New Technologies in Clinical Trials in Corneal Diseases and Limbal Stem Cell Deficiency: Review from the European Vision Institute Special Interest Focus Group Meeting

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Keywords

European vision institute · Diagnostic · New technologies · Cornea · Limbal stem cell deficiency · Dry eye disease · End points · Graft versus host disease · Fuchs endothelial dystrophy · Anterior segment OCT · Confocal laser scanning microscopy

Abstract

To discuss and evaluate new technologies for a better diagnosis of corneal diseases and limbal stem cell deficiency, the outcomes of a consensus process within the European Vision Institute (and of a workshop at the University of Cologne) are outlined. Various technologies are presented and analyzed for their potential clinical use also in defining new end points in clinical trials. The disease areas which are discussed com-

prise dry eye and ocular surface inflammation, imaging, and corneal neovascularization and corneal grafting/stem cell and cell transplantation. The unmet needs in the abovementioned disease areas are discussed, and realistically achievable new technologies for better diagnosis and use in clinical trials are outlined. To sum up, it can be said that there are several new technologies that can improve current diagnostics in the field of ophthalmology in the near future and will have impact on clinical trial end point design.

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Introduction

The European Vision Institute is a renowned “think tank” for ophthalmic research in Europe [1, 2]. Based on the ever-growing variety of new technologies applied in ophthalmology, a panel of international experts and European Vision Institute members gathered at the “European Vision Institute – Special Interest Focus Group – New Technologies for Outcome Measures in Cornea and Limbal Stem Cell Deficiency” (May 16–17, 2019 Department of Ophthalmology at the University of Cologne) to discuss new developments that may be relevant for clinical studies and clinical routine in anterior eye disorders.

This article provides an overview of these developments and their scientific and clinical application in the following ophthalmic subspecialties: dry eye/ocular surface inflammation, imaging and corneal neovascularization, and corneal grafting/stem cell and cell transplantation. The potential and limitations of these new technologies in both clinical studies and clinical routine are discussed. The conclusions drawn may help implement these new technologies in clinical studies and clinical routine and to optimize study outcome measures and clinical processes in the future.

Dry Eye/Ocular Surface Inflammation

Dry eye disease (DED) is an umbrella term to describe the pathological conditions of the ocular surface, which results in tear film instability, ocular discomfort, and potential damage to the ocular surface [3]. DED can arise due to a number of underlying health conditions, including Sjögren’s syndrome, meibomian gland dysfunction and graft versus host disease (GVHD), as well as through environmental stress, idiosyncratic, and idiopathic means [3]. DED is often sub-defined into aqueous-deficient and lipid-deficient or evaporative subtypes. Also lack of mu-

cins can worsen DED [4]. DED has a vast negative impact on quality of life and affects more than 16 million American adults. The prevalence increases with age and is higher among women than men [5]. The ratio of female to male has been shown to be between 1.8 and 2.6 [6].

Several factors were detected that might explain sex-associated differences, for example, physiological ocular differences in the meibomian glands [7], lacrimal gland [8], conjunctiva [9], and cornea [10] that may contribute to the different DED prevalence rates observed in women and men [11]. These can be sex-specific corneal changes during pregnancy, menopause, or menstrual cycle. This changes lead to variations in corneal thickness, sensitivity, or hydration [10].

Corneal Nerve Imaging and Quantification in Pain, Dry Eye, and Inflammation and Their Pathophysiologic Relevance

The revised dry eye workshop (DEWS) II 2017 definition of DED defines DED as a disease with ocular inflammation, neurosensory abnormalities, and tear film deficiency or instability. Current end points are measured by Schirmer’s wetting test, tear break-up time (TBUT), and tear film osmolarity or bulbar redness. However, the reproducibility of these tests is not lineal, and there are several limitations like no accurate diagnostics, no gold standard questionnaires, and various grading systems regarding the corneal fluorescein staining. Moreover, many of these current tests were not validated. Many DED studies, especially immunological studies were performed in mice and the direct transferability to humans has yet to be demonstrated. For example, it has been shown that mice have several unique murine apoptosis and inflammation genes, which do not have human orthologs [12]. Thus, to date, there are no validated biomarkers available to assess ocular surface diseases, such as DED. Patient heterogeneity and poor correlation of clinical measurements to patients’ symptoms complicates assessment. Therefore, the in vivo confocal microscope (IVCM) might be a technology that could allow the provision of future biomarkers in the field of DED [13]. Central and peripheral corneal immune cells can be measured by laser IVCM, which has up to 1 μm axial resolution [14]. Dendritiform cells (DCs) can be detected, quantified, and measured morphologically and quantitatively [15, 16]. Furthermore, the sub-basal nerves can be detected, and changes in morphology (such as presence of micro-neuromas, see Fig. 1) and density were observed and quantitatively assessed [13, 17]. This is particularly of interest in patients with mainly neuropathic corneal pain, as in this condition, and cor-

Fig. 1. IVCM image showing a micro neuroma (arrow) in a patient with neuropathic corneal pain. IVCM, in vivo confocal microscope.

neal staining results and symptom severity may diverge. In a previous study, strong correlation was demonstrated between pro-inflammatory tear film cytokine alterations and DC density [18]. Further, another study assessing DC alterations in subtypes of DED showed that patient with mixed-type DED (both aqueous deficient and evaporative DED) have increased DC density, as compared to evaporative DED or healthy control patients [19]. Moreover, a more recent study of 300 eyes of 150 patients showed that dry eye severity level and DC density are correlated. The greater the severity of DED, the higher the DC density and the larger the DCs (size is a marker for maturation) [20]. Interestingly, patients with DEWS stage 2–4 show no further increase in DC density but demonstrate increased DC area and DC field. Additional studies assessing the peripheral cornea demonstrated that the central cornea mimics the peripheral corneal quadrants in DED, as these findings were seen centrally and peripherally and were not specific for the corneal center [21].

In a phase 4 randomized double-masked controlled trial, topical artificial tears, loteprednol etabonate 0.5%, or loteprednol etabonate 0.5%/tobramycin 0.3% were applied twice daily for 4 weeks and the effect on the sub-basal nerve fiber length compared. In vivo confocal mi-

croscopy of central cornea was performed bilaterally and patients with DED were divided into 2 groups: one with low subbasal nerve fiber length and one with a near-normal nerve fiber length. After therapy, there was a significant improvement in symptoms and corneal fluorescein staining only in DED patients with near-normal subbasal nerve fiber length, but not in patients with decreased corneal subbasal density [22]. Furthermore, DCs assessed in these patients showed significant decrease within 4 weeks by application of loteprednol etabonate 0.5% or loteprednol etabonate 0.5%/tobramycin 0.3% twice daily, but not in the artificial tear group, demonstrating that the efficacy of anti-inflammatory therapy can be assessed by IVCM [23, 24]. Another study demonstrated that Ocular Surface Disease Index score and DC density as measured by IVCM significantly decreased after treatment with loteprednol 4 times daily for 4 weeks ($p < 0.01$) [25]. A more recent randomized double-masked randomized controlled trial compared age- and sex-matched groups of patients with DED, where 1 group was treated with loteprednol etabonate 0.5% tapered dose and the other with artificial tears. This study assessed patients with increased DC density at baseline as an inclusion criterion. The group with loteprednol etabonate showed significant decrease in DC density and size after 2 and 6 weeks of therapy, compared to the artificial tear group. Moreover corneal fluorescein staining was found to be significantly lower in the loteprednol etabonate group compared to the artificial tear group [26, 27].

