to judge the results based on specific inclusion and exclusion criteria. Graft detachment and rebubbling rates have been a huge challenge both during the surgery and also while reporting postoperative data. We highlight the importance of defining graft detachment and rebubbling rates and their surgical relevance, which may also have an impact on graft preparation and insertion techniques.

Keywords: DMEK; graft detachment; rebubble rate.

Abstract in Latvian

Pēc audu sagatavošanas uzlabojumiem, kas tika aprakstīti iepriekšējos pētījumos, sestā pētījuma mērķis ir analizēt transplantāta atdalīšanās un atkārtotas burbuļošanas ātrumu.

Nosaukums: Transplantāta atdalīšanās un atkārtotas burbuļošanās ātrums Descemeta membrānas endotēlija keratoplastikā.

Lai gan donoru sagatavošana un ķirurģiskās metodes joprojām ir izaicinājums, DMEK strauji gūst popularitāti agrīnas rehabilitācijas un vizuālā rezultāta ziņā. Jaunas un uzlabotas donoru sagatavošanas metodes, piemēram, iepriekš noņemti DMEK audi, tiek ātri pieņemtas un izmantotas, jo tas mazina nepieciešamo manipulāciju daudzumu operāciju zālē. Arī donora transplantāta nogādāšana recipienta acī ir uzlabota, pateicoties jauniem produktiem, piemēram, iepriekš noņemtām membrānām. Tā kā DMEK atrodas tikai izpētes sākuma stadijā, līdz šim pieejami tikai dažu pētījumu rezultāti. DMEK joprojām tiek pētīta agrīna transplantāta

atdalīšanās, atkārtotas burbuļošanas ātrums un dažādas neizdošanās, kas saistītas ar DMEK. Ņemot vērā, ka šobrīd transplantāta sagatavošanai un ievietošanai tiek pielietotas dažādas metodes, ir grūti spriest par rezultātiem, pamatojoties uz konkrētiem iekļaušanas un izslēgšanas kritērijiem. Transplantāta atdalīšanās un atkārtotas burbuļošanas ātrums ir bijis milzu izaicinājums gan operācijas laikā, gan arī ziņojot par pēcoperācijas periodu. Mēs vēlamies uzsvērt to, cik svarīgi ir noteikt transplantāta atslāņošanās un atkārtotas burbuļošanas ātrumu un to ķirurģisko nozīmi, kas būtiski var ietekmēt arī transplantāta sagatavošanas un ievietošanas metodes.

Atslēgas vārdi: DMEK; transplantāta atdalīšanās; atkārtota burbuļošana.

6.1 Introduction

From the first studies published, DMEK has demonstrated excellent results in terms of visual recovery and a low rate of postoperative rejection. Different from Descemet stripping automated endothelial keratoplasty, the DMEK surgical procedure has highlighted significant difficulties because of the complexity of handling of the membrane, implanting the graft with the correct orientation, and management of early postoperative complications that may occur, such as the detachment of the graft. Various preparation and delivery techniques have been addressed so far, and there is still no standard technique. At the Veneto Eye Bank Foundation (FBOV) in Italy, we have observed a constant increase in the requests of precut DMEK tissues since 2014 (Fig. 6.1). [93, 94, 95]

DMEK is gaining popularity because of early rehabilitation and better visual outcome. Hence, its validation and standardization in terms of preparation, implantation and postoperative data collection and analysis become essential. In 2016, we published a “proof of concept” paper for preloading DMEK tissues and introduced a modified design for a surgical device that could help to preserve, transport, and transplant a DMEK graft with less manipulation. Preloading DMEK, we assume, will also be useful to standardize and validate DMEKs with advantages like reducing primary graft failure rates due to de-oriented grafts, lowering high surgical skills quotient, high rehabilitation rate with better visual acuity, less surgical time, 33 lower costs, and fewer logistic requirements. DMEK is also gaining popularity in the USA, as per the report of 2015, it was only performed in 748 cases in 2012. This increased to 1522 cases in 2013 to 2865 in 2014 and 4694 cases in 2015.

With the new techniques in the field of DMEK graft preparation and shipment, it becomes essential to standardize the delivery technique, followed by monitoring and reporting the post- operative clinical data in a universally accepted format. This review describes the

results achieved after DMEK highlighting the importance of determining, monitoring, and reporting graft detachment and re-bubbling rates.

6.2 Graft detachment

It is important to monitor the behaviour of the graft during the course of visual rehabilitation and in the late postoperative period once the DMEK graft is implanted inside the recipient's eye. The commonest postoperative complication after DMEK is graft detachment. Usually, DMEK complication rates have been reported separately by detachment rates. The graft detachment can be classified as complete or partial: "complete" when all the donor tissue is completely detached from the recipient and floats in anterior chamber and "partial" when part of the donor tissue is still attached to the recipient.

6.2.1 Diagnosis

It must be noted that all the reports that do not perform anterior segment optical coherence tomography in the follow- up can misreport a partial Descemet membrane detachment (DMDs). If the cornea is relatively clear, the presence of DMDs can be easily detected by slit-lamp biomicroscopy; however, in the presence of severe corneal edema, anterior segment optical coherence tomography remains the best imaging tool to determine the configuration (planar or scrolled) and location of DMD, which are vital for planning the surgical technique. Various classification systems have been proposed for DMD such as planar versus nonplanar, scrolled versus non-scrolled, and peripheral versus peripheral with central involvement. Planar DMDs are those in which the separation between the stroma and Descemet membrane is < 1 mm. When the separation is > 1 mm, the detachment is termed nonplanar. DMDs that exhibit a rolled edge are described as scrolled, and those without a rolled edge are described as non-scrolled. [96, 96, 98, 99]

6.2.2 Rate of graft detachment

The initial report of complete DMDs rate was 30 %. Partial graft detachment rates have been reported in around 62–63 % cases; however, with improved techniques and surgical experience, reported graft detachments have reduced to 34.6 % in a multicentre study and as low as 4 % in one case series. Therefore, a learning curve for DMEK implantation, including the postoperative care, is highly relevant.

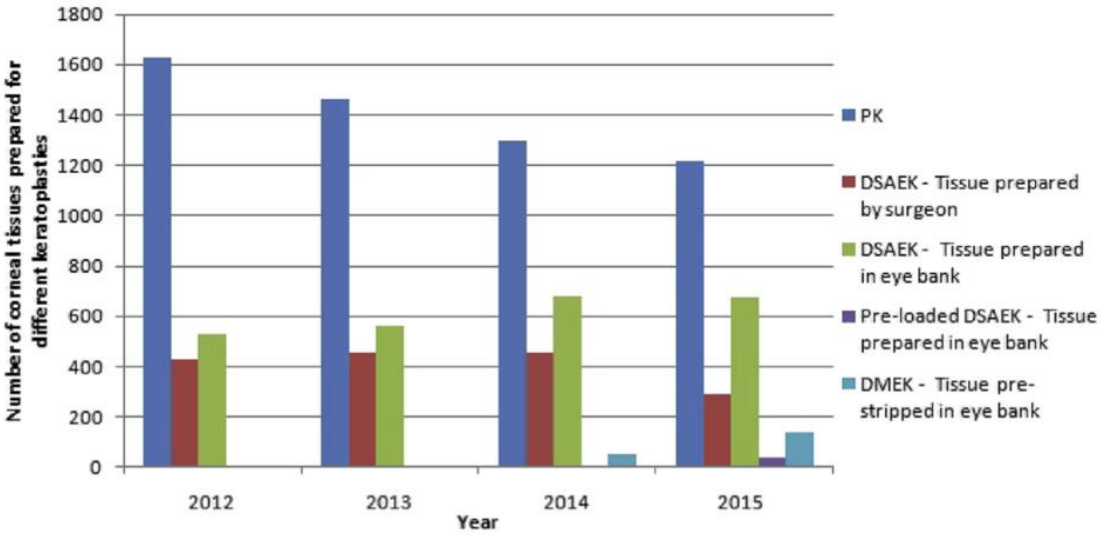

Figure 6.1. **Number of corneal tissues prepared for specific keratoplasties between 2012 and 2015 at the Veneto Eye Bank Foundation (FBOV), Venice, Italy. A consistent decrease in the request for penetrating keratoplasty (PK) has been noticed since 2012, whereas tissues for Descemet stripping automated endothelial keratoplasty (DSAEK) and Descemet membrane endothelial keratoplasty (DMEK) prepared in the eye banks have shown increased popularity**

6.3 Early management or prevention of primary graft failure

Most lamellar keratoplasty complications that threaten graft survival occur in the week or weeks post-transplant and in most cases are evident at the first postoperative clinical examination. The diagnosis and successful management of these early complications is crucial to long-term graft survival. The size of descemetorhexis plays a role to reduce the peripheral postoperative edema and to prevent DMDs. It should be slightly smaller compared with the diameter of the graft. The graft diameter (or chord length) in the eye differs from the diameter of the graft on the trephine block. This difference depends on the disparity between the radius of curvature of the trephine and that of the posterior cornea.

