Robert Wisse

Keratoconus

Inflammatory associations and treatment characteristics

KERATOCONUS
INFLAMMATORY ASSOCIATIONS AND TREATMENT CHARACTERISTICS

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Over de rol van inflammatie en behandeling in keratoconus
(met een samenvatting in het Nederlands)

Proefschrift

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Aan Julia en Roosmarijn

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INTRODUCTION TO THIS THESIS

The field of keratoconus research and treatment underwent major changes is the past decades, owing to increased diagnostic possibilities, improved lamellar grafting techniques, advances in contact lens care, and the advent of corneal crosslinking. The body of literature on keratoconus is therefore rapidly expanding.

This thesis is the result of 5 years of keratoconus research and reflects the consequences of these developments to our department. A shift in patient selection towards crosslinking rather than corneal grafting prompted the exploration of keratoconus treatment beyond surgery. The collaboration between our department and the laboratory of translational immunology enabled the investigation of etiologic factors in the development of keratoconus.

KERATOCONUS; AN OVERVIEW

A brief history of keratoconus

The first scientific report of keratoconus is attributed to the German pioneer in ophthalmology prof. Burchard David Mauchart, who wrote a doctoral dissertation on this subject in 1748.[1] A full century later it was John Nottingham, a British surgeon at the Liverpool St. Anne Eye and Ear Institution, who described the condition in greater detail and distinguished it from other forms of corneal ectasia, most notably the ectasia ex ulcus.[2] He also postulated the notion that the conical shape of the cornea resulted in severe astigmatism, short sightedness and difficulties in prescribing adequate glasses. The increased prevalence of keratoconus in Down's syndrome was described in 1948 by Rados et al[3] and the relationship with an atopic constitution was described by Rahi et al[4] in 1977 and by Gasset et al[5] in 1978.

For long, auxiliary investigations are used to establish a keratoconus diagnosis better and quicker. Bowman in 1859 for instance employed the recently developed ophthalmoscope by von Helmholtz to diagnose keratoconus, using the instruments mirror under an angle to best appreciate the conical shape of the cornea. In 1881 Javal and Scholtz improved their keratometer, where two movable colored reflectors are used to assess corneal curvature. This device proved its value in clinical practice and is still in use today. Placido in 1880 devised his archetypical black and white ringed keratoscopy target to asses corneal shape. The actual inventor of the photokeratoscope is a matter of discussion[6], but Amsler in 1938 published a treatise on a photographic placido disk that diagnosed keratoconus before clinical signs could be detected.[7] The advent of computer-assisted topographical and pachymetric analyses in the nineties have dramatically improved the sensitivity of detection of keratoconus.[8] These analyses have been of great value in refining study populations for genetic studies, the follow-up of disease progression, and is an integral part of the screening examination prior to refractive procedures. Especially the latter can be considered a major driving force behind the development of optical diagnostic devices and cameras, since an ectasia can be induced by a routine LASIK refractive procedure in the wrongly selected patient.[9]

To improve visual acuity in keratoconus patients several treatment strategies can be employed and the mainstay of these treatments has been unchanged for decades, until recently. Firstly, contact lenses can be fitted to correct the astigmatic error in keratoconus eyes. In 1888, keratoconus became one of the first practical applications

of the newly invented glass contact lens by the French physician Eugène Kalt.[10] Improvements in manufacturing and materials led to scleral lenses made from celluloid and PMMA in the thirties, but the lack of oxygen permeability remained an issue. In 1983, Ezekiel et al. introduced an oxygen permeable contact lens.[11] Small rigid lenses and soft contact lenses quickly gained popularity, replacing the existing scleral lens types. Nowadays, major improvements in scleral lens geometry and choice of oxygen permeable materials have led to a reinvention of scleral lenses for keratoconus, and an increased application worldwide.[12]

Advances in surgical instrument making and anesthesia enabled the micro-surgery of corneal grafting procedures in the beginning of the 20th century. Eduard Zirm was the first to successfully transplant a human cornea in 1905.[13] Improvements in ophthalmic microscopes and suture material increased its popularity, and in 1936 Ramón Castroviejo was the first to transplant a full-thickness keratoconus cornea in the Columbia Presbyterian Medical Center, NY, USA. Poor graft survival was a major issue, since the concepts of graft rejection and its inflammatory constituents were largely unknown. The clouding of the graft was attributed to uveitis, for which no effective treatment existed, since prednisolone was only commercially available from 1955 onwards. Nevertheless, this concept of full-thickness corneal transplantation is still in use today. Lamellar surgery regained popularity from the nineties onward with the advent of better mechanized surgical knives to split the cornea in layers.[14] This enabled surgeons to tailor their procedures to the actual localization of the pathology; posterior lamellar surgery revolutionized the treatment for patients with endothelial diseases.[15] The advances in anterior lamellar surgery are covered in the section *Innovations in the surgical treatment of keratoconus.*

Epidemiology

The prevalence of keratoconus is often reported as 1:2000, but may vary widely due to variations in diagnostic criteria and racial predilections.[16] A more realistic estimate is between 50 to 230 per 100.000[17,18], but no data with stratification for disease severity exist. Solid estimates on the incidence, severity and treatment outcomes on population level are mandatory to assess the burden of keratoconus to society.

The vast majority of keratoconus occurs bilaterally, and is often asymmetric.[19] Strictly unilateral cases have been reported, tough incidence plummeted with the advances in disease detection by corneal topography. It has been convincingly shown that unaffected fellow eyes have a much higher risk to develop keratoconus than eyes of

unaffected individuals.[20]

Establishing the diagnosis keratoconus

Keratoconus is an ectatic disease with archetypal corneal thinning, protrusion, and scarring in advanced stages, but its clinical presentation can vary considerably. The effects of these corneal changes on visual acuity can be amazingly variable as well. The mildest cases are often referred to as *form fruste*, suggesting that the disease hadn't set on yet; the most severe necessitate a grafting procedure to restore optical clarity of the cornea. Since keratoconus can present itself so variably, and many treatment strategies exist, a structured ophthalmic exam is proposed.

Typically, the development of keratoconus starts around the first or second decade with an insidious change in manifest refraction and increased (irregular) astigmatism. Naturally, visual acuity is affected in the more severe cases, but its asymmetric presentation can leave many patients asymptomatic; the good eye covers for his affected counterpart and binocular vision is hardly reduced. The optician or optometrist is often the first to suspect the diagnosis of keratoconus when people present with a decreased uncorrected visual acuity, or with changes in manifest refraction. A truly new diagnosed keratoconus is not regularly seen in older patients (i.e. >45 years old). Presumably, an asymptomatic corneal ectasia already existed while an ocular comorbidity causes the symptoms of a reduced visual acuity. Dry eye disease and cataract are the most prevalent comorbidities.

Visual acuity slowly decreases with the progression of the ectatic changes of the cornea, leading to irregular astigmatism and myopisation. Patients can experience a lowered quality of vision as well; a decreased contrast sensitivity, glare and halo's due to increased higher order optical aberrations, and increased complaints of straylight, especially in scotopic/mydriatic conditions.

The patient history should include previous eye surgeries and concomitant presence of associated disorders like an atopic constitution, eyelid eczema, allergic conjunctivitis, and severe eye rubbing. Many syndromes are associated with keratoconus, most notably Down's syndrome, and a high degree of suspicion on the presence of keratoconus should be employed in these cases. A family history is mandatory as well, since the prevalence of keratoconus is increased in first degree relatives.[21] Current (ophthalmic) medications should be noted, especially the use of anti-allergic drug and artificial tears. A complete contact lens history (lens type, achieved quality of vision,

comfort) is helpful in counseling future improvements for the restoration of visual acuity.

The ophthalmic evaluation should consist of the uncorrected and best corrected visual acuities, a manifest refraction, corneal topography (preferably including both anterior and posterior corneal surfaces) and a slit lamp evaluation. Eversion of the upper eyelid can show signs of a floppy eyelid and a papillary conjunctivitis associated with allergic conjunctivitis or contact lens intolerance. Many clinical signs of keratoconus have been described[16], but the relevance of all these signs became rather low, with the advent of modern corneal topography. The presence of a Fleischer ring, Vogt striae and breaks in Descemet membrane, stromal haze, existing limbal vascularization, concomitant dry eye disease, or any other corneal abnormality should be noted. Figure 1 shows an advanced case of keratoconus with striae, nebulae, and an apparent conus. Preferably, a fully dilated exam is performed to assess the clarity of the crystalline lens and any abnormalities of the macula and optic nerve head.

Disease severity can be graded based on clinical and topographic findings. A now commonly used system was devised by Amsler and Krumeich, though it is based on relatively old parameters like corneal steepness, refractive changes, and the presence

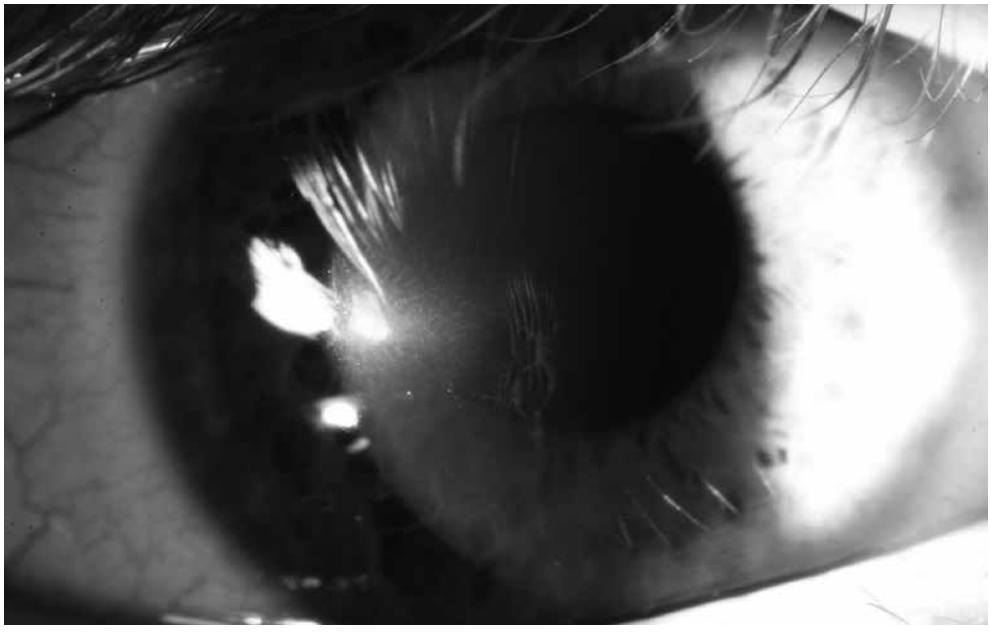

FIGURE 1. Vogt's striae, nebula, and central scarring in an eye with advanced keratoconus.

Grading	Km* (D**)	Thickness (µm)	Spherical equivalent	Cornea
1	<48	>500	< -5	No central scars
2	48-53	400-500	-5 ; -8	No central scars
3	54-55	200-400	> -8	No central scars
4	>55	<200	Not measurable	Central scars

*Km: mean keratometry. **D: dioptre

Table 1. Krumeich-Amsler classification of keratoconus.[22]

of corneal scarring, see table 1.

The Krumeich-Amsler classification has been criticized for a poor correlation with disease impact, since contact lens tolerance is not considered. New modern topography based grading systems have been proposed, but further validation is mandatory to assert their clinical value.[23]

Corneal topography

The importance of corneal imaging in establishing a keratoconus diagnosis cannot be overstated. Abnormalities in corneal thickness and corneal curvature can be appreciated much better and earlier using corneal tomography than with a slit lamp examination only.