Another group of patients should be differentiated from the evaporative and aqueous-deficient DED patients: the neuropathic corneal pain patients with corneal hypersensitivity [28]. This pain is different from inflammatory pain and is caused by a lesion or disease of the somatosensory system. These patients typically do not respond to conventional DED therapies. There are several causes of this disease; a common cause is ocular surgery. Patients often have systemic comorbidities, like depression or anxiety, and studies have shown severely impacted quality of life.

For these patients, clinical testing, questionnaires for symptoms, but also IVCM, are useful diagnostic tools. The corneal nerves show micro-neuromas on IVCM with nerves thought to present with ectopic discharge. In contrast, micro-neuromas are not found in typical DED patients. Therefore, micro-neuromas seem to be specific for neuropathic corneal pain patients [13, 17, 29, 30]. The proposed treatment options are autologous serum eye drops, topical corticosteroids, and amnion membrane placement with good therapeutic effects for patients with

peripheral pain and oral neuromodulators for patients with non-ocular source of pain.

Confocal microscopy analysis is very time-consuming, which might be difficult to implement into clinical practice. Therefore, a future solution may be the use of artificial intelligence for automated image analysis. Artificial intelligence can already detect different corneal layers and can automate DC analysis and identification of micro-neuromas with good sensitivity and specificity (e.g., www.itksnap.org) [31, 32].

With good sensitivity and specificity, confocal microscopy is a good tool for imaging, especially in patients with DED or neuropathic corneal pain, among others. From the experience of Prof. Hamrah and his team, DED patients tolerate the examination well, as they tolerate bright light less, which is not needed here.

Next to IVCMA anterior segment optical coherence tomography (AS-OCT) can be useful in DED patients (for more details on AS-OCT, see below). Here, several important anatomical features can be visualized with AS-OCT. Anatomical landmarks that have been imaged include lid-parallel conjunctival folds [33, 34], meibomian glands [35–37], the lacrimal canaliculus, and lacrimal punctum [38–40] as well as lacrimal glands [41].

Whereas tear meniscus can be measured with commercial OCT systems [42–45], measurement of the pre-corneal tear film requires ultrahigh-resolution prototypes [46–51] because it is only 3–5 μm thick (see Fig. 2). Using *enface* maps of the tear film, it may also be possible to study the evaporation rates of tear film [49] and quantify the thickness of the lipid layer [52]. For both tear meniscus [53–56] and pre-corneal tear film thickness [57], alterations have been reported in patients with ocular surface disease. In addition, treatment with topical eye drops temporarily increases both measures of tear volume [58–63]. Large-scale multicenter and long-term follow-up studies on the effect of dry eye treatment on tear meniscus and pre-corneal tear film thickness are, however, lacking.

Although AS-OCT is not yet regularly used in the clinical assessment of patients with ocular surface disease, the technique may hold significant potential for the future, particularly in the characterization of different phenotypes [64]. A major barrier in this respect is that ultrahigh-resolution systems have not yet been commercialized.

Molecular Profiling of DED

Heterogeneity of DED patient populations makes it difficult to study ocular surface pathology in DED at the molecular level. Strict definition of patient subpopula-

tions enables more meaningful study of underlying pathogenic mechanisms. An exemplar patient subgroup with a moderate-to-severe dry eye phenotype is that with ocular GVHD (oGVHD) after allogeneic hematopoietic cell transplantation.

Allogeneic hematopoietic cell transplantation is the standard of care therapy for various benign and malignant hematological disorders. With ongoing improvement in long-term survival of transplanted patients, there is an increase in the number of patients living with GVHD [65]. GVHD is a common complication of allogeneic hematopoietic cell transplantation occurring in 25–70% of all allogeneic hematopoietic cell transplantation patients, in which donor immune cells attack and destroy host tissues [66]. Chronic oGVHD results from such impairment of the lacrimal functional unit and is associated with an often severe dry eye phenotype. GVHD-associated DED occurs in approximately 50% of allogeneic hematopoietic cell transplantation patients and has a debilitating effect on patient vision and quality of life [67]. Despite this, diagnosis of oGVHD is hampered by variability in clinical presentation and a lack of clinically validated biomarkers. Objective biomarkers to monitor disease state are needed, particularly with regard to future clinical trials, where objective end points are necessary to evaluate compound efficacy. Furthermore, as there are no specifically targeted therapies for oGVHD, current therapeutic regimen depends largely on the experience of the treating physician. Therefore, the ongoing search for new drug targets for oGVHD remains relevant and provides opportunities to move towards a more standardized therapy.

The accessibility of the ocular surface lends itself well to minimally invasive clinical sampling. Hence, many groups have focused their research effort on assessing transcriptional and proteomic changes in conjunctival cells [68–70] and tear film [71–75] in ocular surface disorders. Discovery-based approaches enable the study of possible disease mechanisms as well as the identification of potential disease biomarkers. Numerous studies have focused on the comparison of molecular profiles across DED of varying etiologies [72, 73, 75]. Variation in tear collection approaches and downstream analysis methods (mass spectrometry, protein arrays, and luminex multiplex assays) makes direct comparison of tear proteome studies difficult. However, the 2017 TFOS DEWS II report extensively outlines the current state of the search for tear film biomarkers for DED [76]. This report identified 5 candidates as potential biomarkers for DED, with different expression profiles (up- or downregulated) in dif-

Fig. 2. Ultrahigh-resolution OCT image of the pre-corneal tear film as obtained in a patient with DED; before (a) and 10 min after administration of a lubricant gel (polyethylene glycol 0.4%, propylene glycol 0.3%, and hydroxypropyl guar) (b). The tear film increases from a value of 4.1 mm to a value 11.7 mm. DED, dry eye disease. OCT, optical coherence tomography.

ferent forms of DED. These candidates are, namely, transferrin, lipocalin-1, lysozyme, lactoferrin, and albumin. Multiple research groups have shown that multiplexing of biomarkers into panels has greater diagnostic capacity [73, 77, 78]. The TFOS DEWS II report identifies 3 panels with sensitivity and specificity of >90% [76]. Of these, a 5 peptide panel demonstrated the greatest diagnostic power. This panel included annexin A1, annexin A11, cystatin-S, phospholipase A2-activating protein, and S100A6. Challenges remain to translate these findings into clinically applicable diagnostic tests.

With regard to oGVHD, the search for relevant and clinically reliable end points is hampered by an incomplete understanding of the underlying pathogenic mechanisms. However, it is generally accepted that inflammation is a key driver of ocular surface damage [79–81]. To date, biomarker studies for oGVHD have focused on the analysis of inflammatory markers. For example, using Luminex technology, Cocho and colleagues presented a predictive model based on the concentration of IL-8 and CXCL10 in tears, which had the capability to distinguish oGVHD from Sjögren syndrome's patients [82]. Further, looking at pre- and posttransplant tear cytokine levels in allogeneic hematopoietic cell transplantation patients, they generated a model based on pretransplant levels of CX3CL1, IL-1Ra, and IL-6 that could predict development of oGVHD with 80% sensitivity [83].