DMEK graft is highly transparent and unless marked on its Descemet's side, it becomes difficult to understand the orientation of the graft. Intraoperative and early postoperative 'checking the marks' allows the graft to be implanted with correct orientation and reduces any upside-down orientation, avoiding graft failure due to inverted transplant. Veldman from 20.8 to 10.4 %. Rebubbling the graft could be an option to overcome the et al stamped an "S" on the DMEK donor graft.

Others suggested asymmetric marks on the edges of the graft All these marks ensured correct graft orientation and help in the early postoperative phase to exclude upside down grafting. Melles group proposed an algorithm to predict if a detachment is likely to be transient or lasting: performing anterior segment optical coherence tomography at 1 hour and 1 week post- operative. If at 1 week the graft is completely attached, then it will be detachment-free. Instead, if any detachment is detected, they recommend checking the scan made at 1 hour after

surgery. If this examination also shows the same detachment, then spontaneous re-attachment is unlikely and rebubbling is recommended. [100, 101, 102]

6.4 Management of graft detachment

Management and prognosis vary in the 2 types of detachments. In addition, in the case of partial detachment, the location or the site of detachment plays a significant role along with the conformation, timing in terms of when the detachment occurs (days 1, 3, or later), and percentage of the graft attached. Partial (larger than 33 % of the DMEK-graft surface), central, scrolled, or complete detachment affects the visual recovery and require an intervention, commonly rebubbling. The reinjection of air promotes attachment, improving visual recovery, and preventing fibrosis and shrinkage of Descemet membrane. Cases of free-floating DMEK graft associated with reendothelialization without any air injection have been reported; however, the visual recovery was delayed by months, and the endothelial cell density was low. However, it is still not clear how the endothelial transfer, migration, or regeneration of donor or recipient cells occur.

There are different techniques and algorithm for management of graft detachment. Although there is no literature comparing the efficacy of different gases, most surgeons would choose air or sulfur hexafluoride (15–20 % SF6) or in some cases perfluoropropane (12–14 % C3F8) because of its longer resorption time for cases failing reattachment with the other 2 gases or those detached for a prolonged period.

Complete detachments are the most straightforward regarding management. In these cases, the graft has entirely peeled away from the posterior corneal surface and is usually free-floating in the anterior chamber. It is never likely to reattach, and the entire cornea will remain edematous. These eyes should therefore be reoperated immediately. Most often a re-DMEK or Descemet stripping automated endothelial keratoplasty can be performed with a good clinical outcome. Ham et al recently determined a rebubbling rate of 4.4 % where all the cases were performed within 6 months postoperatively with a graft detachment rate of 11.6 % (> one-third graft detached). The visual acuity levels achieved 6 months after DMEK may remain stable for at least 4e7 years, with complications occurring after the first 6 months in < 5% cases. However, it was reported that minor detachments (< one-third) required secondary intervention, all of them remaining clear at all follow-ups.

Of the major detachments (> one-third), 23 % remained clear and 77 % required a repeat transplantation. Major graft detachment rates decreased over time from 20.8 to 10.4 %.

Rebubbling the graft could be an option to overcome the detachment of partially detached grafts; however, a repeat surgery may have to be performed on the completely detached grafts. [103, 104, 105, 106]

6.5 Rebubbling indications, techniques, management, and success rate

Rebubbling (also called anterior chamber rebubbling) is a procedure to manage graft detachment after DMEK if the graft is correctly oriented. Various techniques for rebubbling have been described with different rate of success. The rebubbling rates of 73.8 % can be found if graft detachment and rebubbling rates are recorded as one unit; however, a 36 % rate of rebubbling has been observed after partial detachments of 15–50 %.

This further highlights the variation in the preferences of the surgeons, as some would observe graft detachments for months before intervention, whereas others may repeat injection after 1–2 weeks. In general, graft detachment rates and time of rebubble have been dissimilar with different methods to rescue the graft.

6.5.1 Area of descemetorhexis may be an important factor for DMEK survival

Further to what is described earlier in the management of graft detachment, Tourtas et al reported that graft detachment rate is related to the extent of descemetorhexis. Of 2 groups, descemetorhexis of 10 mm was performed in group A, whereas group B consisted of 6 mm. 33.3 % graft detachment was found after 4 days in group A, whereas 78.3 % was found in group B. The rebubbling rate was found to be 6.7 % in group A and 30.4 % in group B, which was statistically significant. A larger descemetorhexis in DMEK was found to be correlated with better graft adhesion and lower rebubbling rates, whereas patients with a larger descemetorhexis require less intensive follow-up.

6.5.2 Late intervention resulting in corneal scarring

It has been observed by Baydoun that late intervention may allow the cornea to clear spontaneously; however, visual recovery is longer, while the delayed healing may lead to potential corneal scarring, poorer final visual outcome, and lower endothelial cell count. In a recent study, Muller showed that rebubbling procedures may be performed within 4–6 weeks, before portions of the detached graft scar and graft detachments reattach with interface scarring.

6.5.3 New and improved techniques for rebubbling

Some techniques and algorithms for management of graft detachment have been reported. Sharma et al propose intracameral injection of air in cases of planar DMDs in the superior half of the cornea, and 14 % perfluoropropane C3F8 in all the other cases, reporting a mean time of resolution of corneal edema in 16 days. It is versatile in that it can be performed at the slit-lamp or in the operating room. A 3-year comparative clinical study using 20 % sulfur hexafluoride (SF6) versus 100 % air tamponade to overcome graft detachment in DMEK showed a requirement of rebubble in 2.38 % from SF6 group, compared with 12.8 % from air tamponade group where the detachments were larger than 60 %.

Rebubbling was per formed sooner if more than 50 % detachment was observed. New techniques for DMEK strategies like S-stamping have shown a higher rebubbling rate (13 %), as compared with a non-stamped graft (3 % within first 6 months postoperative). Bimanual technique for insertion and positioning of the endothelium-Descemet membrane graft in DMEK has a reported 6.6 % rebubbling rate.

Busin et al recently reported a new technique for delivery of tri-folded DMEK grafts where the only complication observed was graft detachment (10 of 42 eyes [23.8 %]), which was successfully managed in all cases by single rebubbling within 6 days after surgery.

6.5.4 Rebubbling of the DMEK grafts using eye bank prepared tissues

New techniques from the surgeons and the eye banks have also been evaluated on the basis of rebubbling rates. Eye bankeprepared tissues for DMEK have a reported 27.5 % rebubbling rate. The reported rates of partial detachment of DMEK tissues prepared by surgeons requiring air injection instead range from 9–82 %.

Deng et al noticed that the most common complication after DMEK was partial detachment of the Descemet membrane graft at the periphery after the use of eye bankeprepared tissues. Air injection for partial detachment was performed in 27.5 % cases. No total detachment was observed; however, a single air injection was sufficient for reattachment in 90.9 % cases. Liarakos et al reported the effects of preservation medium on rebubbling rate. This study showed that tissues preserved in hypothermic media show higher detachment rates (35 %) compared with organ culture (27 %); however, the rebubbling rate from corneas previously in cold storage was 24 %, compared with 29 % in organ culture and 18 % when the tissues were excised from fresh globes. [107, 108, 109]

6.5.5 Grading system for rebubbling

Maier et al showed graft detachment with required rebubbling rate of 38.1 %. This study showed a grading system for unfolding and attaching of the graft lamella with regards to its difficulty in four grading groups: 1) graft lamella primarily oriented correctly in the anterior chamber, straight and direct unfolding and centring, which showed a 37 % rebubbling rate due to less manipulations; 2) slightly complicated, indirect unfolding and cantering (duration less than 5 minutes), which showed 44.7 % rebubbling rate; 3) difficult indirect unfolding and centring (duration longer than 5 minutes), repeat air injection with balanced salt solution exchange necessary, which showed a 43.8 % rebubbling rate; and 4) direct manipulation of the graft lamella for unfolding and cantering by cannula or forceps, which showed a 50 % rebubbling rate. This shows that the degree of manipulation may lead to higher rebubbling rates.