Most devices in current corneal practice either use a Scheimpflug principle to acquire a set of corneal images or combine this with a placidodisk based videokeratography. These images are analyzed and elevation maps are calculated. Popular devices are the Orbscan II Topography System (Bausch & Lomb, Orbtek Inc., Salt Lake City, UT, USA), the EyeSys 2000 (EyeSys Laboratories, Houston, TX, USA), the Pentacam HR 70900 (Oculus Optikgeräte GmbH, Wetzlar, Germany), and the Cassini (iOptics, The Hague, The Netherlands). The repeatability of these devices is similar for most parameters, though evidence suggests that measurements are not mutually exchangeable.[24] Most notably is the different representation of corneal thickness in advanced cases.

Figure 2 shows one of the 25 Scheimpflug images on which a corneal tomogram is based. The anterior and posterior corneal surfaces, their reflectivity, and their spatial locations are captured and converted into elevation maps of the cornea.

Many advanced calculations can be performed on these elevation maps, ranging from

FIGURE 2. One of the 25 Scheimpflug images sequentially recorded by the Oculus Pentacam HR.

plain keratometry, to higher-order aberration calculations, and specific keratoconus detection programs. Figure 3 shows a classical representation of these data in four colored maps. The key biometrical indices are given in the left column (anterior keratometry, posterior keratometry, and corneal thickness). The four maps represent the sagittal anterior corneal curvature, corneal thickness, and both front and back elevation maps. Keratoconus is recognized by the irregular astigmatism of asymmetric size and with skewed axes, in combination with a localized thinning of the cornea. The thinnest point in the pachymetry does not necessarily correspond with the location of maximum keratometry, but often they are juxtaposed. The elevation maps can be helpful in judging the aberrant conical shape of the cornea, especially for a keratoconus posterior. Here, a best-fitted sphere is plotted on the elevation data and the deviations in µm are concordantly mapped. Many indices to diagnose keratoconus have been proposed in the past decades (keratoconus index, IS-ratio, KISA%, SRAX etc.), with variable clinical validity.[25,26]

In the Pentacam software, the Belin-Ambrósio analysis can further aid in establishing the diagnosis.[27] Here, changes in corneal thickness at certain paracentral distances are used in combination with various indices to make a prediction on the presence of a keratoconus. A recently developed dynamic bidirectional applanation device (Ocular Response Analyzer, Reichert Ophthalmic Instruments, Depew, New York, USA) provides new insights in the biomechanical changes in the cornea due to keratoconus and might be helpful in the diagnostic process as well.[28,29]

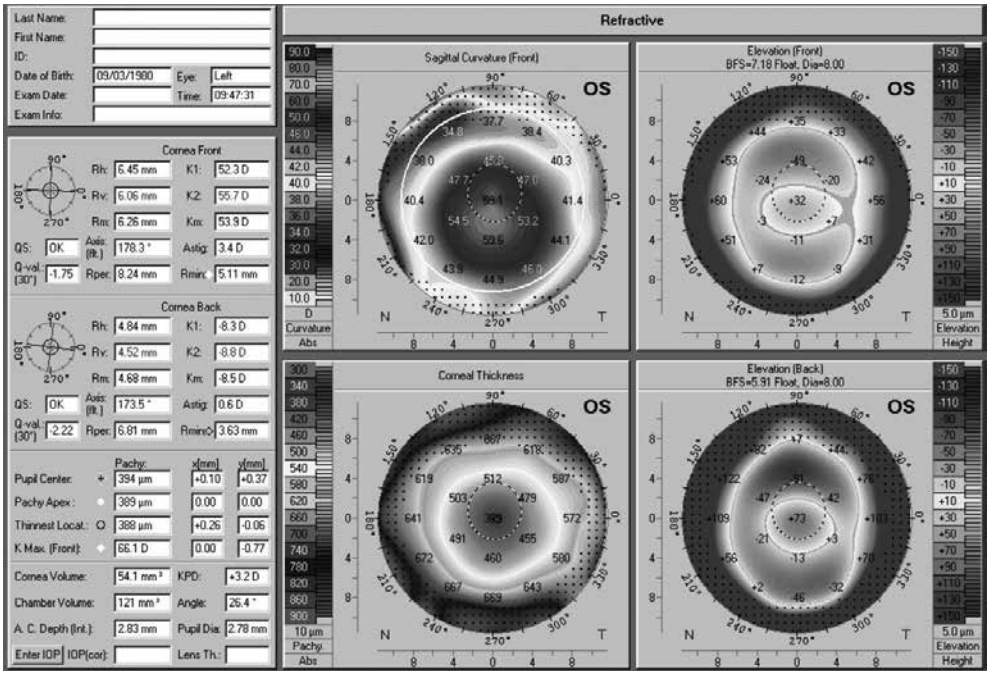

FIGURE 3. Four maps refractive output of the Oculus Pentacam HR. Keratometric parameters are found in the left panel, and the four maps all aid in establishing a keratoconus diagnosis. Upper left: distribution of keratometry readings. Lower left: corneal thickness map. Upper right: elevation map of the anterior surface. Lower right: elevation map of the posterior surface.

Differential diagnosis and relation with other medical conditions

Developmental origins of alterations in corneal shape

Corneal astigmatism is fairly common. It is associated with a decreased visual acuity and a high cylindrical refractive error. An increase in this refractive error might resemble the development of keratoconus, especially in adolescents.[30] Regular corneal astigmatism is not associated with corneal thinning however, and is discernable from keratoconus using corneal topography imaging.

Other ectatic corneal disorders

Several ectatic corneal disorders exist and these conditions are to a varying degree quite similar in clinical presentation. They may actually represent variations in the phenotypic expression of a common pathogenetic mechanism.[31] These conditions share their basic treatment algorithm with keratoconus.

- Pellucid marginal degeneration (PMD) is less common than keratoconus and its age of onset is remarkable higher (2nd-5th decade). It is characterized by

a progressive inferior thinning and protrusion, resembling a beer belly when looked from aside. Corneal topography is needed to distinguish PMD from keratoconus[32], where the inferior located PMD results in a kissing doves sign.

- Keratoglobus is a very rare condition, usually present at birth and often associated with systemic syndromes like connective tissue disorders. Its protrusion is generalized and can be progressive.[33]
- Post-LASIK ectasia is an uncommon late complication of refractive surgery, and is caused by an increased biomechanical instability after the ablation of corneal tissue. It is postulated that these patients might have exhibited a form fruste keratoconus prior to their treatment[34], or share cellular alterations with keratoconus[35].
- The rare brittle cornea syndrome is characterized by severe corneal thinning and semi-spontaneous ruptures, but its autosomal recessive inheritance and clinical picture are easily distinguished from the other ectatic diseases.[36]

Etiology of keratoconus

Although classically defined as a degenerative disease, with mechanically induced trauma accelerating its course, the pathophysiology of keratoconus remains poorly understood. Currently, keratoconus is considered to be a multifactorial corneal disorder caused by the sophisticated interaction of several environmental (eye-rubbing, contact lens wear) and endogenous factors leading to systemic and corneal oxidative stress by hypersensitive response to oxidative stressors that involves mitochondrial dysfunction and mitochondrial DNA damage in genetically susceptible individuals.[37–39]

Prolonged eye-rubbing alone is reported as an independent risk factor for the development of keratoconus, with abundant clinical evidence that vigorous eye rubbing may lead to de novo development of keratoconus.[40,41] However, eye-rubbing without overt keratoconus development will not be clinically recognized if the subject doesn't seek medical attention, and not all patients with keratoconus will exhibit a history of eye rubbing.[42] The role of contact-lens wear on the development of keratoconus remains controversial. A majority of keratoconus patients needs (rigid gas permeable) contact lenses for adequate visual functioning, and all contact lenses alter the corneal shape reversibly by compression to some extent (corneal warpage).[43] Progression of keratoconus is often concurrent with contact lens wear[44], and local tear film alterations could be related to contact lens wear rather than keratoconus alone.[45] In contrast, contact lens wear was not associated with progressive keratoconus in a longitudinal study and a cause-effect relationship cannot be drawn on cross-sectional data.[44,46]

An increasing body of literature points towards an immunological origin, or at least a derailed inflammatory response mechanism, of the development and progression of keratoconus. Traditionally, keratoconus is considered a non-inflammatory disease, and this paradigm is reflected by the myriad references to the seminal articles of Krachmer JH et al in 1984 and Rabinowitz Y et al. in 1998.[16,47] Both articles explicitly consider keratoconus a non-inflammatory condition in their introduction, though Rabinowitz already mentions the discovery of IL-1 in its pathogenesis. Wilson et al. were the first to investigate that the epithelium and endothelium of keratoconus corneas produces interleukin-1 (IL-1) and that keratocytes can be shown to express the IL-1 receptor. On the basis of this, IL-1 is considered to play a role in the regulation of corneal cell proliferation, differentiation, and death.[48] Eye rubbing for instance could provoke an immune response in genetically susceptible individuals leading to the clinical phenotype of keratoconus.[49] The current literature on soluble and cellular inflammatory mediators is rapidly expanding, chapter 9 provides a systematic review hereon.

Recent progress in genome wide association studies (GWAS) have provided critical insights into potential molecular mechanisms underlying keratoconus, and revealed susceptibility loci linked to central corneal thickness, cell metabolism and cellular ageing. More specifically, meta-analyses of large European and Asian cohorts have revealed that variants near FOXO1, FNDC3B, FRAP1/MTOR, and PDGFRA genes conferred relatively large risks for developing keratoconus.[50–52]
However most genetic associations fail to prove a significant functional contribution to disease biology, and the conveyed odds ratio for the development of keratoconus with these polymorphisms is rather low (OR 1.3-1.6).[50]

The natural course of keratoconus

The natural course of keratoconus is well studied and shows an intermittent progressive course. The condition can remain quiescent for prolonged time, before a new period of progression emerges. Extra-corneal factors that accelerate disease progression are vigorous eye-rubbing, and untreated ocular allergy/atopic constitution. Changes in female hormonal status are debated as contributory.[53]

Corneal topography is mandatory to evaluate disease progression and yields a myriad of readings and indices. The value of these quantitative indices is also well studied, and higher KISA, I-S and SRAX readings are associated with the development

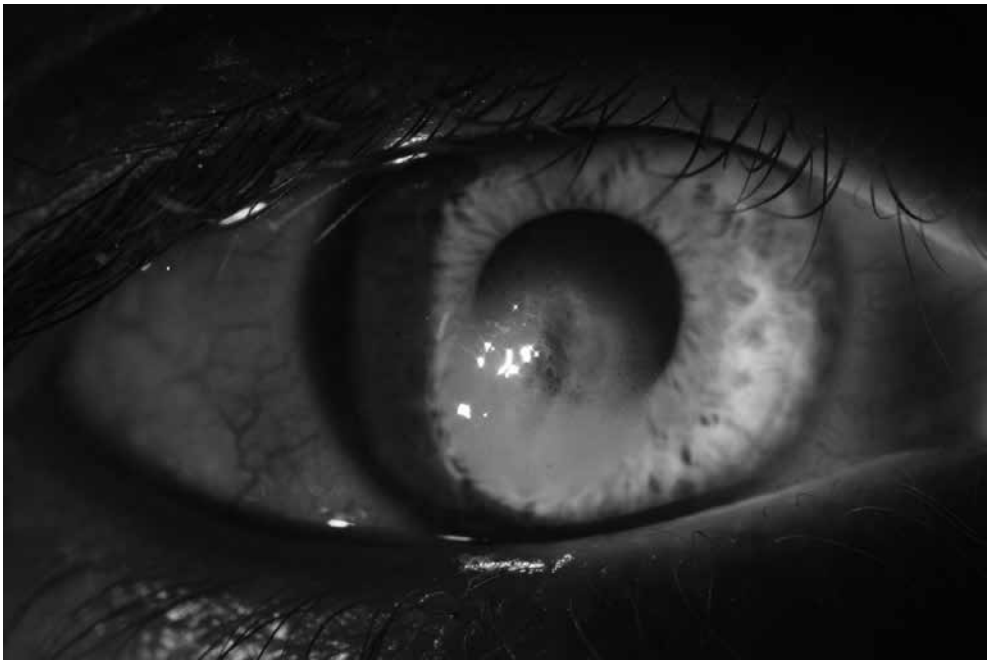

FIGURE 4. Typical presentation of a corneal hydrops, or Kammerwassereinbruch, in keratoconus; note the mild conjunctival redness, and the localized corneal edema with intrastromal cysts. The epithelium in is intact in this case.