A recent cross-sectional observational study compared the proteome of tears collected onto Schirmer strips from patients with and without oGVHD after AHCT [84]. Comparison of proteomes revealed significant differential expression of 79 proteins, with 25 downregulated and 54 upregulated proteins. Comparison of the proteomic profile of oGVHD tears with previously published proteomic profiles of various subtypes of DED re-

vealed broad overlap with aqueous-deficient dry eye. The majority (80%) of downregulated proteins were extracellular or secreted proteins. The downregulated proteins included pro-secretory (lacrutin), defense and immunity proteins (lactotransferrin, lysozyme, lipocalin-1, mam-maglobin-B, secretoglobulin family 1D member 1), and ocular protective factors (proline-rich protein 1).

Upregulated proteins were largely intracellular proteins, with approximately half (48%) being designated as nuclear proteins according to Gene Ontology terms. The profile of upregulated proteins in oGVHD was consistent with pro-inflammatory stress at the ocular surface. The most abundant upregulated proteins were histone proteins, indicating the presence of extracellular DNA and thus widespread cellular death. Gerber-Hollbach et al. [84] hypothesized a key role for histones as a driver of inflammatory pathology in oGVHD, which is in agreement with the previous work of the Jain lab who showed a central role for extracellular DNA in DED pathology [85, 86]. A recent pilot phase I/II trial by Jain and colleagues indicated a promising role for DNase I eye drops in reducing severity of symptoms in aqueous deficient DED patients [87].

The exact mechanisms leading to lacrimal functional unit damage in oGVHD are unclear. The patients analyzed in the report by Gerber-Hollbach et al. [84] had a moderate-to-severe oGVHD phenotype. Therefore, ocular surface pathology was extensive and from this data, it is not possible to infer what proteomic changes occur early in the disease process. Longitudinal studies of allogeneic hematopoietic cell transplantation patients before and after transplant will allow a more careful dissection of the pathways involved in oGVHD. In this way, it may also be possible to identify new druggable targets to lessen the burden of oGVHD.

In summary, much progress has been made in recent years to characterize the molecular profile of the healthy and dry eye human tear film. However, without standardization of analysis and reporting methods, tear film proteomics and transcriptomics will not reach their full potential as diagnostic and prognostic tools for dry eye patients.

Challenges of Drug Development

Challenges of Drug Development in DED

There is an unmet need for novel dry eye therapies since current treatments suffer from low efficacy and a long onset time and require frequent topical instillations. The latter results in low compliance, particularly among the elderly population. As a result, there are over 25 small molecules and biologics currently in clinical development. The fact that only low numbers of novel pharmacotherapies are approved for this disease implies certain challenges in drug development.

Animal Models

The most frequently used model for DED is the mouse desiccating stress model. The lacrimal functional unit is disturbed by exposure to a desiccating stress via continuous air flow and tear production is suppressed via scopolamine. In this model, the readouts are similar to the human clinical signs such as the disturbed integrity of the ocular surface by corneal fluorescein staining and reduced tear production. Inflammation can be assessed in tear fluid, by histology and mRNA expression. Interestingly, this animal model has revealed a high level of translatability when it comes to the pathogenesis of human DED and the efficacy of approved therapeutics for dry eye such as cyclosporine A and integrin inhibitors [88]. The model, however, has a number of technical challenges since the baseline of these readouts can vary due to the age of the animals, the season of the year, and the animal supplier. Consistent implementation of the model between labs is challenging since it depends on several technical details including the incubation chamber, types of air flow, and cages used. These factors warrant standardization in order to ensure comparable results among the scientific community.

Drug Delivery and Residence Time

Current standard of care therapies for DED are instilled via the topical ocular route, resulting in a low (<5% bioavailability), and thus requiring frequent daily administrations. This, however, may result in low patient compliance contributing to low efficacy. The residence time is also very critical during night when the eyes are closed.

During sleep, tear secretion will be reduced due to the lack of sensory stimuli, resulting in decreased levels of tear-derived enzymes and increased levels of non-lacrimal proteins in a hypoxic environment. These conditions are proposed to greatly contribute to epithelial permeability, corneal edema, and recurrent erosions [89]. Drug development teams face the challenges associated with identifying a formulation suitable for the drug modality and physicochemical properties of the molecule and access to broad and cost-effective formulation technologies. Drugs requiring daily topical administration may be improved through the use of ointments, micro- or nanoparticles or micelles, viscosity enhancement, and technologies for mucoadhesion. Long-term administration requires the combination of drugs with devices such as ocular inserts (e.g., rods, lenses, and films), punctal plugs or ocular rings. Finally, long-term efficacy could be achieved with less frequent applications of new modalities such as antisense oligonucleotides or gene therapy of limbal stem cells.

Clinical End Points

DED patients are very heterogeneous and difficult to diagnose. Evaporative dry eye accounts for up to 70% of DED patients and is primarily caused by meibomian gland dysfunction resulting in excessive evaporation of tear film despite normal tear secretion. On the other hand, the remaining 30% of DED patients are characterized as aqueous tear-deficient as a result of reduced tear volume due to loss of lacrimal gland function. Loss of lacrimal gland function can be a consequence of exocrine glands autoimmunity, as in Sjögren's patients, or may not involve autoimmunity, as in non-Sjögren's patients. Current DED diagnosis involves the subjective measure of symptoms using various questionnaires (OSDI, NEI VFQ-25, and VAS) that assess the severity and frequency of symptoms such as burning, stinging, grittiness, foreign body sensation, tearing, ocular fatigue, and dryness. Clinical signs are objective measures of which tear osmolarity is the most reliable measure for disease severity. TBUT, corneal staining, and Schirmer tear test scores are more informative for the severe disease forms [90, 91]. Unfortunately, there is often a low correlation between symptoms and clinical signs likely due to sensory changes that occur on the ocular surface with increased disease severity and age – also known as pain without stain [90, 92, 93]. However, for drug approval, regulatory agencies require signs and symptoms chosen as clinical end points to be significantly and reproducibly improved. In summary, the unknown etiology, a heterogeneous patient population, the low association between signs and symptoms,

and the high variability of clinical tests will have negative implications for significant clinical trial outcomes as long as there are no new standardized outcome measures.

Imaging and Ne(ur)ovascularization

AS-OCT in Corneal and Ocular Surface Disease

It has been 25 years since the first in vivo tomograms of the AS using OCT have been published [94]. Although the development of AS-OCT was not as rapid as that of posterior segment OCT, different time domain, and Fourier domain systems have been commercialized. A full overview of the historical development of AS-OCT is provided in a recent review article [95]. Currently, several commercial systems are available, including spectral domain and swept source OCTs. In addition, several customized systems were realized with an axial resolution of as high as approximately 1 μm by using light sources with very high bandwidth [48, 96–101].

Applications of AS-OCT in corneal disease are wide and clinically well established. This includes evaluation of pterygium [102], management guidance in ocular surface neoplasia [103], characterization of abnormalities associated with keratoconus [104–108], and evaluation of corneal wound healing [109–113]. The role of AS-OCT is also important in preoperative, intraoperative, and post-operative assessment of patients requiring corneal surgeries [111, 114–118]. Recently, application of intraoperative AS-OCT has been proven to be helpful in deep anterior lamellar keratoplasty [119–121], femtosecond cataract surgery [122], as well as during Descemet stripping automated endothelial keratoplasty (DSAEK) [123] and Descemet membrane endothelial keratoplasty (DMEK) [124, 125]. Using deep learning-based technology for segmentation of corneal layers may open further applications for AS-OCT of the cornea [126].

