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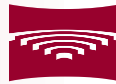

RĪGA STRADIŅŠ UNIVERSITY

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

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

Sector – Clinical Medicine
Sub-Sector – Ophthalmology

Riga, 2021

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The Doctoral Thesis was developed at *Fondazione Banca degli Occhi del Veneto*, Venice, Italy, Rīga Stradiņš University, Riga, Latvia, and at the Department of corneal and external eye diseases, St. Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, United Kingdom

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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
OTU	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.

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.
7. Parekh, M., Romano, V., Franch, A., Leon, P., Birattari, F., **Borroni, D.**, Kaye, S. B., Ponzin, D., Ahmad, S., Ferrari, S. Shotgun sequencing to determine corneal infection. *Am J Ophthalmol Case Rep*. 2020 May 8; 19: 100737. doi: 10.1016/j.ajoc.2020.100737.
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 favorable 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.

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.

1 Study 1

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

Abstract

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.

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 estimated number 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, amplicon based sequencing analysis and data processing

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. The amplification strategy combined amplicon generation with library preparation for Illumina sequencing. 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.

1.3 Results

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. 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.

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 CO2 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 pyrolidone 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 number 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 centers 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

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

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 theater. 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 10×10 reticule fixed inside the eyepiece of an inverted microscope (Zeiss, Milan, Italy) at 100× magnification. An average of 3 readings was recorded to avoid false-positive measurements centrally and paracentrally.

2.3 Results

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 % 6 2.7 % TBPCs after the preparation phase (n = 10). Endothelial cells before stripping (Fig. 2.2 (A)) did not show any TBPC, minimal cell loss was found after stripping (Fig. 2.2 (B)) and after 7 days of preservation (Fig. 2.2 (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. 2.2 (D), (E)) compared with the central endothelium (Fig. 2.2 (F)) where there is no marking.

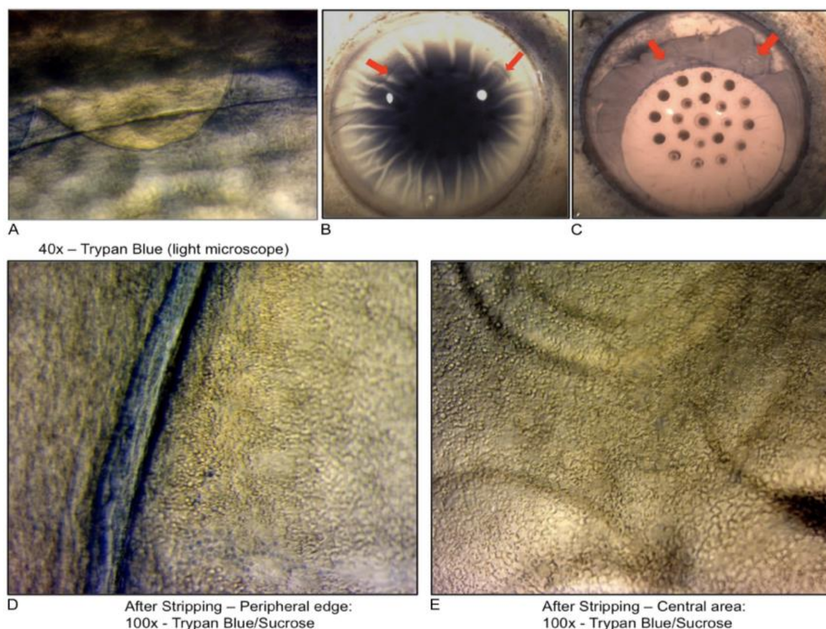

Figure 2.1 **Challenging cases**

(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.