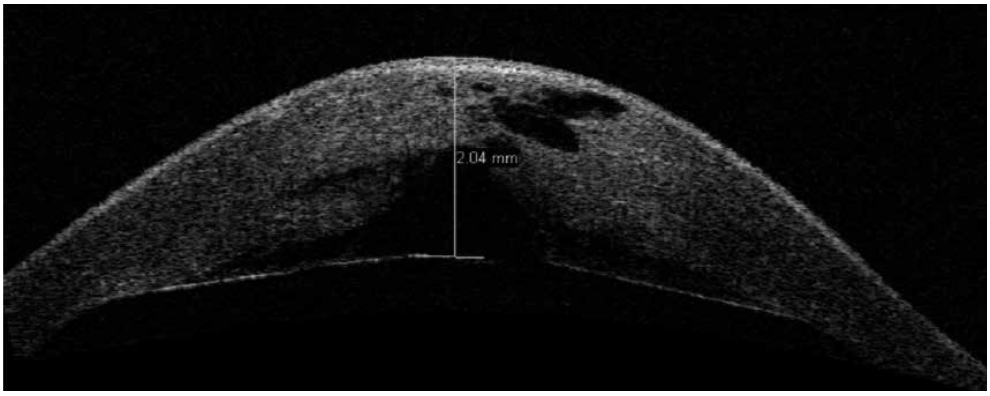

FIGURE 5. Optical coherence tomography image of a corneal hydrops. Note the severely increased corneal thickness and intrastromal cysts (Zeiss Visante OCT).

of keratoconus.[26,54–56] This means that more severe cases of keratoconus show more disease progression on average.

A peculiar inverted relationship between age and keratoconus exists, where pediatric cases are more likely to show progressive disease.[57–59] Evidence exists that keratoconus even progresses at higher age (48-59 years), though this progression is very modest, 0.24D change in corneal curvature on average, and therefore of little clinical significance.[60] Our own 2010/2011 treatment cohort showed 8/96 cases older than 35 year with documented progressive keratoconus.[61] The claim made by some authors that keratoconus progression halts at the age of 35 simply does not hold up. ‘

Progressive advanced keratoconus may lead to a rupture in Descemet membrane with subsequent profound corneal edema, poor visual acuity and acutely increased ectasia. This corneal hydrops, or Kammerwassereinbruch, can be debilitating but typically has a beneficial self-improving course[62], see Figure 4 and 5. The edema mostly resolves, though a corneal scar remains to some extent. Refitting (scleral) contact lenses might achieve adequate visual acuity and prevent a grafting procedure.

Treatment of keratoconus

The treatment options for patients with keratoconus expanded considerably throughout the years, though a number of fundamental considerations are still relevant for a proper patient selection and consultation. The most important question every clinician should ask before any treatment is instigated is whether a relevant benefit for the patient will follow from the proposed strategy. In keratoconus care, as in general, it is therefore of paramount importance to determine which parameters are considered relevant clinical outcomes. Keratometry for instance is the major parameter for the diagnosis of keratoconus and assessment of its progression. From a patient perspective however, visual acuity and manifest refraction are more important, since these factors contribute more directly to the quality of vision. For some patients the dependency on visual aids like spectacles or (scleral) contact lenses is the quintessential outcome. Furthermore, excellent visual functioning and a subsequent quality of life can be attained with a functional monocular status.[63] In conclusion, we should always aim to treat the patient and not a corneal topogram.

The treatment of keratoconus can be subdivided in several entities:

1. Optimizing visual acuity by the prescription of visual aids;
   a. Spectacle correction
   b. Regular contact lens fitting
   c. Advanced (scleral) contact lens fitting
2. Surgically counteracting the induced astigmatic changes;
   a. Intracorneal ring segment implantation
   b. Phakic toric implant lenses
   c. Corneal grafting procedure in contact lens intolerance
3. Restoring the clarity of the scarred cornea after corneal scarring;
4. Prevention of disease progression by corneal crosslinking.

Innovations in the surgical treatment of keratoconus

With the advent of refractive surgery in the nineties, equipment appeared to split a cornea in horizontal lamellae with a fine interface.[14] Mechanized surgical blades (microkeratomes) were able to cut the corneal tissue at a pre-defined depth, effectively splitting it in half. This made partial thickness grafting possible, tailoring grafts according to the nature and location of corneal pathology. For keratoconus, only the affected anterior part of the cornea needs to be transplanted. The posterior (endothelial) part is particularly involved in graft rejections.[64] The chance of graft rejection decreases significantly when the patient's endothelium is left in place.[65] Also, it has been postulated that a closed globe procedure has less risk of a problematic subchoroidal haemorrhage. The eye might be more resilient to blunt corneal trauma as well.

However, merely replacing the diseased anterior half of the cornea with a clear corneal graft led to unwanted side effects: the interface between these layers shows opacification and scarring, leading to a poor visual acuity.[66] Busin showed that the use of a microkeratome and a rather deep cut increases visual acuity one year after a grafting procedure in keratoconus patients.[67] However, the stretched posterior keratoconic stroma and Descemet membrane can have difficulties in adapting the contour of the healthy superficial donor cornea; the concept of a 'keratoconus memory' was postulated. This may lead to wrinkling of the posterior surface, with a profound negative effect on visual acuity, and potential recurrence of ectasia.[67] Leaving remnants

of stroma in place is therefore unwanted and techniques were developed to separate Descemet membrane from the stroma, using either fluid68, viscoelastic devices[69], or air[70]. Melles also described a technique of manual dissecting the complete anterior stroma.[71] These new techniques are all called deep anterior lamellar keratoplasties (DALK). The transplanted anterior corneal thickness is maximized, and the patient retains its own endothelium and Descemet membrane, leading to lower graft rejection rates.

The biggest drawback of a DALK procedure is the risk of inadvertent peroperative corneal perforation. The Descemet is perforated or ruptured necessitating a conversion to a complete thickness graft. Failure and perforation are described in 20% of cases though, leading to poor surgical predictability.[72] The published reports are often based on case series by operated by surgeons experienced in lamellar surgery. The learning curve of DALK techniques is quite long, and the true complication rate might even be higher.[73] This will be addressed in detail in chapter 2.
Techniques that combine the advantage of classical DALK surgery without the high risk of adverse events during surgery are currently being developed. Busin proposed a method in which, in addition to an anterior lamellar keratoplasty, a partial endothelial trepanation (PET) is performed.[74] The endothelium and Descemet are paracentrally and circular loosened, but a certain proportion is left intact. This 'island' is able to mold to the healthy donor curvature. By doing this, the surgeon can retain a graft thickness margin leading to a lowered number of preoperative perforations. The addition of PET is believed to make corneal grafting safer and more predictable. This technique is described in this thesis in chapter 3.

One future development is on creating a central Descemet membrane baring bubble, leaving the peripheral posterior stroma in its place; the small-bubble technique, which shows promising results in terms of surgical predictability.[75] Another interesting development is an isolated Bowman layer transplantation as advocated by the group of Melles from the Netherlands Institute of Innovative Ocular Surgery in Rotterdam. Here, the replaced Bowman is considered to flatten the keratoconus and thereby preventing or postponing more extensive corneal surgery.[76]

However, whether the DALK surpasses the traditional full-thickness grafting technique in terms of cost-effectiveness and long-term survival is open for debate. Van den Biggelaar et al. performed an economic evaluation where a DALK was more costly and more effective as compared with PK. Strikingly, the incremental costs per clinically

improved patient – based on the data of patients without perforation of Descemet membrane – was about as much as the cost of the grafting procedure itself (€ 5250).[77] The Australian graft registry reported a significantly worse survival of DALKs when compared to PKs for keratoconus in the same time-frame (1996-2013).[78]

Crosslinking; a paradigm shift

Progressive keratoconus corneas can be stabilized and strengthened by corneal crosslinking (CXL).[79] The cellular basis of its effectiveness is the creation of chemical bonds between large molecules, inducing a polymerization effect. Crosslinking is used in dentistry to harden filling materials, in the automobile industry to stabilize lacquer, and in many other processes involving polymerization of plastics. The technique of CXL was first applied in human keratoconus corneas in 1998 and consists of an epithelial removal, after which the corneal stroma is saturated with the photosensitizer riboflavin (vitamin B^2) for a certain period of time (normally between 20-30 minutes). Then, ultraviolet-A (UVA, 370nm) radiation is applied which produces reactive oxygen species, which in turn induce interfibrillar covalent collagen cross-links.[80] The effectiveness of epithelium-off CXL has been demonstrated in numerous treatment cohorts and three randomized trials: on average both uncorrected and corrected visual acuity increase, keratometry readings improve slightly, all combined with a low rate of adverse events profile.[81] Treatment effects appear to be stable and no long term side-effects were noted, although the patient numbers in extended follow-up studies is rather low.[82] Other progressive ectatic diseases like pellucid marginal degeneration and iatrogenic post-LASIK ectasia can be successfully treated with CXL as well, although the case numbers are considerably lower.[83,84]

The ease of delivery, its effectiveness, and an attractive safety profile, boosted the popularity of corneal crosslinking in the past decade and led to a paradigm shift in the treatment of keratoconus. National legislators have approved the reimbursement of crosslinking in 2013 the UK[85] and in 2014 the Netherlands.[86] To date, the US Food and Drug Administration denied the approval for crosslinking as applied for by the Avedro company.[87] The potential to prevent a future corneal transplantation is one of the most powerful assets of a crosslinking treatment, the keratoconus itself however is only slightly reduced. A promising development is topo-guided refractive surgery in combination with CXL, to improve keratometry measurements and uncorrected visual acuity in keratoconus patients.[88,89] No comparative trials exist to date however, and a potential pitfall might be a refractive overcorrection due to ongoing flattening of the corneal curvature years after the initial crosslinking.

Aim and outline of this thesis

The aim of this thesis was to describe the new developments in the treatment of keratoconus and to study the etiology of this corneal disease. The thesis has three main sections, subdivided in chapters based on the underlying publications.