A recent extension to OCT is OCT angiography (OCTA), which has been developed to visualize and study the vasculature of the retina, choroid, and optic nerve head [127–131]. OCTA can also be used for the AS and aims at visualizing the vasculature of the conjunctiva, the sclera, and the iris, as well as pathological corneal blood vessels in the case of corneal neovascularization [132]. As compared to invasive angiography using fluorescent dyes, OCTA has many advantages that make the technique more suitable for routine application. OCTA is fast and omits the dye-related side effects associated with the use of fluorescein and indocyanine. Leakage, which may obscure deeper vessels, does not occur with OCTA be-

cause extravascular plasma does not generate an OCTA decorrelation signal. In contrast to classical dye-based angiography, OCTA provides three-dimensional volumetric information of the vasculature that enables visualization of the vasculature at different depths and provides accurate localization of vessels within the tissue.

OCTA has been used to visualize corneal neovascularization [133–137], a pathological condition associated with the risk of severe vision loss [138]. As opposed to dye-based angiography, corneal neovascularization can also be visualized in case of scarring [133]. Corneal neovascularization was documented with OCTA as a consequence of penetrating keratoplasty, herpetic and bacterial keratitis, limbal stem cell deficiency (LSCD), and pterygium [139].

A major limitation of AS-OCTA is the frequent occurrence of motion artifacts. Tracking is commonly used to reduce motion artifacts for retinal imaging; however, this cannot be used for the AS because of the lack of landmarks. Currently, no commercial system has been specifically designed for AS-OCTA, but several existing systems can be adapted for this purpose [140]. Given the potential clinical application of such a system, including surgical planning for corneal transplantation or treatment monitoring after anti-VEGF administration, it may be expected that commercial AS-OCTA systems will become available in the near future.

Software Tools for Objective Analysis of AS Image Data (Corneal Nerves, Blood Vessels, and Lymphatics)

Imaging has become an integral part of clinical and experimental medicine. In ophthalmology, especially optical methods such as OCT or confocal laser scanning microscopy have been established, since they offer microscopic imaging of structures in the anterior and posterior segment of the eye in a noninvasive manner [141]. Sophisticated OCT, confocal laser scanning microscopy, or other approaches operating at very high imaging speeds and resolution capabilities furthermore allow functional investigation of tissue by visualizing, for example, blood and lymphatic vessels [142] or corneal nerves [143]. Image processing and quantification of image data is a crucial step in the research workflow, for which different approaches can be used. Some are introduced in the following with a special focus on methodical objectivity.

In image analysis, quantitative parameters like the number, absolute or relative area, length or shape of structures like blood or lymphatic vessels, nerves, glands, or cells are derived from raw image data. These parameters help describe the presence and constitution of the

Fig. 3. Segmentation of pathologic blood vessels in a murine cornea, 3D image data acquired by OCT. **a** Intensity image of a murine AS. **b** Highlighted blood vessels within the volume, gained by OCT angiography processing. **c** 3D-segmented image of the blood vessels. AS, anterior segment; OCT, optical coherence tomography.

structure of interest. To extract quantitative information from intensity images, segmentation is required, which means labeling of structures of interest of the image. For instance, in an image displaying pathologic corneal vessels in a murine eye, those pixels of the image that represent vessels get the label “1,” or white color, while the background that represents anything else in the image gets the label “0,” or black color. The result is a binary image, showing only the structure of interest, which can be further processed. The segmentation process is shown in Figure 3 for pathologic blood vessels in a murine cornea – suture model [144]. From the segmented image (Fig. 3c), quantitative information can be derived. In the simplest application, the number of white pixels can be referenced to the number of black background pixels, which equals the relative vascularization in the image volume. Depending on the use, a more complex analysis may require vectorization of single vessels in the volume, for example, to analyze vessel branching or tortuosity.

Segmentation is, thus, the crucial step before actual analysis. Depending on the quality and complexity of the image data, different approaches can be used. In artifact-free images with excellent contrast (intensity-based) thresholding may work, which means fading out everything in the image below a certain intensity threshold. However, the exact threshold level is in practice often not clear, requiring a tradeoff between a too low value (all structure represented, but still background artifacts) and a too high value (no background artifacts, but darker parts of the structure faded out). Taking only 1 image parameter (in this case image intensity) into account is a limiting factor, resulting in subjective data. A more intelligent approach is called region growing. Here, the user sets several so-called seed points at representative parts of the structure by selecting, for example, a blood vessel. In

the subsequent region growing calculation, the neighborhood of the marked pixels is investigated dynamically. Similar parts of the image are connected to 1 structure or network. This depends on more than 1 parameter, for example, an intensity range and spatial interconnectivity. Consequently, the region growing is much more robust in detecting the structure of interest. A third approach which is widely used in practice is manual segmentation, which means tracing the structures manually by the use of a mouse and a software pen in the zoomed-in image. This kind of segmentation may be highly accurate in the result, but since it is totally based on user assessment of what belongs to the structure and what does not, it is subjective. Furthermore, this method is very time-consuming and thus not suitable for larger sets of images or 3D data.

To conclude, the amount of required user interaction is critical for the quality and objectivity of the results. On 1 hand, no critical user interaction in the case of histogram thresholding may cause unreliable results. On the other hand, a very high amount of user interaction such as in manual segmentation makes the results subjective. Here, we promote region growing for the following main advantages: (1) low amount of user interaction limits subjectivity and effort, (2) time-effectiveness makes the approach suitable for larger image sets and especially 3D data, and (3) best objectivity and reliability can be reached by outsourcing critical decisions in the segmentation process to the computer, which takes into account 2 or more relevant image parameters. All introduced methods can be implemented in open source and/or free software platforms like java-based ImageJ (National Institutes of Health, Bethesda, MD, USA, <https://imagej.nih.gov>) or MeVisLab (Fraunhofer MEVIS, Bremen, Germany, <https://www.mevislab.de/>).

Fig. 4. Clinical pictures (top) and corresponding morphometric analyses (bottom) of a patient suffering from herpetic viral keratitis. The keratitis is associated with corneal neovascularization. The patient was treated with aganirsen antisense oligonucleotide eye drops to inhibit corneal neovascularization. Compare the clinical picture and morphometric analysis before treatment (**a**), after 90 days (**b**), and 180 days of aganirsen treatment (**c**). Picture from [163].

Cornea Grafting/Corneal Vessel Morphometry/Stem Cell and Cell Transplantation

Morphometry of Corneal Hem- and Lymphangiogenesis as Surrogate Marker for Graft Rejection in High-Risk Keratoplasty

Angiogenesis and lymphangiogenesis are being increasingly recognized as important factors for pathological processes such as tumor growth [145] and corneal graft rejection [146, 147]. Different methods to measure the vascularized areas in corneas used so far are a *semi quantitative* method in which the cornea is divided into several quadrants and each sector is assessed with scores dependent on the number of vessels and the density of the vessel network [148, 149] and a *manual quantitative* method which involves outlining of vascularized areas. Digital pictures of the cornea along with the limbus are taken and analyzed outlining the total corneal area using the innermost vessel of the limbal arcade as the border. Here, the neovascularized area is quantified by manually circumscribing the blood vessel network or each single lymphatic vessel with the cursor [147, 150, 151]. The reading Center CORIC (Cologne Ophthalmological Reading and Image Analysis Center, <https://augenklinik.uk-koeln.de/forschung/arbeitsgruppen-labore/coric/>) was one of the first describing a novel semiautomatic, quantitative method based on threshold analyses. Digital gray value threshold measurements are used to quantify the areas of neovascularization [152–155]. In contrast to the quantitative manual outlining method, this new gray value-based analysis method has 2 benefits: the semiautomatic method measures only the areas, which are in fact covered by the single vessels. The second benefit is the faster and even more precise measurement of single vessels like lym-

phatic vessels. The semiautomatic method allows the detection of all sprouts in the region of interest at once, which is timesaving and even more accurate. This method is already used in several clinical studies (compare Fig. 4) [156–159] and was adapted to other clinical questions like allergic provocation of the conjunctiva [160], meibomian gland loss in ocular GVHD [161], or epithelial defects [162].