6.5.6 Other long- and short-term studies

In a 1-year study by Price et al, the rebubbling rate was 5 %.

Whereas, Rodriguez-Calvo-de-Mora et al reported a one time- rebubbling rate of 13 % and twice in 2% of grafts, where the median duration of follow-up was 18 months (range 3–61 months).

Tourtas reported graft detachment rate of 15.8 % with 3 % rebubbling rate at 6 months postoperative. In another study by Fernandez Lopez et al, rebubbling was performed on an average of 26 days (range 7–92 days) after DMEK. The air cannula was introduced in the area of graft attachment or detachment. After excluding upside-down grafts, the rebubbling success rate in total was 87 % (33 of 38 eyes).

In the attached area approach, it was 92 % (22 of 24 eyes) and in the detached area approach, 79 % (11 of 14). Of 14 eyes that had been rebubbled later than 1 month post-operatively, 11 were successful. In 8 eyes, the graft seemed too stiff / immobile to allow complete unfolding. A recent study by Gerber-Hollbach et al showed, however, that rebubbling a DMEK graft may result in similar visual outcomes as in uncomplicated DMEK when performed within the first 6–8 weeks post-operative.

Rebubbled eyes may have lower endothelial cell density, which may be attributed to additional air bubble trauma and or selection bias through more extensive manipulation during initial DMEK or higher risk of graft detachment in more complicated eyes.

Possible crucial factors in graft detachment include:

1. Air / gas.
2. Previous glaucoma surgery / anterior chamber dysgenesiss.
3. Aanterior chamber depth.
4. Biomechanical behaviour of the graft.
5. Recipient surfaced-graft diameter and descemetorhexis.
6. Prestripped in eye bank and preservation media.

6.6 Future aspects

To increase the efficiency of postoperative care, it would be appropriate to rebubble a tissue regardless of the size of the graft detachment at the earliest possible time and report any graft detachment as a rebubble rate and combine them as one unit. There is a high chance of obtaining true-negative data where detachment rate may not reflect the rebubbling rate and vice versa. This may also promote a DMEK technique that may be better, but because of the rebubbling rate (highlighted same as detachment rate), the efficiency of a particular DMEK technique may not be considered good, although its clinical efficiency may be higher. As different studies have provided different DMEK graft detachment and rebubbling rates at various time points, we believe that, if the rebubbling rate is defined, it would be easier to develop and standardize surgical procedures based on the precise clinical data. Moreover, it has already been reported that a graft that is rebubbled sooner has an earlier rehabilitation and better visual outcome without compromising endothelial cell density and, in addition, this reduces the chance of corneal scarring. So far, we have only found one report in the literature by Terry et al, which has defined the rebubbling rate as “Any tissue that had an injection of air into the anterior chamber in the postoperative period was recorded as a re-bubble.”

This study showed 6 % rebubbling rate between 1 and 3 weeks after the DMEK surgery. It has already been suggested by Muller et al that rebubbling is a feasible procedure to manage graft detachment after DMEK if the graft is correctly oriented. Proper preoperative planning may aid in minimizing intraoperative complications and may increase the success rate. Late interventions > 1 month postoperatively) may still produce graft reattachment, but increased graft stiffness / fibrosis may complicate complete graft unfolding.

Incomplete host Descemet membrane removal may relate to postoperative DMEK graft detachment and wound instability. Graft detachments may reattach with interface scarring. Rebubbling procedures may be performed within 4–6 weeks, before portions of the detached graft scar. We believe that to achieve early rehabilitation rate and a visual acuity of 20 / 20, early rebubbling of the graft is necessary.

The necessity for rebubbling for small peripheral detachments has not been precisely determined. We recommend to reporting graft detachment rates along with rebubbling rates to broaden the knowledge of graft activity in vivo. The definition can be further modified from Terry report and may be redefined as “Graft Detachment and Re-Bubbling (GDRB) rate any tissue that has been detached regardless of the amount of detachment and has an injection of air or other gases into the anterior chamber for reattachment of the graft in the post-operative period.” As early rebubbling of the graft has already shown its benefits in terms of visual outcomes and limiting corneal scarring, reporting of rebubbling rates will also provide a standardized method for surgery and reporting the postoperative data, which will further enhance DMEK surgery. [100, 111, 112]

6.7 Method of literature search

All the articles were searched in PubMed with words “DMEK, Descemet membrane endothelial keratoplasty, rebubbling rate, detachment rate, clinical outcomes.” The year of publication was not considered in the inclusion criteria as DMEK is relatively recently developed.

7 Study 7

After a comprehensive explanation of DMEK surgery in the previous studies, the last study shows tips and tricks for new DMEK surgeons.

Abstract in English

Title: DMEK: tips and tricks.

The learning curve in DMEK has proven to be much steeper than previous endothelial keratoplasty procedures. Hence, we share tips, tricks and a step-by-step guide to improve the learning curve and outcomes in DMEK.

Key Words: DMEK; Learning curve

Abstract in Latvian

Pēc visaptveroša DMEK operācijas skaidrojuma iepriekšējos pētījumos, septītajā pētījumā tiek akcentēti padomi un nianses, kurām pievērst uzmanību jaunajiem ķirurgiem.

Tēzes

Nosaukums: DMEK: padomi un triki.

DMEK apgūšanas līkne ir izrādījusies daudz stāvāka un straujāka nekā iepriekšējās endotēlija keratoplastikas procedūras, tāpēc mēs dalāmies ar padomiem, soli pa solim ceļvedi, apgūstamiem trikiem un niansēm, lai uzlabotu DMEK apgūšanu un rezultātus.

Atslēgas vārdi: DMEK; mācīšanās līkne.

7.1 Introduction

Nowadays endothelial keratoplasty (EK) is the standard of care replacing penetrating corneal transplantations in cases of endothelial dysfunction. Here we share our experience with the aim of making DMEK surgery simpler and more affordable in order to facilitate its uptake.

7.2 Graft preparation

The first step towards successful DMEK surgery is to master the donor tissue preparation technique giving an intact graft to transplant. Although many different techniques have been proposed for DMEK graft preparation, there is no consensus as to which is the optimum. The most commonly used techniques include pneumatic dissection, stripping methods, submerged hydro-separation technique, liquid separation technique and many more

that have been thoroughly evaluated previously. The stripping methods have been the most widely adopted. Briefly, we currently use 2 standard DMEK graft preparation techniques, depending on our setting, which include the following steps to ensure a healthy graft. In an eye bank setting we use a double trephine technique.

It involves the use of 2 punches, a mark on the graft and multiple checks of endothelial cells during the procedure. A DMEK graft prepared in an eye bank setting reduces the surgeon's stress level due to possible failure in tissue preparation before surgery. The second method is used in a theatre setting before the operation. It's cheaper, quicker and it involves the use of a single trephine.

It can be considered a modification of the Melles technique. For beginners, we suggest starting using DMEK tissue prepared in an eye bank setting and planning the first surgeries with the use of pre-stripped tissues.

7.2.1 The double trephine technique

In an eye bank setting, the corneal tissue is washed with sterile phosphate buffered saline (PBS) to remove traces of storage media. The cornea is then checked for endothelial cell mortality using trypan blue stain (0.025 %) and endothelial cell density (ECD) is recorded using a calibrated graticule in the eye-piece of an inverted microscope. Average readings of 5 counts are usually obtained to avoid counting errors. If the tissue shows < 5 % trypan blue positive cells and > 2200 cells/mm^2 then it can be used for transplantation otherwise it is usually used for research purposes only. There are several other factors to check before the tissue is deemed of a 'sufficient quality' for transplant. After ECD and mortality checks, the tissue is fixed on a vacuum block with the endothelium facing up (Figure 7.1 (A)). Using a corneal punch blade (9.5mm), the endothelium is superficially trephined by gentle tapping on the top of the endothelium. The cut margins are visualized using trypan blue stain (Figure 7.1 (B)).

The margin distinguishes the border between the central and the peripheral endothelium. Using sharp acute forceps, the peripheral endothelium is removed, leaving only the central endothelium (Figure 7.1 (C)). To reduce radial tears and peripheral cuts of the tissue, a cleavage hook is used to identify the cleavage plane and separate the periphery of the central endothelium from the stroma (Figure 7.1 (D)). The separated periphery is then grasped using the sharp acute forceps at the superior end and is peeled towards the inferior end (Figure 7.1 (E)). The entire process may take from a few to several minutes depending on the adherence of Descemet membrane (DM) to the underlying stroma.