Section I focusses on the developments of the surgical treatment of keratoconus and the use of contact lenses. Many improvements of existing grafting procedures have been proposed. Naturally, these newest techniques are performed and propagated by their developers and the question arose whether these newer techniques were actually finding their way in the routine corneal practice. We investigated the registries of the Dutch Organ Transplant Registration (NOTR; www.transplantatiestichting.nl) and asked whether lamellar grafting is experiencing the popularity it deservers (**Chapter 2**). One particular development in the surgical treatment of keratoconus was regarded of such importance that a comparative trial was instigated. As mentioned in the section *Innovations in the surgical treatment of keratoconus*, Busin described an anterior lamellar technique where the patients posterior stroma/Descement membrane/endothelium was loosened, but not replaced, after performing a regular anterior lamellar keratoplasty. It promised a lower amount of adverse events during surgery and a shorter learning curve when compared to the current gold standard for anterior lamellar surgery, the DALK technique. The outcomes of this multicenter trial are found in **Chapter 3**. Another important treatment modality for the improvement of visual acuity in keratoconus are (scleral) contact lenses. Our outpatient clinic offers a specialized contact lens service, where most keratoconus and post-transplantation patients are referred to. **Chapter 4** describes a cross-sectional investigation of objective and subjective outcomes of the lens types used in this tertiary setting. Measuring intra-ocular pressure (IOP) can be unreliable in keratoconus, owing to its thinned and irregular corneal contour. Most devices that assess IOP rely on a standard curvature and corneal thickness. We therefore investigated a device that circumvents these drawbacks by assessing IOP transpalpebrally (**Chapter 5**).

Section II reports on the other major novel treatment modality for keratoconus; corneal crosslinking. Many variations on the original treatment protocol by Seiler et al. have been proposed, and whether or not to remove the corneal epithelium has since long been a scientific debate. We performed a non-inferiority clinical trial on the effectiveness of transepithelial vs. epithelium-off crosslinking (**Chapter 6**). In addition, we used our clinical treatment database to investigate potential predictors of treatment effect, specifically for the clinical parameters keratometry and visual acuity (**Chapter**

7). In addition, the effect of complex refractive errors on visual acuity and the effect of crosslinking on these higher-order aberrations are reported in **Chapter 8**.

The final section III is on the immunological aspects of keratoconus. The collaboration with the Laboratory of Translational Immunology enabled us to pursue fascinating research on the fundamental characteristics of keratoconus. To achieve a solid understanding of the current literature of inflammation in keratoconus a systematic review on the soluble and cellular inflammatory mediators was performed (**Chapter 9**). The genetic origin of keratoconus has been known for a long time, and recent authorative genome wide association studies implied several genes significantly associated with the development of keratoconus. We set out to investigate the functional implications of these genes by assessing RNA expression profiles in actual corneal tissue. The results of this study on cellular aging in keratoconus can be found in **Chapter 10**. Furthermore, we assessed the putative role of UV-induced DNA damage for the development of keratoconus. The results are found in **Chapter 11.**

A general discussion of the thesis is given in **Chapter 12**, followed by a summary in Dutch in. Finally, a list of contributors, acknowledgements, abbreviations, publications, and the résumé of the author are given in **Chapter 13.**

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2

Does lamellar surgery for keratoconus experience the popularity it deserves?

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ABSTRACT

Aim

To analyse developments in surgical treatment for keratoconus by assessing rates and types of corneal surgery from 2005 - 2010.

Methods

The Dutch Transplantation Foundation supplied data on all keratoplasty procedures for keratoconus performed from 2005 - 2010 in The Netherlands. Registration was carried out by the eyebank at allocation and by the surgeon at the time of surgery. The type of surgery was categorized as either a penetrating or a lamellar procedure.

Results

575 anonymized records were received, with excellent data-completion (99%). Patients undergoing penetrating surgery had on average a lower visual acuity, higher k-readings, and were slightly older compared to the lamellar group. A previous corneal hydrops was recorded for 19.1% of patients. Regular penetrating keratoplasty decreased in popularity from 79.7% in 2005 to 43.7% in 2010, due to the increased rate of lamellar surgery (42.5% in 2010) and 'mushroom' penetrating keratoplasty (13.8% in 2010). When hydrops cases were excluded, popularity became equal (47.6% penetrating vs. 52.4% lamellar surgery, in 2010).

Conclusions

Lamellar surgery is gaining in popularity, though regular penetrating keratoplasty is still the more commonly performed procedure. Only when hydrops cases are excluded do transplant rates become comparable.

INTRODUCTION

Penetrating Keratoplasty (PKP) has traditionally been the standard procedure for corneal transplantation in patients with keratoconus (KC).

History of penetrating corneal surgery

For over 70 years, a penetrating technique has been used in which a circular donor disc is cut with a trephine and sutured in a concordantly prepared recipient. One major drawback is the replacement of the healthy host endothelium. Keratoconus patients in general are young when grafted, with a long life expectancy. Graft failure will eventually occur, mainly due to endothelial cell (EC) rejection and EC failure, potentially necessitating a second (or third) grafting procedure during the course of their life. Other disadvantages of PKP are that it is an 'open-sky' procedure, that surgical wound healing has a prolonged course necessitating tight suturing, the risk of suture-related infections, and the persistent risk of wound dehiscence. A new development in penetrating keratoplasty surgery is the PKP with a mushroom-shaped (PKPm) wound configuration (Chan et al. 2010, Saelens et al. 2008). The diameter of the anterior surface is made larger than the posterior surface. The larger anterior diameter of the corneal button potentially lowers induced post-operative astigmatism, whilst the smaller interior diameter preserves relatively more healthy endothelial cells of the host. Saelens et al. report a mean astigmatism at one year of 2.67D ±1.95 (n=15). No trials comparing regular PKP and PKPm are available for comparison however. Suturing complies with regular penetrating surgical norms.

New developments in lamellar corneal surgery

Visual outcomes after LKP were generally inferior to PKP, due to the stromal interface created. The desired optical clarity to compete with PKP visual outcomes was provided by baring Descemet's membrane (Terry 2000). A visco-dissection technique to separate Descemet's membrane was proposed. (Melles et al. 2000) The 'Big Bubble Technique' described by Anwar and Teichman in 2002 (Anwar & Teichmann 2002a) led to a more consistent and reproducible technique with much lower rates of perforation compared to earlier developed techniques (Anwar & Teichmann 2002b). Retaining the host endothelium has beneficial effects on graft-survival and rejection rates (Sarnicola et al. 2012), though DALK procedures are considered technically more demanding with a long learning curve. Up to a third of cases are complicated by the perforation of the Descemet membrane, with a subsequent conversion to PKP in the majority of cases

(Cheng et al. 2011).

A modified Anterior Lamellar Keratoplasty (ALKP) technique has been advocated as an alternative to the DALK technique. Busin in 2012 described a technique using a microkeratome to dissect an anterior lamella of maximum thickness, circumventing the risk of perforation of Descemet. The remainder of the host cornea is para-centrally loosened, permitting its adaptation to the curvature of the healthy donor lamella (Busin et al. 2012).

Incidence of corneal surgery in keratoconus

Several sources describing practices in corneal surgery are available, mostly eyebank registration databases (Boimer 2011, Eye Bank Association of America 2010, Ting et al. 2011, Xie et al. 2009) and national registration databases (Cunningham et al. 2011, Keenan et al. 2012, Stenevi et al. 2012, Williams et al. 2012). Not all databases supply surgical data stratified per diagnosis, i.e. data necessary for calculating lamellar and penetrating surgery rates. Little data is available on the true incidence of keratoconus surgery. An overview of extracted data is given in table 1.

Country	Timeline	KC specific	Registry	Outcome		
				n	PKP	LKP/DALK
UK (Keenan et al. 2012)	1999-2009	Yes	Yes	ca. 5200	88% -> 57%	8.8% -> 40.1%
Australia (Williams et al. 2012)	1985-2011	Yes	Yes	5412	90%	10%
Canada (Boimer et al. 2011)	2000-2009	Yes	No	1070	100% -> 82.2%	0% -> 17.8%
US (Eye Bank Assoc. 2010)	2010	Yes	No	5422	87%	13%
Scotland (Ting et al. 2011)	2001-2010	Yes	No[$]	264	100% -> ± 42%	0% -> ± 58%
China (Xie et al. 2009)	1996-2007	Yes	No[&]	674	37.6% -> 38.6%*	0% -> 61.4%
Sweden (Stenevi et al. 2012)	2005-2011	Yes	Yes	745	NR[#]	NR
New Zealand (Cunningham et al. 2011)	2000-2009	No	Yes	938	NR	NR

#: NR: Not recorded. $: retrospective analysis of all histopathological records of submitted donor corneas. &: retrospective analysis of two Northern-China-based hospitals. *: in earlier years epikeratophakia accounted for all non-penetrating surgeries., numbers not shown. This technique was completely replaced by lamellar surgery, with a stable amount of penetrating surgeries.

TABLE 1. Summary of available literature on surgery rates for keratoconus

Dutch health care organization and graft registration

Dutch hospital health care is organized around a system of obligatory health insurance with private insurance companies. Corneal surgery, being lamellar or penetrating, is a designated insured treatment. All costs involved with the procedure are covered by the insurance companies and hospitals. There is no financial consequence for the individual patient in choosing a certain treatment regime.

The Netherlands has a national eyebank registration (Dutch Organ Transplantation Register,NOTR,http://www.transplantatiestichting.nl/over-de-nts/organisatie-en-taken/notr). The NOTR is a program of the Dutch National Transplantations Foundation (NTS). This program collects data of tissue and organ transplantations to improve the quality and efficiency of these transplantations. The NTS allocates the donated corneal tissue to national and international eye banks. With the patient's consent, the NOTR receives their data directly from the corneal surgeon performing the transplantation. Computerized, standardized forms provided by the NOTR are completed at the time of surgery and at regular intervals postoperatively. High data completion rates have been attained since NOTR registration is obligatory to obtain allocated corneas. One Dutch corneal surgery centre has its own eyebank (Amnitrans, http://www.niios.com/content.php?na=25), and does not participate in the national registration program. The number of transplants performed by the non-participating corneal centre could not be retrieved.

Goal of study

In view of the long-term advantages of lamellar keratoplasty, the surgery of choice in eyes with a healthy endothelium is a Deep Anterior Lamellar Keratoplasty, with maximal depth (Han et al. 2009, Javadi et al. 2010, Shimazaki et al. 2002). A shift from penetrating to lamellar surgery could have been expected. Is this expected shift reflected in daily practice, in a developed country with a well-organized healthcare system, lacking financial constraints confounders? To assess whether these proposed techniques are truly gaining popularity, we analysed the frequencies of various surgical modalities for all KC transplantations in the Netherlands from 2005-2010.

MATERIALS AND METHODS

Data

Data were investigated of patients with KC who underwent corneal transplantation between 2005 and 2010 in the Netherlands, through analysis of the prospective database of the Dutch Organ Transplant Register (NOTR).

The baseline data which was extracted consisted of age, gender, presence of previous hydrops, best corrected visual acuity in LogMar (BCVA) and Keratometry (K) readings. BCVA could either be obtained with spectacles or contact lenses; the highest visual acuity was noted. Furthermore, surgical information (eye bank case number, surgical procedure performed, date of surgery, and any surgical remarkts) was recorded. The surgeon registered the surgical procedure by choosing one of the following procedures from a set drop-down menu: (PKP regular (PKPr), PKP mushroom (PKPm), anterior lamellar keratoplasty (ALKP), DALK with maximal depth (DALKmax), DALK with residual stroma (DALKrs), DALK of unspecified depth (DALKns) and Other. In the DALKns-cases, a DALK was performed, but the depth was not registered. No information was available on the nature of contact lens correction (regular rigid gas permeable, scleral, piggy-back etc).