Lymphatic vessels are essential for maintaining the homeostasis of tissue fluids, transport of antigen, and migration of immune cells under physiological and pathological conditions. However, following organ or tissue transplantation, lymphangiogenesis triggers the rejection of transplanted organs or tissues and thereby limits transplant survival [164, 165]. Furthermore, the formation of lymphatic vessels during tumor growth increases the risk of tumor metastasis to adjacent lymph nodes and beyond [166]. The precise molecular and cellular interactions governing these important cell-vessel interactions are only poorly understood until now. Corneal lymphatic vessels are clinically invisible because of their thin walls and clear lymph fluid. There is no easy and established method for in vivo imaging of corneal lymphatic vessels so far. Steven et al. [167] developed a novel method using noninvasive two-photon microscopy to simultaneously visualize and track specifically stained lymphatic vessels and autofluorescent adjacent tissues such as collagen fibrils, blood vessels, and immune cells in the mouse model of corneal neovascularization in vivo.

Since this method requires a multiphoton confocal microscope, this approach is not easy to be translated into clinical practice. Therefore, currently new methods to visualize lymphatic vessels in the cornea are investigated. One very promising approach is the intrastromal injection of fluorescein and subsequent imaging with scanning

Fig. 5. Representative pictures of mOCT show a longitudinal cross-section of a physiological corneal lymphatic vessel (**a**) and a corneal blood vessel (**b**). While the lymphatic vessel is dark and mainly cell-free, the blood vessels are showing a high amount of cells, and the vessel wall and endothelium is hyperreflective. Picture from [142]. mOCT, microscopic optical coherence tomography.

laser angiography (HRA + OCT). Le et al. [168] could show experimentally that by this method corneal lymphatic vessels drain the injected fluorescein and can thereby be visualized in vivo. Here, both the device as well as the dye are clinically validated. Thus, this method can be very easily translated into clinical practice and be helpful to assess whether the cornea of a patient contains lymphatic vessels or not.

More elegant would be a completely noninvasive method with a clinically available device. The OCT technology is already heavily used in ophthalmology. The next generation of this technology is the so-called *microscopic* optical coherence tomography [169]. With this high-resolution OCT, we were able to visualize blood- and lymphatic vessels in the vascularized cornea of mice without any kind of dye or invasive treatment [142] (see Fig. 5). Lymphatic vessels were discriminated from blood vessels as dark vessel-like structures with the lumen lacking a hyperreflective wall and occasionally occurring, individual, slowly moving particles, which were presumably immune cells.

In conclusion, the detection and quantification of blood and lymphatic vessels in the cornea is an essential parameter for clinical studies to observe the success of antiangiogenic and immunomodulatory therapies in different corneal diseases and corneal transplantation. Vessel regression has already been shown to improve graft survival in patients with corneal neovascularization and, therefore, offers new therapeutic options [170]. The so far clinically invisible lymphatic vessels are already very close to being clinically detectable. Future studies should answer which vessel parameters (e.g., area vs. length vs. end points) are the most clinically important, as data on this

question is missing. Furthermore, it is still difficult to clinically differentiate between mature and immature vessels, which might also be of relevance in terms of responsiveness to treatment approaches.

Patient-Reported Visual Disability in Fuchs' Endothelial Corneal Dystrophy Measured by the Visual Function and Corneal Health Status Instrument

Comprehensive evaluations of ocular diseases are incomplete without each individual patient's perspective, in addition to anatomical, functional, and optical parameters. Patient-reported outcome measures (PROMs) offer such a perspective. PROMs, generally in questionnaire format, help assessing and interpreting the patient's vision-related disability. To achieve these goals, PROMs use validated and standardized questions characteristic to the specific disease. PROMs may assist in routine care to tailor an individual treatment plan that meets the patient's needs, in research, and in the evaluation of health care processes and systems [171, 172]. PROMs can be useful for screening, diagnostic staging, or monitoring diseases. PROMs can be used to quantify patient-relevant outcomes when assessing physicians' performance or justify reimbursement, such as after surgical intervention. For example, Catquest-9SF, a cataract-specific PROM instrument [173], measures vision-related outcomes after cataract surgery [174].

Clinicians and investigators interested in using a PROM for corneal and LSCD must identify a suitable instrument [175]. Khadka and colleagues [175] provide an overview of existing ophthalmic PROMs and a guide for quality assessment of non-listed instruments. For

Fig. 6. Development of a patient-reported outcome measure. As an example, the V-FUCHS instrument is shown. First, the instrument's items and layout were developed. Second, V-FUCHS (version 0) was pretested. Third, the modified V-FUCHS was tested for reliability and validity. The final V-FUCHS instrument allows for standardized and rapid assessment of Fuchs' dystrophy-specific visual disability [156]. V-FUCHS, Visual Function and Corneal Health Status.

some clinical settings, suitable instruments of sufficient quality do not exist yet [175, 176]. To fill such gaps, the development of a new PROM instrument needs to identify relevant content areas in the population of interest. After framing questions in patients' words using an 8th grade language level, the preliminary instrument must be pretested and thereafter validated. For example, for patients with endothelial dysfunction, Wacker and colleagues rigorously developed and validated the Visual Function and Corneal Health Status instrument (V-FUCHS) to quantify Fuchs' dystrophy-specific disability (Fig. 6) [177]. V-FUCHS assesses visual disability because of diurnal variation of vision or glare before and after endothelial keratoplasty. V-FUCHS is available in English and German (<https://www.uniklinik-freiburg.de/augenklinik/fol/expo/agwacker.html>) [177, 178] and may help assessing disease stage and prognosis in addition to improved optical and morphological outcome measures [179–183].

Only high-quality PROMs can fulfill the promise of converging the clinician's and patient's view of clinical reality. It is essential to confirm that the instrument is measuring just 1 underlying construct, called latent trait (validity) and that the instrument measures this construct consistently (reliability) [175, 176]. Inherent to most measures of psychophysical health is its dependency on the patient's ability to estimate abstract concepts such as glare and to transform these concepts to numerical scales; interpretations need to account for the individual's cultural background [177, 184]. Modern analysis techniques of item response theory allow assessment of item difficulty and the respondent's ability level on a linear logit scale.

Taken together, PROMs are promising tools that can improve patient care and patient-provider relationship. Intriguingly, most PROMs are easy to administer. Once an adequate instrument has been identified or developed, it can be used by patients in preparation of a clinic

visit. In this setting, PROMs may help synthesizing patient-relevant data quickly in a standardized manner and may allow for investing more time into shared decision-making.