The tissue is peeled leaving approximately 10 % of the inferior peripheral hinge. The hinge protects the DMEK tissue from free floating or forming a roll in the media during the transport phase from an eye bank. It is also helpful to allow stamping of the DMEK tissue on the DM side to avoid the tissue being transplanted upside down. A biopsy punch is used to create a small stromal punch (Figure 7.1 (F)) and the peeled DMEK tissue is replaced back on the stroma (Figure 7.1 (G)). The vacuum is released, and the tissue is inverted on the vacuum block with the corneal epithelial side facing up. The punched stromal piece is then removed from the epithelial side. This allows gentian violet dye on the tip of a cleavage hook to be used to mark the letter 'F' (with correct orientation) on the DM (Figure 7.1 (H)). The stromal piece is returned and the tissue is inverted back and fixed on the vacuum holder. Although we have used letter 'F', other letters like 'S' can also be used. Once the tissue is ready, the endothelium is re-stained using trypan blue (Figure 7.1 (I)) for final quality assurance of the graft in terms of ECD and mortality before shipping to the surgeon.

The surgeon can then choose the diameter required for the patient and use a second trephine for excision of the graft before the transplant. We have observed minimal mortality and a high success rate using this technique. Slight modifications such as oscillating movements, different points of initiation and use of peripheral DMEK grafts have allowed us to manage challenging cases with tight adherence, cut / horse-shoe shaped tears and post-cataract surgery tissues.

These steps have reduced our tissue wastage dramatically for DMEK grafts.

We have also noted that certain techniques are useful in generating high quality grafts but require a more substantial amount of time and instruments compared with other techniques that provide slightly less quality, although acceptable for a transplant, and they do not require long working hours or special instruments that could be a financial concern. The technique used for DMEK graft preparation is very subjective depending on the end-user's requirements. Therefore, it is recommended to use as many standard procedures and instruments as possible to avoid complications during graft preparation.

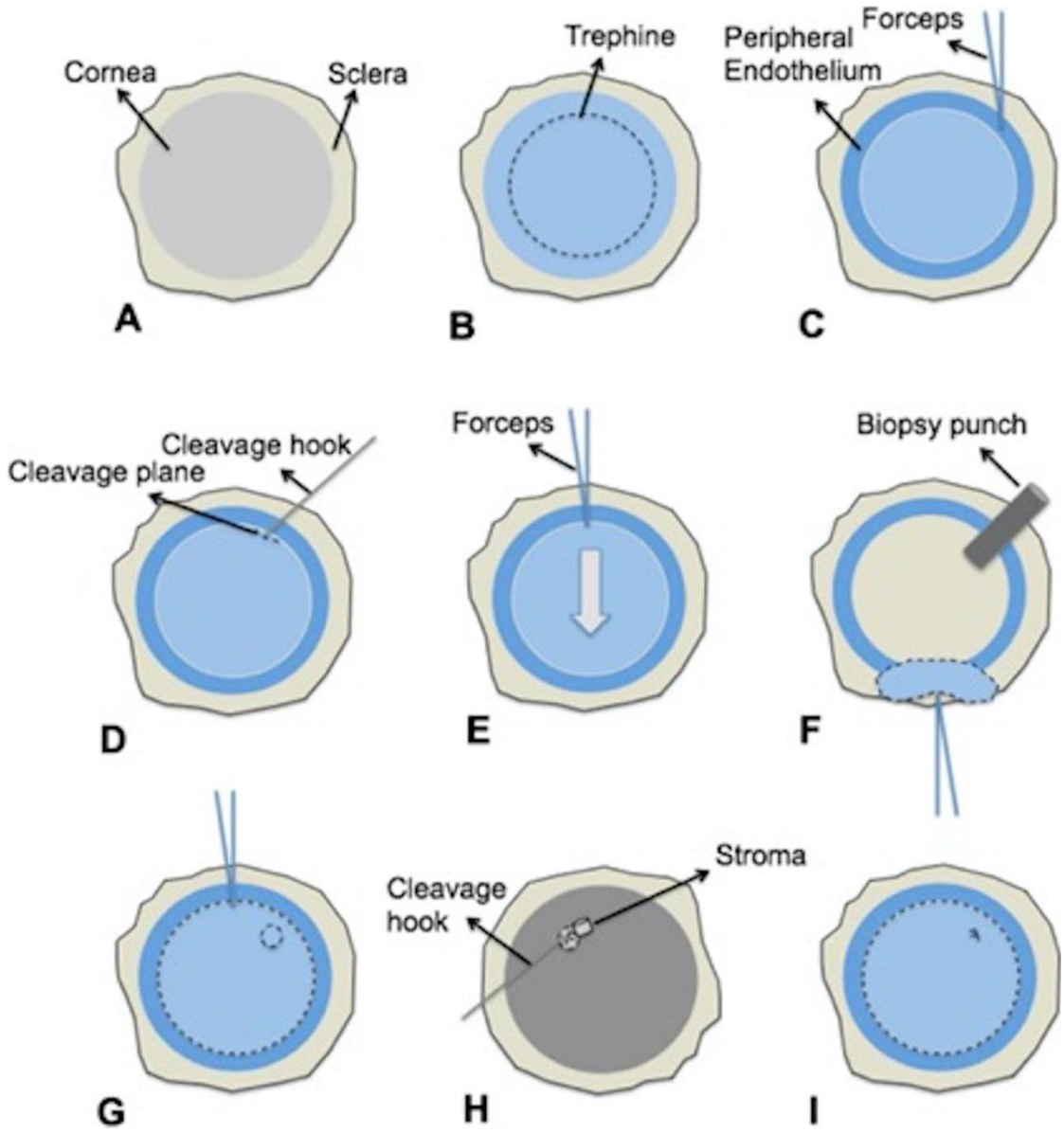

Figure 7.1 **DMEK tissue preparation in Eye-Banking setting**

7.2.2 The single trephine technique

For preparation of the graft in the operating theatre, it is possible to use a different technique. It involves the use of only 1 trephine and, therefore, it is more cost effective.

The cornea is centred on a punch base using active suction. The vacuum is created with a syringe and the tissue is secured on the base. We start by staining the endothelium with trypan blue 0.06 % (Vision Blue®; DORC, Zuidland, The Netherlands) for 15–20 seconds. Thereafter we identify an area in the periphery of the trabecular meshwork (TM) without damage, residual uveal tissue or previous corneal incisions to start peeling the DM (Figure 7.2 (A); marked in red).

DM is peeled from TM (Figure 7.2 (B)) by gently swiping the DM layer from its periphery towards the centre (Figure 7.2 (C)) using a pediatric crescent knife 2.3 mm, angled bevel up (Alcon Laboratories, Inc., Fort Worth, Texas). During this step it is important to be careful not to apply to much pressure. If the crescent blade is too deep in the stroma, it will compromise the stripping by cutting into the stroma.

The blade should be used perpendicularly to the cornea and at an area of 100 degrees should be peeled for 2–3 mm toward the centre of the cornea. (Figure 7.2 (C)).

The peeled endothelium is then replaced back on the corneal stroma using BSS in a syringe.

The diameter of the punch is selected as required for the patient. The punch is placed on the graft and before punching, the stripped and stained endothelium should be visible through the centre of the punch (Fig. 7.2 (D)). If the trypan blue staining is not visible the graft should be replaced in a different position or the peeled area should be increased. A donor cornea punch is then used to cut the graft. The size of the graft usually ranges from 8.25 to 9.5 mm in diameter. Once the graft is cut, the cornea scleral rim is removed and the stripped area should be visible and possible to grasp. The forceps shouldn't be grasped too strongly. Higher grasping force could break the grasping point leading to a loss of tissue and need for regrasping potentially leading to higher loss of EC. The suction of the punch should be kept on throughout the procedure. The stripping is then completed with a longitudinal movement trying to avoid any damage or tears.

During the peeling, high tension on the graft should be avoided to minimise the risk of ruptures. Sometimes however, during stripping movement of the cornea could occur due to tension forces. In this case, additional toothed forceps could aid in keeping the corneal stroma in position. Once fully stripped, the tissue is placed on the corneal stroma and drops of preservative medium are placed on top of it. At this point, the patient should be called to theatre to start the surgery.