If a surgeon reported conversion from a lamellar procedure to a penetrating procedure, the case was labelled as regular penetrating surgery. Reporting conversions or surgical complications was not obligatory. Permission for anonymized data extraction was granted by the NOTR scientific/ethical council (the Dutch Cornea Workgroup, a subcommission of the Dutch Ophthalmic Society).

Sample size and data quality

For this study anonymized records were supplied by the NOTR. Surgeons and treatment centers were anonymized as well. Patients were excluded if the surgical procedure performed was not in accordance with the procedures performed on patients with keratoconus (for example procedures mainly for removing the endothelium, such as a top-hat procedure). In these cases data was insufficient to confirm a proper keratoconus diagnosis. Additional remarks on the surgical procedure were not suitable for computing conversion rates.

Statistical Analysis

To analyse the frequencies of various surgical procedures with respect to time, procedures were grouped per calendar year and the various surgical modalities were clustered. Outcomes were presented in two main groups: penetrating keratoplasty (PKP) versus lamellar keratoplasty (LKP). Secondly, data was presented into the more specific, surgical groups PKPr, PKPm, ALKP,DALKmax, DALKrs, and DALKns. Cases in which the type of surgery was registered as 'other' , were classified separately. Statistical analysis was performed using IBM SPSS Statistics 20.0.

RESULTS

Baseline Characteristics

Five hundred and seventy-five records were received. Data of 569 eyes of 523 patients were suitable for analysis (99%). In six records data was insufficient to confirm a proper keratoconus diagnosis; a penetrating 'top-hat' procedure was registered, indicated for endothelial disease. The K-readings were all within normal ranges in these six cases. Diagnosis, gender, age, sex, and date of transplantation were recorded in 100% of cases. BCVA was recorded in 94.9%, previous corneal hydrops in 82.1% and corneal K-readings in 35.0%. The latter could often not be determined due to advanced keratoconus.

Mean age at time of surgery was 37.6 years (±13.2), 68% of patients were male and 19.1% of eyes had a previous hydrops. Baseline characteristics specified per treatment modality (penetrating vs. lamellar surgery) are given in table 2. Data specified per surgical technique is given in table 3.

Developments in penetrating vs. lamellar surgery

Absolute number of transplantations per year was 75 in 2005, 103 in 2006, 97 in 2007, 114 in 2008, 98 in 2009 and 82 in 2010. Penetrating surgery rates changed from 80.0% in 2005, 74.8% in 2006, 76.3% in 2007, 80.7% in 2008, 69.4% in 2009 to 56.1% in 2010. These numbers are visualized in Figure 1.

Developments in specific keratoplasty procedures

When stratified per operation technique, multiple developments over time can be

	PKP (n=417)	LKP (n=146)	Other (n=6)
Age (SD)	37.8 (13.7)	35.8 (11.2)	37.5 (18.1)
Gender (% male)	70.0%	63.7%	50.0%
Previous hydrops	20.6% (NR*=21.3%)	2.1% (NR=7.5%)	0% (NR=33.3%)
BCVA logMAR (SD)	1.09 (0.69, NR=2.6%)	0.79 (0.50, NR=11.0%)	0.67 (0.45, NR=33.3%)
K-mean (±SD)	54.6 (6.9, NR=71.7%)	52.7 (±6.6, NR=44.5%)	NR

PKP: penetrating keratoplasty. LKP: lamellar keratoplasty. BCVA: Best-corrected visual acuity. K-mean: average corneal curvature in dioptres. * NR: Non-recorded data. Age and gender were always recorded. Hydrops data was missing in 17.9%. BCVA pre-transplant data was missing in 5.1%. K-mean readings pre-transplant were missing in 65.0%.

TABLE 2. Baseline characteristics per treatment modality

	PKPr (n=361)	PKPm (n=57)	DALKmax (n=73)	DALKrs (n=39)	DALKns (n=7)	ALKP (n=27)
Age (±SD)	37.9 (13.9)	37.2 (12.4)	36.5 (12.2)	34.5 (10.7)	31.4 (8.1)	37.3 (9.7)
Gender (% male)	69.5%	71.9%	63.0%	74.4%	57.1%	51.9%
Previous hydrops	20.5% (NR=22.7%)	21.1% (NR=12.3%)	1.4% (NR=1.4%)	5.1% (NR=0%)	0% (NR=100%)	0% (NR=11.1%)
BCVA logMAR (SD)	1.09 (0.66, NR=3.0%)	1.10 (0.83, NR=0%)	0.85 (0.50, NR=2.7%)	0.84 (0.47, NR=12.8%)	NR	0.55 (0.46, NR=7.4%)
K-mean (SD)	54.8 (6.9, NR=74.0%)	53.7 (6.7, NR=57.8%)	53.1 (6.9, NR=34.2%)	53.7 (8.4, NR=69.2%)	NR	51.1 (4.4, NR=22.2%)

PKPr: Regular penetrating keratoplasty. PMPm: Mushroom keratoplasty. DALK: Deep anterior lamellar keratoplasty. DALKmax: Maximal depth DALK. DALKrs: DALK with residual stroma. DALKns: DALK of unspecified depth. ALKP: Anterior lamellar keratoplasty. BCVA: Best-corrected visual acuity. K-mean: average corneal curvature in dioptres. * NR: Non-recorded data. Age and gender were always recorded. Hydrops data were missing in 17.9%. BCVA pre-transplant data were missing in 5.1%. K-mean readings pre-transplant were missing in 65.0%.

TABLE 3. Baseline characteristics per type of surgery

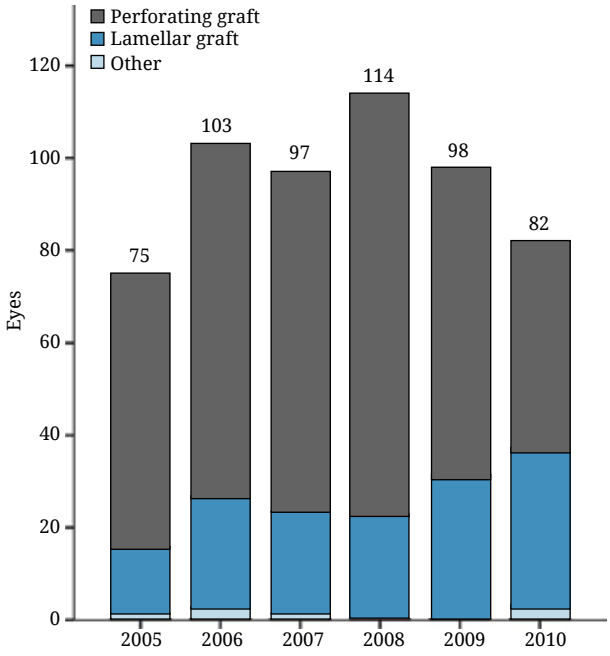

FIGURE 1. Distribution of corneal transplantations for keratoconus in the Netherlands from 2005 – 2010.

identified. The overall lowered penetrating surgery rate is due to the regular PKP's decreased frequency, partly compensated for by the novel mushroom PKP technique (n=57; 15.9% of all penetrating surgeries). Overall though, the most performed procedure, even in 2010, remains the regular PKP (2010: 43.8%).
Distribution of lamellar procedures is more variable, with an initial even distribution of techniques. ALKP rates remained low (n=27) and even show a decreasing trend. The DALK technique (n=119), especially with maximal stromal depth (n=73) is gaining popularity (DALKmax 2010: 27.5%). Distributions are visually represented in Figure 2.

Corneal hydrops and choice of treatment

In cases with a recorded previous corneal hydrops, a penetrating approach was chosen in 96.6%, three lamellar procedures were performed. If cases with previous corneal hydrops are excluded, overall DALK rates increase from 20.7% to 28.3% and DALK surpasses PKP in 2010 as treatment of choice (34.9% vs. 31.7%).

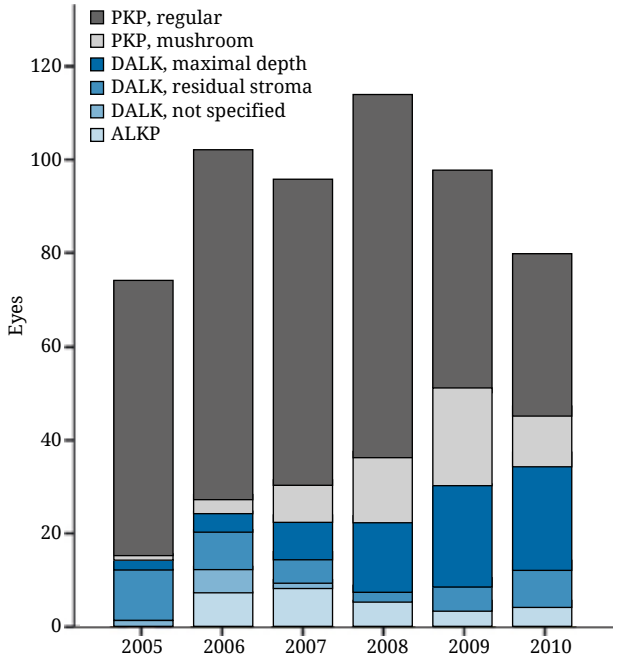

FIGURE 2. Specified distribution of corneal transplantations for keratoconus in the Netherlands, from 2005–2010

DISCUSSION

This study analysed the frequencies of various keratoplasty techniques for the indication of keratoconus, from 2005 to 2010 in the Netherlands. Lamellar techniques are gaining popularity; lamellar keratoplasty rate for KC increased from 25.4% in 2005 to 42.5% in 2010. Nevertheless, regular penetrating keratoplasty is still the most performed procedure in 2010 (43.8%). Furthermore, we report on the incidence of a previous corneal hydrops (19.1%) and its effect on the choice of surgery (96.6% penetrating surgery).

Corneal surgery underwent major changes during the last decade. Keenan et al. were the first to publish a comprehensive paper on these changes, using longitudinal national data (Keenan et al. 2011). Various other studies (Boimer et al. 2011, Cunningham et al. 2011, Ghosheh et al. 2008, Ting et al. 2011, Williams KA et al. 2007, Xie et al. 2009) investigated trends in corneal surgery but to our knowledge this is the first study especially on keratoconus. This is of particular interest since the technique of anterior lamellar surgery is rather different from posterior lamellar keratoplasty. Other data sources combine lamellar surgery regardless of location (anterior vs. posterior), and posterior grafting experienced a high popularity in itself (Cunningham et al. 2011). Our data are more in line with UK national registry data (40.1% lamellar surgery) than with US eyebank data (13.0% lamellar surgery). One strength of this study is the use of an obligatory nationwide registration database, rendering these data representative of the true ophthalmic practice in a developed country. Data completion on demographic entries is 100%. High data completion rates were attained since NOTR registration was obligatory for obtaining the allocation of corneas. Though one treatment centre did not participate in registration, we do not believe this materially alters our conclusions considering their limited number of keratoplasties for keratoconus.