Limbal Stem Cell Growth on Recombinant Collagen Membranes

The corneal epithelium is directly being replenished by the limbal (epithelial) stem cells (LESCs). The limbal epithelial stem cells are housed at their own 3D stem cell niche, more specifically the limbal crypts, limbal epithelial crypts, and focal stromal projections. These types of stem cell niche are located in close proximity to one another at the superior and inferior limbal region [185]. The exact mechanism of maintaining LESCs in their progenitor genotype at the limbal niche remains to be elucidated; however, there is growing evidence that specific interactions with other types of niche cells as well as the limbus-specific composition of extracellular matrix. In recent years, there has been growing evidence that LESCs form a vast but limited number of interactions with melanocytes, mesenchymal stem cells (MSCs) and immune cells (leucocytes) within the stem cell niche and that loss of contact with the limbal basement membrane results in the proliferation and differentiation, that is, loss of “stemness.” Under physiological condition, the LESC is a slow-cycling cell that, when stimulated, will proliferate and differentiate centripetally according to the XYZ axis (upward [i.e., more superficial], centrally [toward center of the cornea], and toward the nasal and temporal regions of the limbus as well). Damage to the LESCs or the limbal niche and/or loss of the LESC pool will result in LSCD, an orphan disease with an estimated prevalence of up to 9/100,000. This clinical condition is characterized by chronic and recurrent corneal epithelial erosions, ocular surface inflammation, conjunctivalization of the cornea, and corneal neovascularization; all of which result in a painful red eye with decreased visual potential. LSCD can be caused by a wide variety of diseases, ranging from thermal and chemical burn, genetic disease (e.g., aniridia), infection (e.g., trachoma), immune-diseases (e.g., Steven-Johnson syndrome), iatrogenic (previous cornea/limbal surgery), contact lens wear, and others [186].

Historical treatment of choice for LSCD was keratolimbal transplantation (allogeneic cadaveric) or conjunctivolimbal transplantation (either allogeneic [i.e., cadaveric – living related] or autologous). As large pieces of limbal tissue were transplanted, these surgical treatments often resulted in (i) life-long immune suppression in the acceptor, (ii) tissue rejection, and (iii) iatrogenic LSCD in

the healthy donor eye. Ever since its introduction in 1997, cultivated limbal stem cell transplantation (CLET) has become the standard treatment of choice for LSCD [187] with a reported success rate of 67% [186]. In CLET, a small limbal biopsy (1–2 cm²) is harvested and expanded ex vivo on a carrier material. The carrier material most frequently used in CLET is the human amniotic membrane (HAM). Other carrier membranes that have made it into human application for CLET are fibrin gels and siloxane contact lenses. In February 2015, Holoclar (Chiesi) was EEMA approved as the first stem cell-based medicine to receive authorization for commercial use throughout the European Union. Holoclar is a commercially available CLET therapy in which a fibrin membrane is being used as the carrier membrane. However, the application for Holoclar is limited to patients suffering from unilateral LSCD as a result of chemical or thermal burn. Therefore, a vast majority of patients cannot be treated with Holoclar.

CLET techniques making use of HAM as the carrier membrane have only been applied in clinical trials and have yet to be commercialized. Nonetheless, more patients have received HAM-assisted CLET than fibrin-based techniques. Furthermore, HAM-assisted CLET has been proven to be effective in a wider range of pathologies causing LSCD, as well as in cases of allogeneic CLET. Moreover, HAM has been used for over 2 decades in ocular surgery, resulting in significant expertise of the material in ocular surgery. Also, HAM has favorable biochemical characteristics (presence of anti-inflammatory mediators and various growth factors) and elicits only minimal immunological reaction. However, HAM has unfavorable optical characteristics limiting visual outcome after surgery, and there is significant inter- and intra-donor variation, and there is a theoretical risk of disease transmission. Therefore, there remains a need for optimized carrier membranes that combine the advantages of fibrin and HAM, while tackling its shortcomings. A very promising approach is the application of collagen hydrogels as a carrier for LESCs in CLET as collagen is characterized by inherent biocompatibility and cost-effectiveness [188]. After all, collagen is the most abundant protein of the human body, and more than 90% of the human cornea consists of collagen type I. Collagen hydrogels have already been applied successfully in corneal tissue engineering [189–191]. In 2009, the group of Fagerholm et al. [189–191] were the first to successfully implant acellular Recombinant Human Collagen type III (RHC III) hydrogels, as a corneal substitute in humans. In subsequent reports, RHC III-based corneas were im-

planted in 20 patients, with collagen being sourced from yeast in each of these cases. After surgery, implants supported full epithelial regeneration, though, slow re-epithelialization rates could be noted, with full epithelial regeneration taking up to 1 year. These results indicate the potential of collagen-based corneal regeneration. Moreover, it should be stressed that collagen hydrogels are highly tunable, creating opportunities previously unseen in CLET [188].

In recent years, simple limbal epithelial transplantation (SLET) has risen to the attention as well. During SLET surgery, a small strip of donor limbal tissue (e.g., 2 × 2 mm) is divided into several smaller pieces, which are then distributed evenly over a HAM placed on the cornea. SLET targets the same niche of LSCD patients as with Holoclar, that is, autologous stem cell transplantation for unilateral cases of chemical and thermal burn; however, long-term follow-up has yet to be performed for SLET-treated patients. Furthermore, SLET does not challenge major hurdles observed with CLET, including HAM-assisted surgery and lack of tackling deeper corneal opacities in a single surgery. Also, SLET technique is more frequently complicated by biopsy detachment.

End Points in Using ATP-Binding Cassette, Sub-Family B, Member 5-Positive LESC for Clinical Trials

An intact corneal epithelium is necessary for transparency and refraction. As mentioned in the previous chapter, this epithelial layer is constantly maintained by a quiescent LESC population located in the basal layer of the limbus, the vascularized junction between the corneal and conjunctival epithelium. The limbal epithelial cells reside in the basal layer of the limbus, but upon injury or due to normal wear and tear of the corneal epithelium, the cells enter the transient amplifying state, while they migrate to the site where they are needed.

Recently ATP-binding cassette, sub-family B, member 5 (ABCB5) [192, 193] emerged as a highly specific LESC marker [194]. Ksander et al. [194] demonstrated that ABCB5 marks LSCs and is required for LESC homeostasis, corneal development, and regeneration. Furthermore, the authors showed that prospectively isolated human or murine ABCB5-positive LSCs possess the exclusive capacity to fully restore the cornea upon grafting to LSC-deficient mice in xenogeneic or syngeneic transplantation models [194]. Overall, the authors demonstrated that ABCB5 loss of function in ABCB5 knockout mice causes depletion of quiescent LSCs due to enhanced proliferation and apoptosis and results in defective corneal differentiation and wound healing [194]. Based on

these data, ABCB5 can be used as a tool to identify the role of LESC populations in neovascularization and inflammation, and the ABCB5KO mouse is a reliable LESC deficiency model.

A dysfunction or depletion of LSCs in combination with the destruction of their SC niche may lead to a LSCD that significantly alters tissue homeostasis due to a persistent tissue inflammation which changes the integrity of the corneal surface. Thus, corneal neovascularization with recurrent epithelial defects, corneal scarring, corneal conjunctivalization, and formation of a fibrovascular pannus occurs. Corneal transparency and visual function is no longer maintained, resulting in patient discomfort and partial or total blindness.

The contrast between the avascular cornea and the densely vascularized conjunctiva highlight the role of the limbus as a “barrier” for vascularization and immunity. Notara et al. [195, 196] have reported that loss of LSC phenotype due to UVA and UVB irradiation coincides with the pro-inflammatory shift in the limbal niche cells which may set up the conditions for (lymph)angiogenesis and compromise the limbal barrier. These studies indicate that LSC and their niche are key for the maintenance of the corneal immune and angiogenic privilege [195–197].