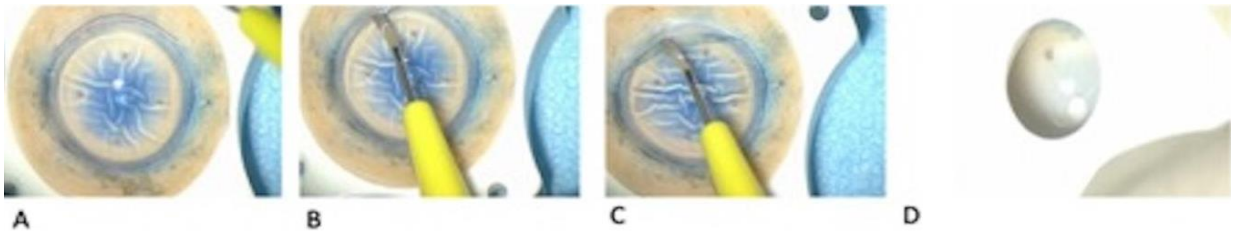

Figure 7.2 **Peeling (A–C) and punching (D)**

7.2.3 Graft size

In endothelial keratoplasty the aim is to replace the dysfunctional endothelial layer. Therefore, the graft diameter can vary as the size of the defective area changes. Corneal endothelial cell density (ECD) is higher in the periphery compared to the central cornea, especially beyond 9.00 mm. It has been hypothesized that there may be stem or progenitor like corneal endothelial cells with proliferative potential in the transition with the trabecular meshwork. It has been established that postoperative corneal clearance and graft survival depend largely on a sufficient postoperative ECD.

Whereas in penetrating keratoplasty (PKP) larger grafts are more prone to rejection due to the proximity to the limbus, this does not seem to be the case with either endothelial keratoplasty techniques, DSAEK or DMEK.

Moreover, the diameter of arc of the posterior cornea is larger, thus allowing the use of oversized grafts without compromising the limbus.

Intuitively, delivering larger grafts would provide not only a higher number of transplanted cells, but the possibility of an area containing cells of high proliferative potential, which could potentially increase graft survival in penetrating keratoplasty (PKP), DSAEK and DMEK.

Interestingly, neither Anshu, nor Terry, found differences in postoperative ECD in DSAEK grafts diameters from 8.0 to 9.0 mm. However, Romano found that using DSAEK grafts sized 9.5 mm or larger was associated with improved graft survival and Ishii similarly found that smaller DSAEK graft diameter was a significant factor for lower postoperative ECD.

Regarding DMEK, a recent report failed to find an association between graft diameter (between 8 to 10 mm) and central postoperative ECD. The authors hypothesize that the lack of difference in postoperative ECD could be attributed to the higher rebubbling rate in the 10.0 mm group; that each graft size was systematically used by a different surgeon, which could be a confounding factor; and that larger grafts required larger descemetorhexis and thus, a larger host surface to cover. Postoperative peripheral ECD data was not provided in this study. Quilendrino et al. routinely used 9.5 mm DMEK grafts and provided an interesting hypothesis on why an ECD decline was seen for large grafts.

Postoperative corneal deturgescence after DMEK affects almost exclusively the posterior surface, leading to an increase of its arc length, so that the endothelial cells must “stretch” and cannot be accurately measured with the fixed frame of the specular microscope.

Preparing a DSAEK graft of 9.5 mm requires a modification of the preparation technique, whereas larger DMEK grafts can be obtained rather straightforwardly. DMEK surgical technique using large grafts has proven feasibility with proper training. Care must be taken to ensure that the graft does not end up stuck in the angle.

Although the use of a large DMEK graft is desirable in order to deliver more endothelial cells, the size of the graft must be carefully customized by measuring the white-to-white distance in cases that are not straightforward, such as Asian populations, high hyperopic eyes and narrow anterior chambers, where smaller grafts are preferred. On the contrary, myopic and buphthalmic eyes can benefit from grafts larger than 9.5 mm. Interestingly, in a DMEK multicentre study 83 % of surgeons used DMEK grafts with diameters that ranged from 8 to 9 mm, and only 6 % used grafts larger than 9 mm. In our experience, graft unfolding is more difficult when using DMEK grafts larger than 9mm. Due to this, we suggest that inexperienced surgeons who are new to the procedure should undertake their first cases using smaller graft diameters. Further studies are required to definitively assess if the use of larger DMEK grafts is related to higher post-operative ECD and higher postoperative survival.

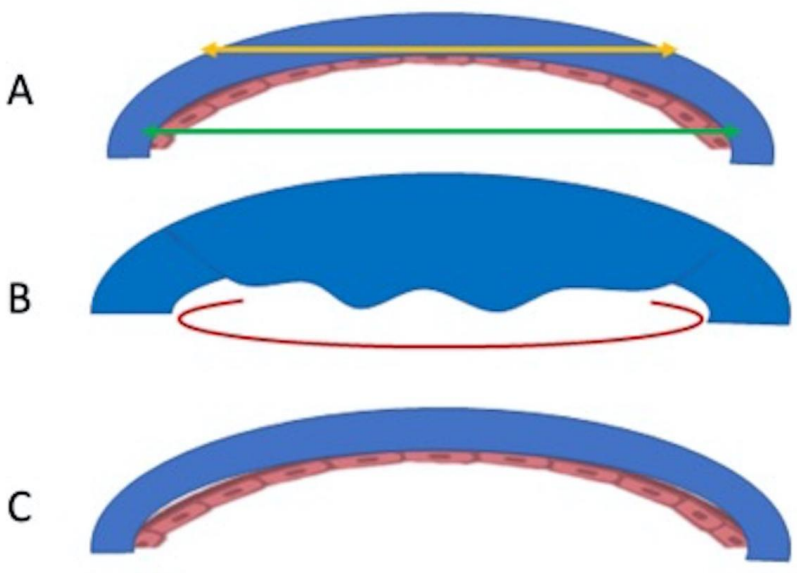

Figure 7.3 **Position of the DMEK graft**

7.3 Pre-operative assessment and patient preparation

7.3.1 Anaesthesia and dilating drops

We prefer to perform DMEK surgery under topical anaesthesia using Minims Proxymetacaine hydrochloride 0.5 % w/v eye drop solution (Bausch & Lomb House, Surrey, UK) combined with a peribulbar anaesthesia with lidocaine 2 % and bupivacaine 0.5 % in

a 3:2 ratio. Oral premedication with 15 mg midazolam or 10 mg diazepam prior to local anaesthesia could be considered in anxious patients.

We strongly discourage sub-Tenon's anaesthesia to avoid any bleeding at the surgical site, which can spread into the eye and lead to the formation of fibrin in the anterior chamber (AC) making the surgery more complex. Diuretics such as d-mannitol or acetazolamide are usually not required before surgery.

We recommend dilating the pupil with drops like tropicamide 1 % or atropine 1 % 30 minutes before surgery to obtain maximum dilatation. Dilated pupils help to optimize red reflex, reduce the surface contact between iris and graft, reduce the risk of pupillary block avoiding a peripheral iridectomy and aids the visualization of the endothelium during descemethorexis (Figure 7.5 (A)).

7.4 DMEK surgery

7.4.1 Incisions

The original DMEK technique described by Melles in 2006 starts with a 3.5 mm, superior, clear cornea, tunnelled, self-sealing, sutureless main incision and three auxiliary paracenteses. The technique for the main incision and auxiliary paracentesis has remained unchanged in the subsequent standardization of the technique. Other publications describe the creation of a clear corneal main incision that ranges from 2.2 to 3.2 mm (Figure 7.4), depending on size and nature of the insertion device for the DMEK graft: specific glass injectors (Melles pipette [DORC International] and Geuder [Geuder AG, Heidelberg, Germany]), used mainly in Europe; a modified Jones tube (Straiko–Jones tube [Gunther Weiss Scientific Glassblowing Co, Portland, OR]) or an intraocular lens cartridge.

We suggest placing the first 2 side ports 80–90° away from the main wound. They should be horizontal allowing the injected BSS to leave. This will also simplify the unfolding process. The third side port should be more perpendicular as it will be useful at the end of the surgery to manage the level of air in the AC. The location and the placement of a suture on the main incision after the graft insertion also varies greatly depending on the surgeon.

Nevertheless, DMEK can be considered a virtually sutureless procedure, increasing the postoperative refractive stability and decreasing the suture-related complications compared to previous keratoplasty techniques.