Another strength of our data results from Dutch health care and health insurance organization. There are no financial consequences for the patient in choosing between LKP and PKP. Financial consequences are little if any for the doctor and his hospital; the insurance companies should cover the expenses. The costs of the procedure are therefore unlikely to influence treatment choice. The main contributing factor in the choice of surgery seems to be the surgeon's preferences and abilities and the presence of a previous corneal hydrops. This impact of a corneal hydrops on the choice of surgery is debated by Anwar et al., who describe near-Descemetic techniques to perform

successful lamellar surgery after a corneal hydrops. (Anwar & Anwar 2011). However their lack of suitable donor corneas for penetrating surgery is important, though this is of less concern in The Netherlands. Incomplete baring of Descemet's membrane has a negative effect on visual acuity, which favours a penetrating approach for eyes with a previous corneal hydrops.(Fontana et al. 2011)

The study design leads to certain considerations, since all data were anonymized. We were unable to review the initial patient records, if recorded values were ambivalent. Registration of corneal hydrops, and refractive status were subject to less than perfect registration, although both attained a more than 80% completion rate. Few details on the type of surgery are recorded, and ALKP-registration especially is non-specific. Our data give no insight into the type of ALKP or the popularity of Busin's microkeratome-assisted ALKPs (Busin et al. 2012). We could not calculate DALK to PKP conversion rate, since seldom a remark on the surgical procedure was recorded and adding surgical remarks was not obligatory. Regarding published conversion rates up to 20% (Cheng et al. 2011),, our registration is lacking in this aspect.

A DALK procedure baring Descemet's membrane is considered the surgery of choice in eyes with a healthy endothelium (Han et al. 2009, Javadi et al. 2010, Shimazaki et al. 2002). In this light it is remarkable that in our data, eyes without a previous hydrops are as likely to receive penetrating as lamellar surgery, even in 2010. Surgeon preferences and abilities seem to be of major influence in the treatment choice. The increasing acceptance of the lamellar technique brings hope, since more and more surgeons are becoming familiar with these novel techniques.

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3

Partial endothelial trepanation in addition to deep anterior lamellar keratoplasty in keratoconus patients. The PENTACON trial.

Robert PL Wisse, Cathrien A Eggink, Bart TH van Dooren, Allegonda van der Lelij

INTRODUCTION

Keratoconus is a progressive, corneal disease in which irregular refractive properties of the cornea result in loss of visual acuity. Keratoconus usually arise in adolescence, is bilateral and has an estimated incidence of 1:2000.[1] The aetiology of keratoconus is largely unknown, genetic predispositions are currently under investigation. Treatment is aimed at improving vision, principally using (rigid) gas permeable contact lenses (RGPs). With progression of the disease non-correctable refractive abnormalities and/or corneal scars arise. For these advanced stages of keratoconus, and in contact lens intolerance, a corneal transplant is the only viable treatment modality.

The first corneal transplant for keratoconus was conducted in 1936 by Ramon Castroviejo in New York's Columbia Presbyterian Medical Centre. Ever since, corneal grafting is subject to many technical developments. For over 70 years, a technique is used in which a circular donor disc is cut with a trephine and sutured in a concordantly prepared recipient, called a perforating keratoplasty (PK). With the advent of refractive surgery in the years 1990[2], equipment appeared to split a cornea in horizontal lamellae. This made partial thickness grafting possible, tailoring grafts according to the nature and location of corneal pathology. For keratoconus, only the affected anterior part of the cornea needs to be transplanted. The posterior (endothelial) part is particularly involved in graft rejections.[3] Therefore, the chance of a graft rejection decreases significantly when the patient's endothelium is left in place.[4] A technique where the transplanted anterior corneal thickness is maximized up to Descemet membrane is called a deep anterior lamellar keratoplasty (DALK). The biggest drawback of a DALK procedure is the risk of inadvertent peroperative corneal perforation, since the fragile Descemet membrane is easily ruptured, which might warrant conversion to a complete thickness graft. To prevent inadvertent perforation, several techniques are described to split the stroma from the posterior lying Descemet membrane and corneal endothelium, using either fluid[5], viscoelastic devices[6], or air[7]. Failure and perforation are described in 20% of cases though, leading to poor surgical predictability.[8] DALK techniques require a long learning curve, and the reported perforation rates might be an underestimate.[9]

To circumvent this problem a technique was developed in which, in addition to a mechanized anterior lamellar keratoplasty , a partial endothelial trepanation (PET) is performed. This technique was first performed by Prof. Massimo Busin, Villa Serena

Hospital, Forli, Italy.[10] The endothelium and Descemet membrane are paracentrally and circular loosened, but a certain proportion is left intact. This 'island' is able to mould to the healthy donor curvature. By doing this, the surgeon can retain a safer graft thickness margin leading to a lowered number of preoperative perforations. The addition of PET is believed to make corneal grafting safer and more predictable.

Here, we study the outcomes of this new technique in a randomized clinical trial, with the DALK technique as comparator technique: Partial ENdothelial Trepanation in Addition to anterior lamellar keratoplasty in keratoCONus patients, the PENTACON-trial. The primary goal was to assess the surgical safety of both techniques. Secondly, we assessed secondary treatment outcomes in terms of visual acuity, manifest refraction, corneal astigmatism, and endothelial cell density at one year post-treatment.

METHODS

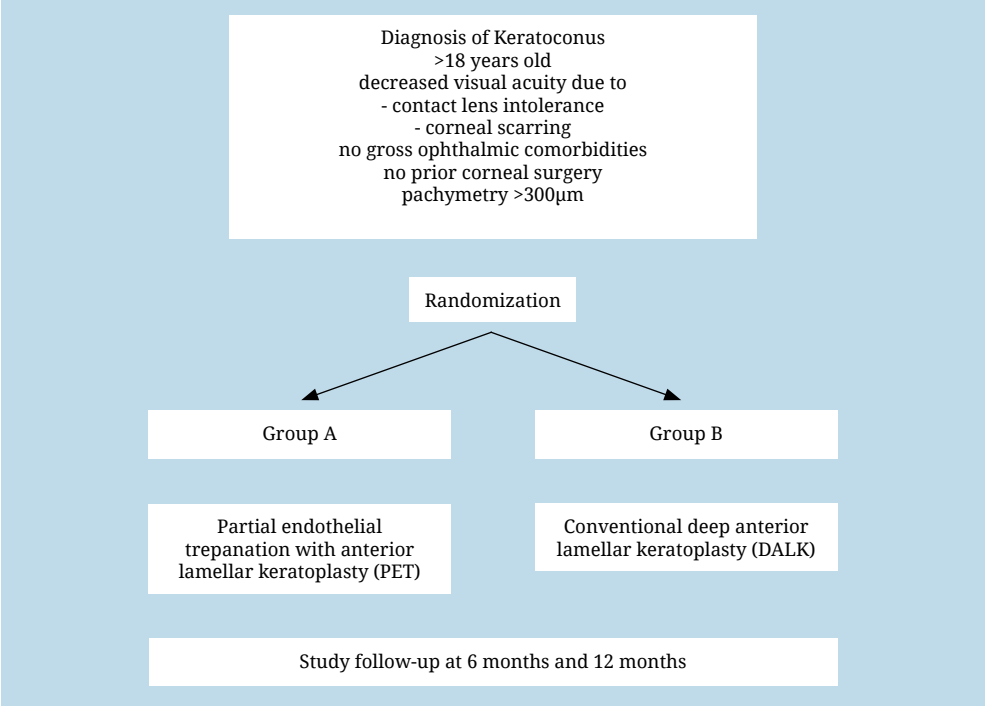
Study design

This multicentre randomized clinical trial was conducted from March 2011 until June 2015. Study participation was granted by the University Medical Center Utrecht, University Medical Center Nijmegen St. Radboud, Rotterdam Eye Hospital, Amphia Ziekenhuis Breda, and Westfries Gasthuis Hoorn. The conduction of this study was approved by the Ethics Review Board of all participating centres and was performed in accordance with local laws, the European guidelines of Good Clinical Practice, and the tenets of the Declaration of Helsinki. The study was registered at ISCRTN (no° ISRCTN39068025) and clinicaltrials.gov (no° 30756.041.10).

Patients eligible for study participation were randomized using a permutated block size and were stratified for the presence of atopic diseases. The web-based randomization tool was hosted by our institutions biostatistical department (UMCU Julius Center).

Patient selection

Inclusion criteria were formulated as follows: age equal or above 18 years, keratoconus as defined and classified by presence of corneal thinning and protrusion on slit-lamp



examination and topographic criteria according to KISA% index[11,12] (>100%) and mean corneal curvature map, and a decreased best corrected visual acuity due to corneal scarring or contact lens intolerance. Exclusion criteria were prior corneal or refractive surgery, a (localized) corneal thickness < 300 µm, corneal steepness to severe for proper suction ring placement, associated corneal endothelial disease on specular microscopy as defined by, gross ophthalmic pathology surpassing keratoconus as cause of decreased visual acuity.

Primary and secondary outcomes

The event of a surgical complication necessitating conversion to a full-thickness corneal graft was considered as primary outcome parameter. Hereto, all surgical and post-operative adverse events and protocol deviations were recorded in study specific case report forms.

Secondary study objectives focussed on the effectiveness of both techniques at six months and one year follow-up: uncorrected and best spectacle corrected visual acuity (UCVA/BCVA), manifest refraction, corneal astigmatism, contact lens use (soft/rigid/scleral) or spectacle use, graft rejection and failure rate, corneal endothelial function, and correlation of outcomes with atopic constitution. Graft rejection was assessed by slit lamp examination.[13] Graft failure is related to endothelial cell dysfunction and graded concordantly as corneal endothelial disease. Atopical constitution is defined by the presence of allergic conjunctivitis at time of screening or confirmation of atopy (e.g. allergy, asthma, eczema, laboratory testing with elevated IgE levels) by patient history. All patients are routinely screened for total IgE serum levels.

Clinical protocol and used equipment

Patients were examined at baseline, and at 6 and 12 months follow up. The ophthalmic examination consisted of a brief history, use of (ocular)medication, use of visual aids (spectacles/contact lenses)and the occurrence of adverse events. UCVA and BCVA were assessed using an EDTRS visual acuity chart. Manifest refraction was taken by an optometrist or ophthalmic assistant. Slitlamp examination focussed on the presence of corneal pathology, corneal clarity, and suture related complications. Hereto, dedicated case report forms were employed. A dilated fundus exam assessed the incidence of cataract, glaucoma or macular disease.

Corneal topography and pachymetry were acquired using the Oculus Pentacam HR Type 70900, Oculus Optikgeräte GmbH, Wetzlar, Germany. Endothelial cell counts

were acquired with the Topcon Sp-3000p, Topcon Corporation, Tokyo, Japan. Intra ocular pressure was measured using the Topcon CT-80. If unattainable, the Goldmann applanation tonometer was used.

Surgical technique and donor preparation

All donor corneas were supplied by the Euro Cornea Bank Beverwijk, conform EEBA medical standards.[14]

Group A - Partial endothelial trepanation in addition to anterior lamellar keratoplasty (PET):

After the patient is sedated the lamellar graft is prepared. The donor cornea is mounted on an artificial anterior chamber (ALTK, Moria S.A., Antony, France) with the epithelium up and an anterior corneal lamella is cut with a 350µm microkeratome head and a hand-driven microkeratome (CBm, Moria S.A., Antony, France). Then the anterior corneal lamella of the recipient is prepared by applying the suction ring to the eye of the patient and the intraocular pressure is increased to >65 mm Hg. Balanced salt solution (BSS, ALCON, Fort Worth, Texas, USA) is instilled on the corneal surface and the same hand-driven microkeratome is advanced in the tract until the anterior lamella was completely severed from the underlying recipient stroma. This should be done very prudent to create an even cut and avoid the forming of buttonholes. The size of the head depends on the corneal thickness measured pre-operatively. At least 100µm should left in place. Dependent on the corneal curvature 4 different suction rings (-1, 0, +1 and +2) can be used.