Conditions leading to LSCD include chemical and thermal burns, inflammatory eye disease (e.g., Stevens-Johnson syndrome or ocular cicatricial pemphigoid) and contact lens-related hypoxia [198]. Some LSCD cases, such as aniridia, are congenital and range from 1 in 40,000–1 in 100,000 people [199]. Strict upgrading in safety regulations in the Western world have limited the LSCD cases, for example, prevalence in the UK is calculated to be 240 per year [200]; however, in developing countries, including some African and Middle Eastern ones as well as India, the prevalence is estimated to be approximately 1.5 million [201].

End Points in Limbal Stem Cell Transplantation for Clinical Trials

Therapeutic options for LSCD depend on the etiology, severity, extent, and laterality of the disease. While treatment of mild and moderate cases aims at control of symptoms and causes, in more severe cases, where no or insufficient amounts of LSCs are present, the LESC pool needs to be restored. Earlier procedures involved transplantation of limbal tissue, either from the patient's healthy or less affected contralateral eye [202], or in bilateral LSCD, from a living or deceased donor [203, 204]. Newer, tissue-sparing techniques such as CLET [187] or SLET [205, 206] have significantly reduced the amount of

donor tissue required and, thus, decreased the risk of harming the donor eye. However, as transplantation success strongly depends on the percentage of LESCDS within the transplanted cells [207], a major challenge in the further development of transplantation techniques remains the identification of the LSCs, which would allow enrichment of the stem cell content of the transplant.

Recently, thanks to the identification of ABCB5 as LESC marker [194] and the development of an antibody directed against an extracellular loop of the plasma membrane-spanning ABCB5 molecule [192], the first potential molecular surface marker for prospective LESC enrichment by antibody-based cell sorting has become available. Therefore, a novel treatment strategy based on the utilization of allogeneic ABCB5⁺ LSCs derived from cadaveric ocular tissue expanded in vitro and manufactured as an advanced-therapy medicinal product (ATMP) was developed.

Currently, this LESC-based ATMP is being tested in a first-in-human multicenter phase I/IIa clinical trial (ClinicalTrials.gov NCT03549299) to evaluate the safety and efficacy of ascending doses of allogeneic ABCB5⁺ LSCs for the treatment of LESCDS. Cells are topically applied on the entire corneal and limbal area following surgical dissection of conjunctival pannus tissue from the corneal surface. For pre- and postoperative local antiangiogenic therapy, patients receive subconjunctival injections of the anti-VEGF antibody bevacizumab until engrafted ABCB5⁺ LSCs have restored limbal barrier function protecting the reconstituted cornea from conjunctival blood vessel ingrowth. Concomitant immunosuppressive medication includes topical and systemic corticosteroids and long-term ciclosporin.

Primary efficacy end point of the clinical trial is the response rate at 12 months after LSC transplantation, with response defined as no or mild corneal neovascularization and no or mild epithelial defects. Secondary efficacy measures include the response rate at 3 months after LSC transplantation and neovascularization, epithelial defects, ocular symptoms (pain, photophobia, and burning), ocular inflammation, corneal opacity, visual acuity, and quality of life (as per visual function questionnaire 25) throughout 12 months after LSC transplantation. Primary safety measures are adverse events throughout 24 months after LSC transplantation. Secondary safety measures include physical examinations, vital signs, and tonometry.

Taken together, an advanced LESCDS treatment strategy based on transplantation of ex vivo-expanded ABCB5⁺ LSCs, which are derived from cadaveric corneal tissue and manufactured as a GMP-conforming ATMP, is being

developed. Based upon the cornea-restoring capacity of human ABCB5⁺ LSCs observed in animal models of LESCDS [194, 208], this ATMP holds promise for replenishment of the patient's LSC pool, recreation of a functional barrier against invading conjunctival cells, and restoration of a transparent, avascular cornea.

Clinical End Points in Endothelial Keratoplasty

In the current era of evidence-based medicine (EBM), the formulation and selection of clinical end points is very important since the outcomes of studies will be used to define whether a new treatment policy should lead to relevant (added) value for the patient in comparison to the standard or usual treatment. David Sackett MD (1934–2015) was the father of EBM and according to the British Medical Journal “arguably the most important movement in medicine in the past 25 years” [209, 210].

In The Netherlands, the application of the principles of EBM is a mandatory request by the National Health Care Institute (Zorginstituut Nederland; ZINL) for assessing whether care fulfills the “established medical science and medical practice.” The concept “established medical science and medical practice” is normative. This means that “practice” is not “health care that individual professionals (usually) provide” nor is “practice” the opinion of individual care-providers (and individual patients) about the value of the intervention. In a recent editorial, the relationship between evidence-based and EBM was addressed and concluded that over the years, a shift was seen from “trust in experts” to “trust in numbers” [211].

The assessment may eventually include what the professional group as a whole feels is regarded as a representative and/or correct treatment. ZINL advises the Dutch Minister of Public Health, Welfare and Sport on the content of the basic insurance package. To approve the admission of a surgical innovation for medical practice, ZINL uses fixed EBM steps consisting of searching for and selecting information (e.g., PICOT analysis) and then assessing and grading the quality of the evidence. ZINL first consults the scientific associations of professionals and patients' organizations to explore which clinical end points and outcome measures should be chosen. The insights and experience obtained by professionals and patients in practice – depending on the quality of the evidence found and subject to certain conditions – play a decisive role in determining the final conclusion. The ideal EBM approach integrates individual clinical expertise with the best available external clinical evidence from systematic research. This principle of integration of clinical

expertise with scientific evidence is very alive: registries (big data), artificial intelligence, and image analysis will help us answer clinical questions more efficiently in the near future [211].

As a practical example of the approval process of endothelial keratoplasty procedures in The Netherlands, DSAEK was approved by ZINL in 2008, but DMEK was rejected in 2008, 2010, and 2014 because DMEK did not yet fulfill the abovementioned established medical science and practice criterion [212]. This clearly addresses the need for designing appropriate studies with well-defined clinical end points.

Over the years we have seen multiple meta-analyses, systematic reviews and randomized clinical trials (RCTs) on various endothelial keratoplasty techniques. Initially, the discussion in the literature focused on the relationship between graft thickness and visual outcome. In 2013, the results of a prospective non-comparative case series were published which showed that ultrathin (UT) DSAEK grafts, intended to be thinner than 130 μm , result in better visual acuity compared with the literature [213]. However, studies supporting and rejecting a relationship between graft thickness and visual acuity are limited by retrospective design, heterogeneity in graft thickness measurement techniques, and non-standardized visual acuity measurements [214]. In 2016, we published the UT-DSAEK study, a multicenter prospective double-masked RCT that was designed to compare visual and refractive outcomes, endothelial cell (EC) loss, and incidence of complications after DSAEK (donor thickness 209 μm ; range 147–289 μm) and UT-DSAEK (donor thickness 101 μm ; range 50–145 μm) [215]. Best spectacle corrected visual acuity (BSCVA) was significantly better after UT-DSAEK compared with DSAEK at 3 months (0.17 vs. 0.28 LogMAR; $p = 0.001$), 6 months (0.14 vs. 0.24 LogMAR; $p = 0.002$), and 12 months (0.13 vs. 0.20 LogMAR; $p = 0.03$). However, refraction, EC loss (40% at 3 months, $p < 0.001$), donor loss, and graft dislocation did not differ between UT-DSAEK and DSAEK. We recognize that the clinical impact of the difference in visual acuity of 0.07 LogMAR (nearly one Snellen line) between DSAEK and UT-DSAEK at 12 months in our study may be limited. Also, there is a significant increase in BSCVA in the DSAEK group over 12 months, thereby illustrating the influence of defining the end point for measuring visual acuity on outcomes after an endothelial keratoplasty procedure. It was concluded that UT-DSAEK results in faster and better recovery of BSCVA with similar refractive outcomes, EC loss and incidence of com-

plications. In terms of improvements of quality of vision, UT-DSAEK also results in faster recovery of contrast sensitivity and lower posterior corneal higher order aberrations compared to DSAEK [216].