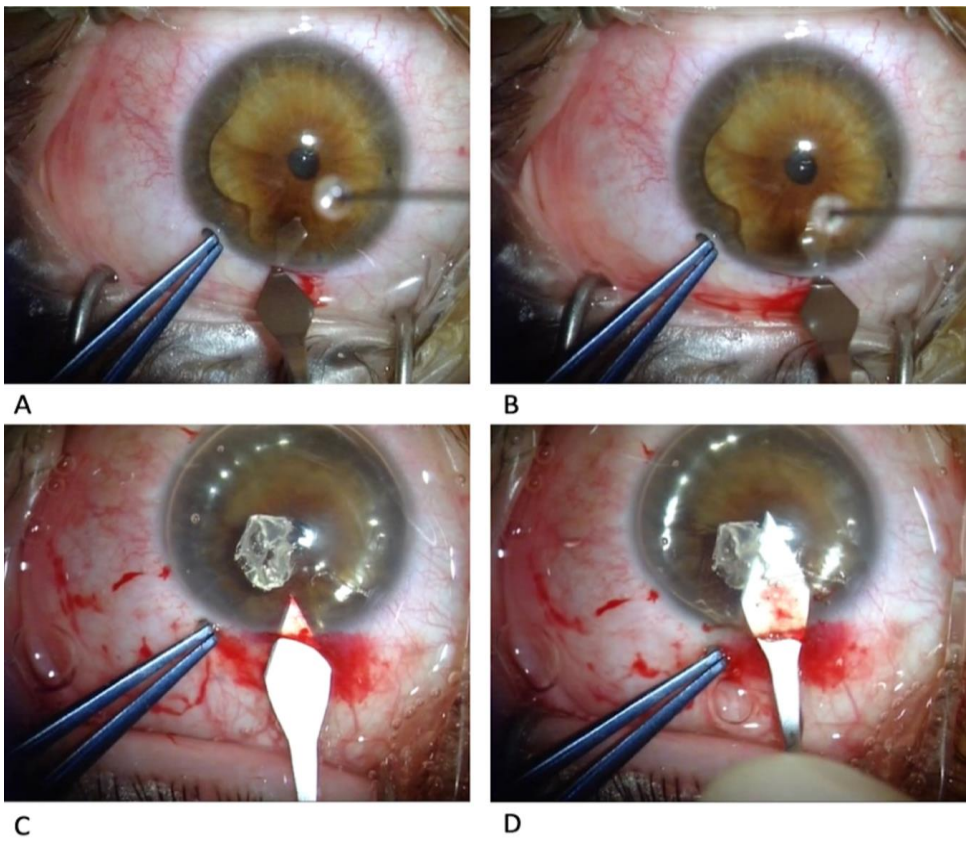

Figure 7.4 **A–D The main corneal incision**

7.4.2 Descemetorhexis

Descemetorhexis can be performed under air or with the help of ophthalmic viscosurgical devices (OVD). Descemetorhexis under OVD has advantages such as a more stable anterior chamber, reduction of flare and iris fluctuation.

We suggest performing at least a 180° descemetorhexis followed by the peeling of the recipient Descemet's membrane and endothelium with forceps (GRIESHABER® Asymmetrical Forceps, Alcon, Fort Worth, TX, US) (Figure 7.5°– (C)). To facilitate the insertion of the forceps in the AC we suggest bending it 60° in the middle.

This is to avoid any contact / damage with the recipient corneal stroma. When required, forceps could aid with the refining and enlarging of the descemetorhexis reducing the donor-recipient overlaps (Figure 7.5 (D)), in order to reduce the requirement for rebubbling. It is important to completely remove OVD before inserting the graft (Figure 7.5 (E)). To double check if the AC is free from OVD, it is possible to insert a bubble of air and record its expansion. If OVD remnants are present, the air bubble will not expand in the AC. Additionally, air in the AC prevents swelling of the recipient cornea during the graft staining and loading phase. At this point a 10 / 0 nylon suture is placed on the main incision. Performing this step before the

insertion of the graft will facilitate faster suturing at the end of the surgery, avoiding major complications like expulsion of the graft from the AC or loss of air (Figure 7.5 (F)).

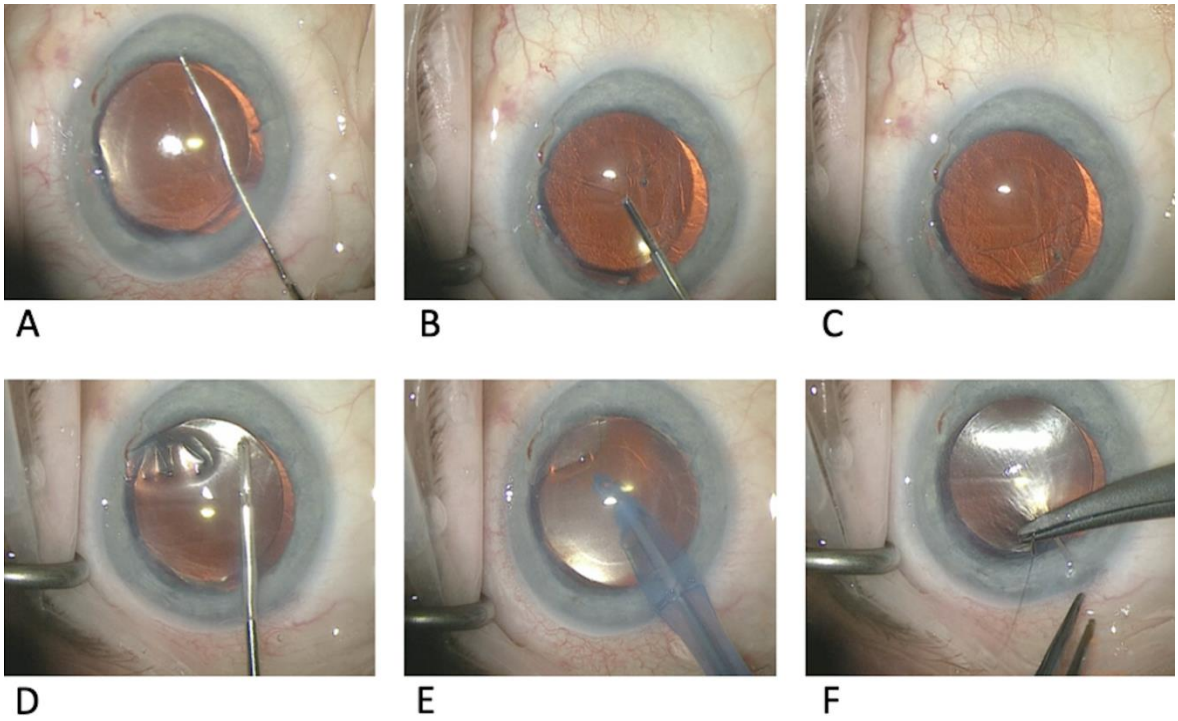

Figure 7.5 **Step-by-Step Descemethorexis (A–F)**

7.4.3 DMEK graft staining, loading and insertion

Staining the graft before its insertion into the AC is an important step in a successful surgery. A well stained graft aids its visualization during unfolding. As a standard approach we suggest staining the graft with membrane blue (0.025 %) for 30 seconds. The graft is then placed in a pot with BSS to facilitate the loading process. To insert the prepared DMEK graft into the AC requires a specialized injector. Ideally, it should facilitate the loading of the graft, cause minimal cell loss / damage to the endothelium and preserve the AC volume upon insertion. Surgeons may choose from a range of different insertion devices available commercially.

In our experience we found it was easier to start with glass injectors such as the Geuder glass tube (GEUDER AG, Heidelberg, Germany). During the loading of the graft, the injector should be full of BSS and its tip completely submerged under the BSS to avoid air being taken up. If the graft is loaded but air is present in the injector, we suggest trying to remove as much as possible. If air is accidentally injected in the AC, it could complicate the unfolding process and it should be removed. When injecting the graft, it is important to have a low AC pressure and flat AC. This is because when injecting the graft, BSS is also being injected and an elevated AC pressure could result in a torpedo reaction that will push the graft back outside.

7.4.4 DMEK unfolding and air injection

DMEK unfolding is the most variable step in terms of time in DMEK surgery. The DMEK graft, when peeled and submerged in BSS, will spontaneously rolls outwards. This requires additional unfolding manipulation once the tissue is injected into the AC. At the end of the surgery, DM should be well attached to the stroma in order to maintain the transparency of the tissue. If the DM is rolled inwards, it needs a complete opening of the graft before the attachment can be performed using a standard air tamponade or SF6 gas.

The patient selection is always a critical step. If the AC is very deep or very shallow, it could be a major challenge. Tissue from older donors tends to form wider graft rolls, which consequently require less manipulation during surgery, and where possible they should be considered for complex surgery. The presence of coexisting ocular pathologies, such as glaucoma tubes, anterior synechiae, iris malformations and anterior chamber intraocular lenses increases the risk of complications. These cases should be avoided in the when first learning the DMEK procedure.