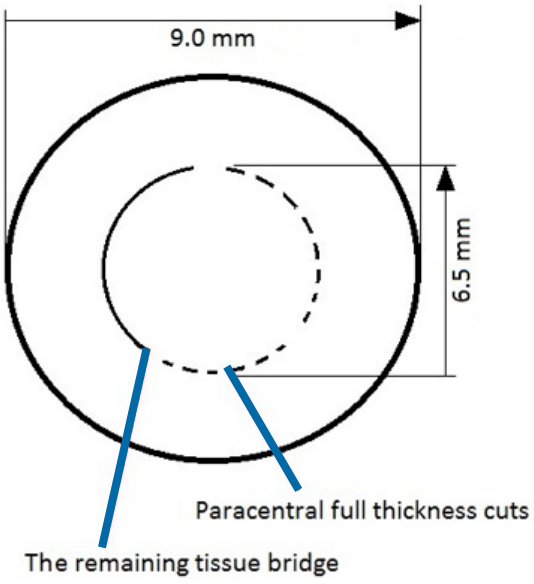


FIGURE 1. Schematic representation of the Partial Endothelial Trepanation (PET) after removal of the anterior lamella

Thereafter a partial trepanation with a 6.5 mm disposable hand

trephine is made into the remaining stroma. In this grove of the remaining tissue, including Descemet's membrane and endothelium, a cut is completed manually and oblique with a Thornton knife over 180-270°, see figure 1. This small 'island' will stay in place. The diameter of the exposed stromal bed is measured with a calliper and the diameter of the donor graft is chosen accordingly. Finally, the lamellar graft is sutured in place under tension of 16 interrupted 10-0 nylon sutures. After removal of the speculum the eye is patched. Part of the described technique is published by Busin.[10]

Group B - Conventional deep anterior lamellar keratoplasty (DALK) type big bubble technique according to Anwar and Teichmann.[7]

Statistical analysis and power analysis

Baseline measurements between the treatment groups were compared using an independent samples t-test. Fischer's exact test (two tailed) was used to determine the relation between treatment and risk of conversion to a perforating keratoplasty. Decimal visual acuity was converted to the logarithm of the minimal angle of resolution (logMAR). Normality and homoscedasticity of the residuals were tested visually, and in a Q-Q plot and scatterplot, respectively. A *P*-value <0.05 was considered statistically significant. Data are recorded as mean ± standard deviation. All tests were performed in SPSS version 22.0 for Windows.

With an expected perforation risk reduction of 85 % (current DALK ratio = 20%, 3% perforation reported by Busin et al.[10]), incorporating a sequential power calculation with a two-sided alpha 0.05 and beta 0.80, approximately 30 patients need to be included in each treatment arm.[15,16]

RESULTS

Clinical characteristics

A total of 14 eyes from 14 patients were enrolled in this trial. Two external centers participated (Radboud UMC n=2, Amphia Ziekenhuis Breda n=1).One patient postponed his surgery after randomization and was excluded from analysis. Six DALK procedures and 7 PET procedures were therefore included. Two randomized cases (one DALK, one PET) developed a corneal hydrops while on the waiting list for surgery, and following the intention-to-treat analysis both were included. Mean age was 38.3 ±12.1y and 61.5% of the patients was male. At baseline, mean logMAR UCVA and BCVA were 1.59 ±0.35 and 0.89 ±0.69 respectively. Mean refractive astigmatism was 3.4 ±2.0D, mean IOP 10 ±2.1mmHg, and mean thinnest pachymetry 322 ±66µm. Endothelial cell counts were only attainable in two cases (2110 and 2558 cells/mm^2). Topographic indices on average were a Kmax of 76.7 ±14.1D, Kflat 58.6 ±6.17D, Ksteep 64.8 ±8.5D, and an astigmatism of 4.0 ±2.6D. Baseline characteristics did not differ significantly between both groups, see table 1.

Donor characteristics

All patients received their donors from the European Cornea Bank, Beverwijk, The

	PET	DALK	*P**
Gender (% male)	86%	33%	0.06
Age	36.4 ±10.8	40.5 ±14.2	0.56
Atopy	57%	67%	0.97
UCVA (logMAR)	1.76 ±0.21	1.36 ± 0.39	0.14
BCVA (logMAR)	1.12 ±0.79	0.74 ±0.65	0.42
Manifest refraction			
Sphere (D)	-9.25 ±6.33	-4.17 ±5.41	0.21
Cylinder (D)	-2.88 ±2.22	-3.71 ±1.96	0.55
Keratometry			
Kflat (D)	58.65 ±6.25	58.45 ±6.69	0.96
Ksteep (D)	66.45 ±10.12	63.23 ±7.00	0.54
Kmax (D)	78.05 ±17.43	75.37 ±11.24	0.76
Corneal astigmatism (D)	3.32 ±1.99	4.77 ±3.08	0.36

PET: partial endothelial trepanation. DALK: deep anterior lamellar keratoplasty. UCVA: uncorrected visual acuity. BCVA: best corrected visual acuity. logMAR: log of the minimal angle of resolution. D: diopter. *independent students t-test

Table 1: Baseline characteristics of both treatment groups

Netherlands. Mean donor age was 61.3 ±9.8y and the average time between death and enucleation was 15½ hours. On average the difference between the age of the donor and patient was 21.4 ±12.2y, range 4-42 . Mean ECD was 2692 ±170 cells/mm^2, range 2400-3000. Donor characteristics did not differ significantly between both groups (data not shown).

Primary outcome

The primary study outcome was defined as the incidence of surgical adverse events necessitating conversion to a penetrating keratoplasty. Adverse events occurred in 10 of 13 surgeries. Five of 13 surgeries were converted to perforating keratoplasties (DALK:PET 3:2, P = 0.592, Fisher's exact test), including both cases with a previous corneal hydrops. Only two surgeries reported no complications at all, both PET. A wide range of protocol deviations was noted: full perforations (5), microperforations (2, both DALK), poor microkeratome cuts (3, all PET), post-operative rebubblings (3, DALK:PET 1:2), and pre-Descemetic preparations (2, both DALK).

Secondary outcomes

Secondary outcomes were assessed at 6 months and 12 months post-operatively. Overall, at 6 months mean logMAR UCVA and BCVA increased to 0.93 ±0.27 (*P*=0.02) and 0.48 ±0.27 (*P*=0.15) respectively. At 12 months this further increased to 0.52 ±0.20 (*P*= 0.003) and 0.26 ±0.36 (*P*=0.03). The following parameters are only reported at the 12 months assessment since topographic data and manifest refraction were often not attainable at the 6 months' time point. Due to the low number of cases only overall outcomes were reported; a valid comparison between both techniques was not feasible. All cornea's were clear at the final follow-up visit, and all sutures were removed. Though two PET cases had some Descemet folds (Snellen BCVA 0.45 & 0.55). One case (DALK) was suspected of an epithelial rejection and treated subsequently (Snellen BCVA 0.7). Two thirds of the patients used scleral contact lenses after their surgery. Endothelial cell densities were to often unattainable or not recorded; only three viable measurements were recorded, data not shown. Mean refractive astigmatism was 3.8 ±2.2D, with one case (DALK) of 8D astigmatism. On average the topographical indices were a Kmax of 53.0 ±2.8D, Kflat 42.4 ±5.1, Ksteep 46.5 ±3.5D, and an astigmatism of 4.9 ±3.4D. No significant differences were observed between both treatment groups for any of the secondary outcomes parameters. No long term sequelae like suture related complications, cataract, glaucoma, or ocular hypertension were noted during trial follow-up.

DISCUSSION

In general, no solid conclusions can be drawn with regards to the primary outcome based on this underpowered clinical trial. Whether the partial trepanation technique proposed by Busin is superior to the regular DALK technique in terms of surgical safety is still open for debate. On average, UCVA and BCVA improved significantly after 12 months, and visual acuity improved in all eyes. Post-operative mean refractive and topographic astigmatism were in line with other studies.[8,17] No long term sequelae from corneal surgery were recorded, though two-thirds of the patients used (scleral) contact lenses at the 12 months follow-up. Some findings of this study however deserve to be discussed.

Firstly, this trial was heavily underpowered and in analogy to Tolstoj on (un)happy families[18], many disrupting events were encountered in the course of this trial. Most notable were 1) problems in implementing this trial in clinical practice, despite local ethical approval, 2) the advent of corneal crosslinking during the course of the trial[19], and 3) the narrow indication for this type of surgery (e.g. less severe cases respond well to contact lenses, more severe cases are often scarred after a corneal hydrops and thereby poor candidates for trial participation). During the four years that the trial was open for participation only 14 eyes were included, and we considered it unrealistic that the pre-defined power of 60 inclusions could eventually be met. Finally, the scientific equipoise, where we honestly believe that both treatments are equal, could not be held; The PET technique was not as safe as expected, though a learning curve effect might interfere this finding. The combination of a low trial inclusion rate and serious doubts on study safety prompted the termination of this trial in June 2015.

Both treatments arms were confronted with a remarkable high rate of adverse events (AE), and these can be viewed from different perspectives. From a trial perspective, conversion to a perforating surgery was the most relevant AE. From an ethical/juridical perspective the AEs that require a re-operation could be considered the most severe, i.e. the detached Descemet membranes. From a patient perspective however, the AEs that negatively influence optimal visual acuity can be regarded the most burdensome, i.e. the Descemet folds that impair visual acuity on the long term. Apart from the intrinsic difficulties and long learning curve associated with lamellar surgery[9], the degree of keratoconus in this study was very severe, with an average Kmax of 76.6D and an average pachymetry of 322µm. If these two mean values are considered a compound

index of the staging of keratoconus severity, interesting comparisons can be made with other surgical studies[7,10,20], should the baseline characteristics be adequately reported. The increased availability and the clinical experience with scleral contact lenses in The Netherlands can be considered a contributing factor for this difference; with adequately fitted scleral lenses virtually all clear keratoconus corneas can achieve a good visual acuity.[21]

Another consideration is that the treatment protocol did not formally exclude scarred corneas. In clinical practice however, lamellar surgery in these cases pertains an even higher risk of Descemet perforation/rupture, and lamellar surgery after a sustained hydrops is rarely successfully completed.[22] During the course of the trial, cases with a sustained corneal hydrops were not considered suitable for trial participation. Mainly because performing a successful Descemet baring DALK becomes increasingly technically demanding, and secondly, because the scarred residual stroma in a PET procedure might preclude optimal visual recovery. Apart from abovementioned alterations, the surgical and clinical protocol remained virtually unchanged. This could be considered a strength of this study, in the light of the difficult equilibrium between trial obligations and surgical innovation. Researchers recently debated that the timeframe of a well-conducted trial spans many years[23]; years in which the investigated technique can be adjusted and improved. What then is the value of a trial if it provides evidence based medicine for yesterday's procedures? The latter is of particular relevance in corneal surgery. Busin himself recently published an improved technique for keratoconus surgery which renders the previously reported PET technique obsolete.[24]

In conclusion, a significant increase of uncorrected and corrected visual acuity was recorded for the group as a whole 12 months after corneal transplantation surgery for keratoconus. The added value of the PET over the DALK technique in terms of surgical safety cannot be deducted from these data, nor could we assess differences in the secondary outcomes (e.g. visual acuity, endothelial cell loss). However, in either treatment arm the incidence of intra-operative adverse events was higher than expected.

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4

Objective and subjective evaluation of the performance of medical contact lenses fitted using a contact lens selection algorithm.

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Submitted

ABSTRACT

Purpose

To evaluate the objective and subjective performance of medical contact lenses (CLs) fitted for a broad range of clinical indications using a lens selection algorithm.

Design

Prospective observational study.