Nowadays, corneal surgeons are increasingly adopting DMEK for the treatment of corneal endothelial dysfunction [217]. The primary advantage of DMEK over previous techniques has been suggested to be superior visual recovery [218]. In 4 meta-analyses, DMEK showed superior BSCVA compared to DSAEK [219–222], but RCTs comparing DMEK to UT-DSAEK are scarce. The first RCT, the DETECT study, reported superior BSCVA following DMEK compared to UT-DSAEK [223]. However, in that RCT, 70% of corneal transplantations were triple procedures, which hinder attributing visual recovery to corneal transplantation only. Both eyes of twelve subjects were enrolled in the study, leading to a dependency between eyes, and the visual recovery in the UT-DSAEK arm was reduced in the first 6 postoperative months compared to previous studies assessing UT-DSAEK and DSAEK [213, 215]. Recently, we performed an RCT in The Netherlands to compare BSCVA, endothelial cell density, refraction, and complications of DMEK versus UT-DSAEK in pseudophakic eyes with Fuchs' endothelial corneal dystrophy in a multicenter setting [224]. BSCVA did not differ significantly between DMEK and UT-DSAEK at 3 months (0.15 vs. 0.22 logMAR; $p = 0.15$), 6 months (0.11 vs. 0.16 logMAR; $p = 0.20$), and 12 months (0.08 vs. 0.15 logMAR; $p = 0.06$). However, 12 months after surgery, the percentage of eyes reaching $\geq 20/25$ Snellen BSCVA was higher in DMEK compared to UT-DSAEK (66 vs. 33%, $p = 0.02$). Endothelial cell density did not differ significantly 12 months after DMEK and UT-DSAEK (1,870 vs. 1,612 cells/ mm^2 ; $p = 0.12$). However, the rebubbling rate in DMEK was 24% as compared to 4% after UT-DSAEK. The DETECT study reported similar rebubbling rates in DMEK and UT-DSAEK of 24 and 4%, respectively.

In comparison with the DETECT study variations in design with the current RCT may explain the differences in mean visual acuity. Visual recovery was reduced in the UT-DSAEK arm of the DETECT study compared to previous studies and to the UT-DSAEK arm of our study during the first 6 months. In our RCT, eligibility criteria were limited to patients with Fuchs' endothelial corneal dystrophy, and no surgeries were combined with cataract extraction and intraocular lens implantation (triple procedures). To avoid dependency between 2 eyes, only the first eye was included in patients fulfilling the eligibility criteria in both eyes. These differences underscore the importance of de-

fining inclusion criteria and selecting clinical end points when designing and comparing outcomes of RCTs that will be used by governmental institutions for approving new surgical innovations for medical practice. A standardized and objective approach for defining the disease state being treated and outcomes reported is needed to make the results of future trials applicable to clinical practice. Tomographic pachymetry of the central and peripheral cornea [225], posterior float maps [226], and corneal backscatter [227] provide simple and relevant classification for clinical practice and research purposes. In endothelial keratoplasty, graft detachment is one of the main complications. Intraoperative OCT can aid visualization in opaque corneas and evaluate graft orientation during surgery [228]. In the postoperative period, graft dislocation can also be objectively quantified using artificial intelligence [229]. We feel that the formulation of standardized data sets for reporting outcomes, as has been done in the past in the field of refractive surgery, would be highly recommendable for endothelial transplantation techniques.

Conclusion and Future Perspectives

The aim of this consensus meeting was to discuss and evaluate novel technologies for the better diagnosis of ocular surface diseases. Several new technologies have been presented and evaluated for their potential clinical use to improve current diagnostics and to also define new end points in clinical trials.

Currently, clinical end points in dry eye/ocular surface inflammation consist of various questionnaires, Schirmer's test, TBUT, and tear film osmolarity or bulbar redness. However, patient heterogeneity, poor correlation of clinical signs and patients' symptoms, and the lack of validated biomarkers limits the use of these end points in clinical assessments. Corneal nerve imaging by IVCN is a well-tolerated and promising technology especially in patients with DED or neuropathic corneal pain. In the past decade, much progress has also been made in the molecular profiling of patients with ocular surface disease. However, these molecular methods might not reach their full potential as diagnostic and prognostic tools due to the lack of standardization. Thus, the heterogeneity of patients and the low association between signs and symptoms as well as the high variability of clinical tests might have negative implications for clinical trial outcomes in dry eye/ocular surface inflammation. This warrants novel, validated, and standardized outcome measures.

In terms of imaging, OCT-based imaging has been well established and is meanwhile used in a plethora of diseases at the ocular surface. With the recent development of OCTA and its application at the AS, imaging can potentially become even more important and might provide valuable clinical end points in the near future. Image processing and data quantification is crucial, and determining the right amount of user interaction will be critical for the quality and objectivity of the results.

Great progress has also been made in measuring corneal blood and lymphatic vessels as surrogate marker for graft rejection in high-risk keratoplasty. The detection and quantification of corneal blood and lymphatic vessels is an important parameter to assess the success of antiangiogenic and immunomodulatory therapies. However, in the clinical setting, it is still unclear which vessel parameters are the most important, and it is still challenging to differentiate between mature and immature vessels, which might be relevant in terms of responsiveness to novel treatment strategies.

In recent years, tremendous progress has also been made in the field of LESC transplantation. A very promising approach is the application of collagen hydrogels as LESC carriers for CLET rather than HAM, due to their transparency and lack of cytotoxic effects. Furthermore, ABCB5 has been identified as a highly specific LESC marker that is required for LESC homeostasis, corneal development and regeneration. Thus, ABCB5 might be used as a tool to identify the role of LESC populations in corneal neovascularization and ocular surface inflammation. In addition, ABCB5 is the first molecular surface marker for prospective LESC enrichment by antibody-based cell sorting. Ex vivo-expanded ABCB5⁺ LSCs hold promise for replenishment of the patient's LSC pool to re-establish cornea transparency in LESC patients, and this approach is currently evaluated in an international, multicenter clinical trial.

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Statement of Ethics

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/

or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from the participants involved in the here summarized studies.

Conflict of Interest Statement

The authors have no conflicts of interest to declare. O.O., S.F., and C.U. are employees at F. Hoffmann-La Roche Ltd., Basel, Switzerland. H.S. is an employee at TICEBA GmbH, Heidelberg, Germany; RHEACELL GmbH & Co. KG is the clinical trial sponsor and a subsidiary of TICEBA GmbH.

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Author Contributions

All authors contributed to the conception of the work by writing sections of the manuscript and drafting and revising it critically as well as final approval of the published version.

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