An AC free from OVD, air and fibrin remnants, is the first prerequisite for a successful and safe unfolding. The surgeon must take care not to dislocate the DMEK graft in the vitreous chamber. In post-vitrectomy eyes a temporary hydrophilic methacrylate sheet can be useful. Different techniques to unfold DMEK grafts are reported in the literature. For beginners, we suggest the tap technique. After the insertion of the graft, the suture on the main wound is closed and bordered. Short taps with 2 cannulas on the corneal surface and bursts of BSS from the side ports help to open the graft and position it in the correct orientation (Figure 7.6).

Usually, the fluid waves within the AC through the side ports also help to open the graft. In cases of a very tight scroll, an air bubble injected inside the roll's lumen with a 30G-cannula may help the graft to unfold.

It is better to keep the AC shallow but not completely flat. When the surgeon is more experienced in the technique, to avoid the requirement for extra manipulations inside the anterior chamber, the endothelium could be manually tri-folded to preserve the endothelium inwards leaving the DM exposed. DMEK tissue tri-folded with endothelium inwards shows similar endothelial cell loss compared to the endothelium-outward technique and the spontaneous unfolding of the graft inside the recipient eye reduces overall time and surgical manipulation.

Pre-loading of DMEK grafts was introduced recently and aimed to avoid complications in surgery, reduce tissue wastage due to preparation errors, minimalize overall surgical time and to transplant a quality assured graft. When comparing the endothelium-inwards method with the endothelium-outwards method there was no major differences in endothelial cell loss

between the groups and no significant differences in clinical outcomes. The correct orientation of the graft should be checked as soon as possible. The direct observation of the Moutsouris sign or the F mark is usually quick and helpful.

If the orientation is correct, we can proceed to completion of the unfolding process. In cases where the orientation of the graft is upside down, the anterior chamber can be deepened and a BSS flush can be used to invert the graft in the AC. Sometimes during unfolding, high posterior pressure could increase the possibility of flush out of the graft or its aberrant fixation in the corneal wounds making the surgery more complex. In these cases, we suggest placing a tight suture on the main incision and increasing the pressure in the AC with BSS via side ports. When the tissue is completely scrolled and centred, air can be inserted under it to attach the graft to the recipient stroma.

We prefer air to SF-6 tamponade. After air is injected in the anterior chamber, if needed, the centration of the graft can be corrected with forceps (GRIESHABER® Asymmetrical Forceps, Alcon, Fort Worth, TX, US) pulling the graft into the desired position. To increase graft mobility, we suggest performing this procedure with no more than 50 % of air in AC. With this movement we could lose some endothelial cells but it will improve the centration of the graft. At the end of the surgery, the vertical incision can be used to fill the AC with air. If available, Intra operative Optical Coherence Tomography (OCT) can improve all surgical steps increasing visualization of the graft. If the graft detaches in the early post-op hours, then it needs to be re-attached using a re-bubbling technique.

Although, the re-bubbling of the tissue depends on the detachment of the graft, it has not yet been standardized to reduce the re-bubble rates completely.

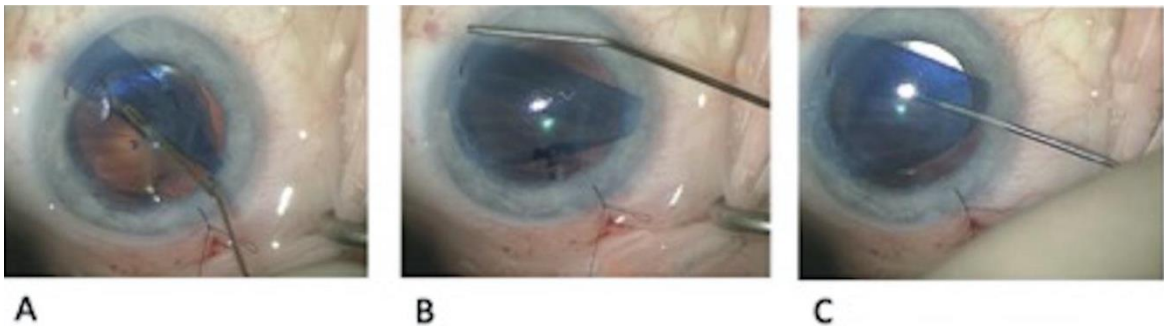

Figure 7.6 **DMEK graft unfolding (A–C)**

Simple tips can help surgeons new to DMEK to improve their outcomes and facilitate the uptake of DMEK surgery. DMEK is a safe procedure to replace a damaged or dysfunctional corneal endothelium. A well prepared DMEK graft and different surgical techniques has helped to improve the desired surgical outcome.

Conclusions

DMEK has become a gold standard for the treatment of endothelial failure. Its minimally invasive nature, fast optimal visual recovery and low occurrence of post-operative immunologic rejection have made it a choice for many surgeons. The current thesis adds new features to the development of DMEK procedure.

In our studies we have seen how the new tools of metagenomics sequencing can improve the graft preservation, giving a better starting point for DMEK procedures. The costs, presence of live organisms, turnover time, downstream processing and data analysis are improving every year. Given the current trends in genomic technology development, more standardized results will be obtained in the near future. NGS could therefore be of significant value for checking the microbiological load in industrial production to ensure the safety of healthcare products.

To improve outcomes in DMEK surgery, preparing a larger diameter graft would allow the transplantation of more cells especially the so-called putative stem cells that are assumed to be at the periphery of the cornea.

In this thesis we demonstrated that by changing the speed of the graft stripping we can influence the tightness of the graft scroll. The slower the speed is, the wider the graft scroll width will be in the final conformation. This finding may be due to the longer duration of stress that the tissue sustained during the peeling process and its effect on subsequent elastic recoil of the graft.

Simple tips can help all surgeons to improve their outcomes and facilitate the uptake of DMEK surgery. The tips and tricks described could be beneficial for new and experienced corneal surgeons.

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Summary of publications

1. Gallon P, Parekh M, Ferrari S, Fasolo A, Ponzin D, **Borroni D**. Metagenomics in ophthalmology: Hypothesis or real prospective? *Biotechnol Rep (Amst).* 2019 Jun 26; 23: e00355. doi: 10.1016/j.btre.2019.e00355. eCollection 2019 Sep. Review. PubMed PMID: 31312608; PubMed Central PMCID: PMC6609782.

2. Rocha-de-Lossada C, Prieto-Godoy M, Sánchez-González JM, Romano V, **Borroni D**, Rachwani-Anil R, Alba-Linero C, Peraza-Nieves J, Kaye SB, Rodríguez-Calvo-de-Mora M. Tomographic and aberrometric assessment of first-time diagnosed paediatric keratoconus based on age ranges: a multicentre study. *Acta Ophthalmol*. 2020 Dec 30. doi: 10.1111/aos.14715. PMID: 33377591.

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30. Rocha-de-Lossada C, Rachwani-Anil R, **Borroni D**, Sánchez-González JM, Esteves-Marques R, Soler-Ferrández FL, Gegúndez-Fernández JA, Romano V, Livny E, Rodríguez Calvo-de-Mora M. New Horizons in the Treatment of Corneal Endothelial Dysfunction. *J Ophthalmol*. 2021 Jul 9; 2021: 6644114. doi: 10.1155/2021/6644114.

Grants, scholarships and awards

1. Latvian state scholarship – VIAA (eng: SEDA) scholarship for Academic year 2017 / 2018 – 7.370 Euro.
2. Erasmus+ scholarship 2017 for PhD students – 4.800 Euro.
3. Venice Camera di Commercio Research Grant Award 2017 10.000 Euro.
4. Best PhD student – Rīga Stradiņš University (RSU) 2018.
5. European Society of Cataract and Refractive Surgeons (ESCRS) Clinical Research Awards 2018 – META-Cor project – Principal Investigator 199.000 Euro.
6. ESCRS Trainee Bursary Belgrade 2018 – Best Paper – 750 Euro.
7. Ophthalmology Star Award prize – ESCRS 2019 Paris for the paper "A comparative study on different Descemet membrane endothelial keratoplasty graft preparation techniques".
8. 2021 – "Paladini della Salute" Award – Italian Ministry of Education.

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My greatest gratitude to my family for continuous support during my adventure.

Supplement

Ethical approvals and informed consent

Veidlapa Nr. E-9 (2)

RSU ĒTIKAS KOMITEJAS LĒMUMS NR. 29 / 29.09.2016.