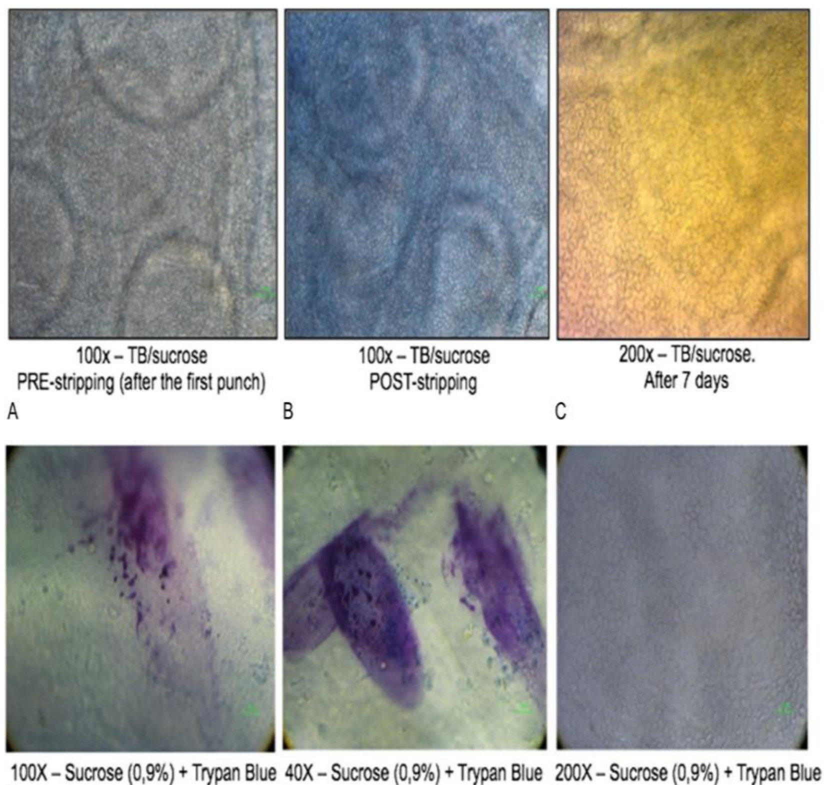

Figure 2.2 **Endothelial cells during the validation study**

(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 center 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 millimeter 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

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.

4 Study 4

Study 4 shows a novel technique for DMEK tissue preparation.

Abstract

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 ≥ 0.5 mm. The mean preparation time was 6.21 ± 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.

Keywords: Descemet membrane endothelial keratoplasty; DMEK graft preparation; preparation technique.

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.
- 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.
- The above-described DMEK punch has a circular guarded blade missing 1 clock hour, creating an uncut hinge on the donor cornea.
- 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 (eg, Sinskey hook) is used to identify the end of DM at the level of Schwalbe line in the uncut hinge area.
- DM with overlying endothelium is peeled off from the underlying corneal stroma using a curved spatula or a crescent blade (Fig. 4.1 (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. 4.1 (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. 4.1 (C)).
- The DMEK graft is grasped with forceps (tying, jewelers, or other DMEK forceps) from the triangle marking and further stripped in a single-peel technique (Fig. 4.1 (D)).

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

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).

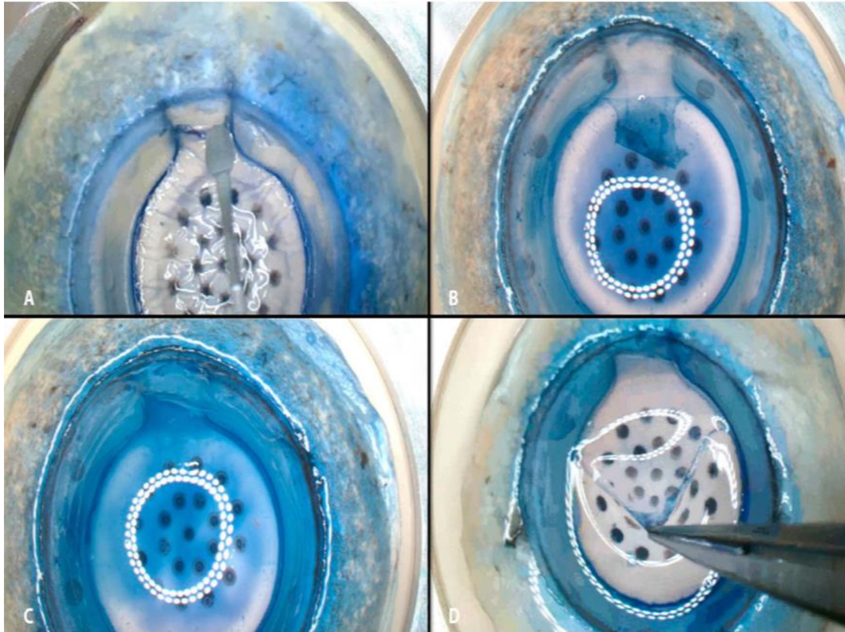

Figure 4.1 **DMEK graft preparation technique**

(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 (×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

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.

Keywords: Cornea; peeling; DMEK; speed.

6 Study 6

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

Abstract

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.

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

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.

Keywords: DMEK; Learning curve.

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, J. M., Romano, V., **Borroni, D.**, Rachwani-Anil, R., Alba-Linero, C., Peraza-Nieves, J., Kaye, S. B., 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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29. **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.

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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 – Riga Stradins 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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Supplement

Supplement 1

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

RĪGAS STRADIŅA UNIVERSITĀTE ĒTIKAS KOMITEJA

Ētikas komitejas sēdes datums: 29.09.2016.

29

Veidlapa Nr. E-9 (2)

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

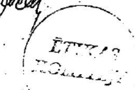
Izskaidrošanas formulārs: ir

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)

I dati personali contenuti nel presente modulo sono trattati nel rispetto e nei limiti stabiliti dal Regolamento (UE) 2016/679 del Parlamento Europeo e del Consiglio del 27 aprile 2016 (General Data Protection Regolation – GDPR), secondo l'informativa conservata presso la sede legale dell'Azienda n. ______________________ e pubblicata nel sito internet, accessibile al seguente link: _____________________

Data________________ Firma ____