Rīga, Dzirciema iela 16, LV-1007
Tel. 67061596

	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Profesore Vija Sīle	Dr.phil.	filozofs
3.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
4.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5.	Profesore Regīna Kleina	Dr.med.	patalogs
6.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
7.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
8.	Docente Iveta Jankovska	Dr.med.	
9.	Docents Kristaps Circenis	Dr.med.	

Pieteikuma iesniedzējs: **Dr. Davide Borroni ar pērnieku grupu**
Medicīnas fakultāte, doktorantūra

Pētījuma nosaukums: RECOVER-ME REfractive, Corneal, Ocular surface and VitrEo-Retinal infections treatment – MEtagenomic guided

Iesniegšanas datums: 29.09.2016.

Pētījuma protokols: Izskatot augstāk minētā starptautiskas sadarbības pētījuma pieteikuma materiālus (protokolu) ir redzams, ka pētījuma mērķis tiek sasniegts veicot ar acs infekcijas pacientiem, bez kāda apdraudējuma viņu drošībai, veselībai un dzīvībai, klīniski-analītisku darbu (audu paraugu ņemšanu un atbilstošas analīzes), iegūto datu apstrādi un analīzi, kā arī izsakot priekšlikumus. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst medicīnas pētījuma ētikas prasībām.

Izskaidrošanas formulārs: tiek nodrošināts

Piekrišana piedalīties pētījumā: tiek nodrošināta

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., prof.

Paraksts

RIGAS STRADIŅA UNIVERSITĀTE ĒTIKAS KOMITEJA

Ētikas komitejas sēdes datums: 29.09.2016.

Veidlapa Nr. E-9 (2)

29

RSU ĒTIKAS KOMITEJAS LĒMUMS NR. 49 / 26.11.2015.

Rīga, Dzirciema iela 16, LV-1007
Tel. 67061596

	Komitejas sastāvs	Kvalifikācija	Nodarbošanās
1.	Profesors Olafs Brūvers	Dr.theo.	teologs
2.	Profesore Vija Sīle	Dr.phil.	filozofs
3.	Asoc.prof. Santa Purviņa	Dr.med.	farmakologs
4.	Asoc.prof. Voldemārs Arnis	Dr.biol.	rehabilitologs
5.	Profesore Regīna Kleina	Dr.med.	patalogs
6.	Profesors Guntars Pupelis	Dr.med.	ķirurgs
7.	Asoc.prof. Viesturs Liguts	Dr.med.	toksikologs
8.	Docente Iveta Jankovska	Dr.med.	
9.	Docents Kristaps Circenis	Dr.med.	

Pieteikuma iesniedzējs: **Davide Borroni, 1. studiju gada doktorants**
Doktorantūras nodaļa

Pētījuma nosaukums: „ Translācijas izpēte medicīnā: ģenētika, oftalmoloģijā."

Iesniegšanas datums: 30.10.2015.

Pētījuma protokols: Izskatot augstāk minētā pētījuma pieteikuma (protokolu) ir redzams, ka pētījums tiek veikts Bērnu Klīniskās Universitātes slimnīcā un sadarbībā ar Insubria University Varese, Itālijā, Mērķis tiek sasniegts veicot pacientu medicīniskās dokumentācijas (medicīnas vēstures) izpēti, kā arī iegūstot Latvijā, Bērnu Klīniskās Universitātes slimnīcā, no pacientiem (bez kāda apdraudējuma veselībai un dzīvībai) DNS paraugus. To sekvenēšanu un izvērtēšanu veic pētījuma pieteicējs kopā ar kolēģiem Insubria University Varese Itālijā. Pēc iegūto datu apstrādes un analīzes, iegūtie dati nonāk atpakaļ Latvijā un pētnieks izsaka savus priekšlikumus un rekomendācijas. Atļauja klīniskā pētījuma veikšanai Latvijā no BUK slimnīcas saņemta. Personu (pacientu, dalībnieku) datu aizsardzība, brīvprātīga informēta piekrišana arī no bērnu vecākiem piedalīties pētījumā un konfidencialitāte tiek nodrošināta. Līdz ar to pieteikums atbilst pētījuma ētikas prasībām

Izskaidrošanas formulārs: ir

Piekrišana piedalīties pētījumā: ir

Komitejas lēmums: **piekrist** pētījumam

Komitejas priekšsēdētājs Olafs Brūvers Tituls: Dr. miss., prof.

Paraksts

Ētikas komitejas sēdes datums: 26.11.2015.

REGIONE DEL VENETO
Coordinamento Regionale Trapianti

Coordinamento Ospedaliero Trapianti

Azienda _____

MANIFESTAZIONE DI VOLONTÀ AL PRELIEVO DI
BULBI OCULARI O CORNEE AI FINI DI TRAPIANTO TERAPEUTICO
(Ai sensi della L. 1 aprile 1999 n. 91 "Disposizioni in materia di prelievi e trapianti di organi e tessuti)

Il sottoscritto/a _____, ai sensi dell'art. 23 della L. 01 aprile 1999 n. 91, ha informato alle ore _____ del giorno _____ il Sig./Sig.ra _____ abitante in via _____ n. ____ CAP _____ città _____ Tel. _____ che il Sig./Sig.ra _____ nato/a a _____il _____ e deceduto/a in data _____ alle ore _____ nel reparto di _____ dell'Ospedale di _____ Medico di Famiglia _____ per il/la quale è stata accertata la morte ai sensi della L. 29.12.93 n. 578 e del D.M 11.04.08 che aggiorna il D.M 22.08.94. n. 582,

è stato riconosciuto come potenziale donatore di ❑ bulbi oculari ❑ cornee a scopo di trapianto.

Previa consultazione del Sistema Informativo Trapianti, ha altresì informato che:

☐ **NON RISULTANDO** alcuna volontà espressa in vita dal loro congiunto, essi possono presentare **opposizione scritta** al prelievo;

☐ **RISULTANDO volontà** ☐ **favorevole o** ☐ **non favorevole** al prelievo di organi e di tessuti espressa in vita secondo le modalità di legge, essi possono presentare una **dichiarazione di volontà successiva**, sottoscritta dal potenziale donatore, opposta a quella precedentemente espressa.

Ha inoltre informato che:

- in caso di non opposizione al prelievo di bulbi oculari o cornee, la Fondazione Banca degli Occhi del Veneto eseguirà accertamenti chimico-clinici, sierologici ed anamnestici volti a valutare l'idoneità del tessuto prelevato ed in particolare a prevenire la trasmissione di patologie da donatore a ricevente;
- i tessuti oculari saranno conservati per un periodo massimo di 5 anni e saranno utilizzati in modo anonimo, salvaguardando la riservatezza e la tracciabilità di campioni e informazioni;
- potrà negare il suo consenso all'utilizzo dei tessuti oculari a fini scientifici anche successivamente e avrà diritto di accesso alle informazioni generate da tali attività;
- i tessuti oculari non sono oggetto di lucro e saranno smaltiti nell'osservanza delle norme vigenti.

_____

Descrizione situazioni particolari

_____

_____

_____

data_____ IL PROFESSIONISTA SANITARIO _____

L'avente diritto, Sig./Sig.ra _____

1 Coniuge non separato **2 Convivente "more uxorio"**

3 Figlio/i di età non inferiore agli anni diciotto **4 Genitore/i**

5 Rappresentante legale _____

☐ **SI OPPONE** ☐ **NON SI OPPONE** al prelievo di bulbi oculari/cornee.

☐ **ACCONSENTE** ☐ **NON ACCONSENTE** che i tessuti oculari siano utilizzati per studi scientifici e volti a migliorare la conoscenza e le possibilità di cura.

data_____ L'AVENTE DIRITTO _____

Stante l'impossibilità a ottenere senza ritardo l'opposizione dell'avente diritto in forma scritta, il sottoscritto _____ consapevole delle sanzioni penali, nel caso di dichiarazioni non veritiere e falsità negli atti, richiamate dall'art. 76 D.P.R. 445/2000, dichiara che il/la Sig./Sig.ra _____ ha manifestato telefonicamente

☐ **OPPOSIZIONE** ☐ **NON OPPOSIZIONE** al prelievo di bulbi oculari/cornee.

data_____ IL PROFESSIONISTA SANITARIO _____

Informativa ai sensi degli artt. 13 e 14 del Regolamento (UE) 2016/679 del Parlamento Europeo e del Consiglio del 27 aprile 2016 (General Data Protection Regolation – GDPR)

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