Subjects

A total of 281 eyes were evaluated from 281 patients who visited the contact lens service at a tertiary academic clinic (University Medical Center Utrecht, the Netherlands) in the period from August 2014 through October 2014.

Methods

We obtained each patient's medical history, CL history, and visual acuity; in addition, patients completed a questionnaire.

Main outcome measures

Clinical indications for CL wear; CL type; change in corrected distance visual acuity (CDVA) with CL use; CL wearing duration; CL wearing time; subjective measurements on a visual analog scale (VAS) questionnaire (score range: 0-100); and the effectiveness of the lens selection algorithm.

Results

The most common indications were keratoconus (25%), dry eye disease (23%), and keratoplasty (20%); the most common CL types were scleral lenses (53%) and soft lenses (either conventional soft lenses or silicone hydrogel lenses; 35%). The use of CLs significantly improved CDVA compared to the use of spectacles (the median change was -0.15 logarithm of the minimal angle of resolution (logMAR) (range: 1.00 to -2.10; $P<0.001$)). Daily-wear CLs were worn by 77% of patients for a median of 15 hours/day (range: 5-18 hours), 7 days/week (range: 1-7 days); the remaining 33% of patients wore their lenses continuously. With respect to the questionnaire, the patients generally reported high scores for comfort, visual quality, lens handling, and overall satisfaction, with similar results between the scleral lens and soft lens groups. The lens selection algorithm was found to be generally effective, as indicated by an overall satisfaction

rating ≥70 in 81% of patients.

Conclusions

CLs fitted using the lens selection algorithm yield satisfactory clinical results, including improved visual acuity, satisfactory wearing time, and satisfactory overall subjective performance. Moreover, subjective performance was similar between scleral lens users and soft lens users. This study underscores the importance of using scleral lenses and the need for offering a variety of CL types in tertiary eye clinics.

INTRODUCTION

To treat a wide range of ocular diseases, modern-day eye-care practitioners have a growing arsenal of medical contact lenses (CLs). The primary optical indication for fitting a patient with medical CLs is to improve visual acuity in cases of high refractive error and/or irregular astigmatism;[1] less common indications include anisometropia, nystagmus, and occlusion.[2] In a clinical setting, another important indication for CL use is for therapeutic purposes (e.g., in the case of a corneal bandage, in which the cornea is physically protected from the environment in order to improve hydration, promote corneal healing, and relieve pain).[3-10] Often, several effects are desired.[4,6] All of these applications have specific requirements with respect to the lenses' design and material. A wide variety of CL types are currently available, including conventional soft lenses, silicone hydrogel lenses, rigid gas-permeable (RGP) corneal lenses, scleral lenses, hybrid lenses, occlusive lenses, iris print lenses, filter lenses, piggyback systems, and scleral prosthetics. Tailoring a CL to adequately fit the patient's needs requires a trained eye-care practitioner.

Clinical applications for CLs have expanded due to improvements in the materials used (for example, more permeable lens materials)[3] and recent innovations in lens design, including custom-made specialized lenses,[11,12] and toric- and tangential scleral lens designs.[13-15] In turn, these developments have altered the prescription habits of eye-care practitioners. For example, the improved material properties of silicone hydrogels has led to a major shift from conventional soft lenses to silicone hydrogel lenses.[5,8] More interestingly, the increased availability of custom-designed contact lenses for patients with keratoconus or keratoplasty[11,16-20] has been accompanied by a large increase in the use of scleral lenses.[21-23]

Scleral lenses play an important role in medical CL practice, particularly in cases in which other lens designs have suboptimal results, for example in the case of unstable lens fitting, poor tolerance, unsatisfactory visual improvement, and/or corneal bandage. However, the ability to fit scleral lenses requires specific skills and training. Another factor that has hampered the popularity of scleral lenses is prejudice with respect to poor handling of scleral lenses and a lack of comfort for the user. Recently, Van der Worp et al.[21] and Schornack[22] reviewed the outcomes of studies using scleral lenses, and several studies have evaluated the fitting of medical CLs in specific settings.[1,3,5,7,19,24] However, no overarching, evidence-based method for fitting the

optimal CL type in more challenging clinical cases is currently available. In addition, the patients' subjective experiences based on these various treatment strategies also warrant attention.

Our goal was to evaluate the experiences of CL practitioners and patients in a large, tertiary clinic. Thus, we prospectively evaluated the effectiveness of a practical lens selection algorithm, and we examined the clinical outcomes and patient satisfaction in response to the strategies chosen. Importantly, the comprehensive lens selection algorithm enables practitioners to achieve desirable results.

METHODS

In this prospective observational study, we included all consecutive patients who visited the Contact Lens service (Visser Contact Lens Practice) at the University Medical Center Utrecht from August 2014 through October 2014 for a follow-up for a medically indicated CL. The inclusion criteria were ≥18 years of age and CL use for ≥3 months prior to enrollment. The exclusion criteria were patients who came for an emergency visit or patients who were unable or unwilling to participate. Our institution's Ethics Review Board ruled that approval was not required for this study; however, all participating patients provided written informed consent. All procedures were performed in accordance with the Declaration of Helsinki and with local laws regarding research on human subjects.

During the study visit, the primary and secondary clinical indication for CL use, CL type, and CL history were recorded; in addition, the following data were obtained from the patients' medical history: the presence of allergies and/or eczema, the use of topical eye drops (e.g., lubricants, prophylactic antibiotics, steroids, glaucoma eye drops, anti-allergy eye drops, or other eye drops), and average CL wearing time. Best corrected distance visual acuity (CDVA) was measured as Snellen visual acuity both with (CL CDVA) and without (spectacle CDVA) CLs.

All patients were also instructed to complete a questionnaire covering the following four specific topics: lens comfort, visual quality, lens handling, and overall satisfaction with their lenses. Scores were obtained on a visual analog scale (VAS); the scores ranged from 0 (unacceptable performance) to 100 (excellent performance). This questionnaire was used in our previous studies, and approval for using it here was granted by the Research and Ethics Committee of the City University, London, United Kingdom.[25,26] Patients with a visual acuity score of <1/300 (i.e., <distinguish hand motion) did not complete the questions regarding visual quality; CVDA was also not evaluated in these patients. Patients with continuous-wear bandage lenses were omitted from the lens handling section of the questionnaire, as their lenses were replaced by our contact lens service; lens wearing time was also not determined in these patients.

Patients with continuous-wear CLs visited the practice every 4-6 weeks to either replace or clean their lenses, and they were prescribed prophylactic antibiotic eye drops (chloramphenicol 0.5%, minims BID; Bausch & Lomb). All other patients were

monitored at an interval that met their specific clinical needs.

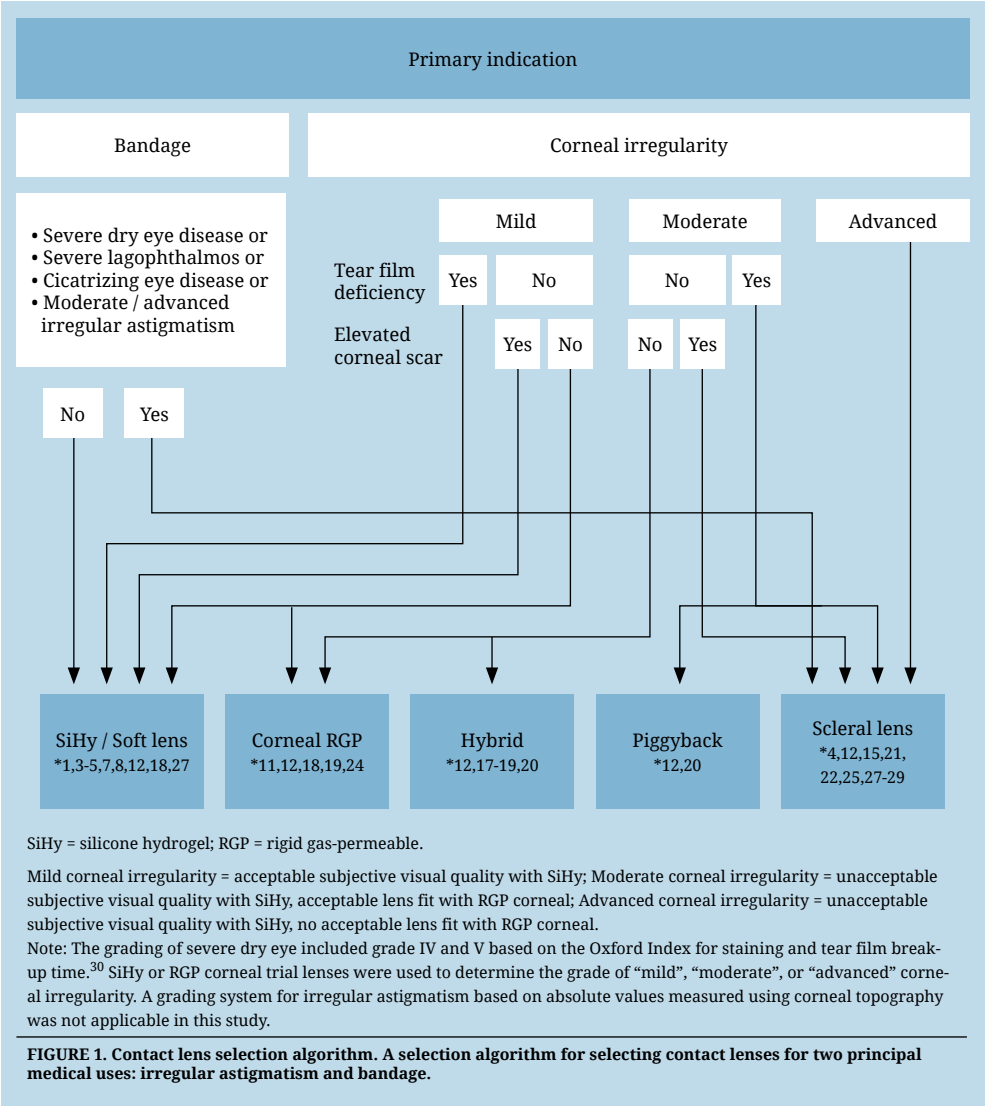
Contact lens selection

The selection of a specific CL type was based on the severity of the disorder and the presence of additional indications and/or other complicating factors.

Our CL selection algorithm was developed for two principal uses for medical CLs: irregular astigmatism and bandage (Figure 1). The grading of severe dry eye included grade IV and V based on the Oxford Index for staining and tear film break-up time.[30] A grade of mild, moderate, or advanced corneal irregularity was determined based on CL performance and acceptable visual quality: SiHy or RGP corneal trial lenses, which were fitted in accordance with the manufacturer's guidelines, were used to assess the effects of corneal irregularity. The grade "mild" refers to acceptable subjective visual quality with a SiHy lens; the grade "moderate" refers to unacceptable subjective visual quality with a SiHy lens and an acceptable lens fit with a RGP corneal lens; and the grade "advanced" refers to unacceptable subjective visual quality with a SiHy lens and an unacceptable lens fit with a RGP corneal lens. A grading system for irregular astigmatism (based on absolute values measured using corneal topography) was not applicable in this study, as the actual location of the corneal irregularity or cone (i.e., central or peripheral) can have a significant influence on CL fitting. For example, an advanced centrally located keratoconus might benefit from a RGP corneal lens, whereas a less advanced inferiorly located protrusion might impede the fitting of an RGP corneal lens, thus requiring a scleral lens.

Our approach to select the appropriate type of soft lens (including conventional soft lenses or silicone hydrogel lenses) is summarized in Figure 2. Indications beyond this scope (e.g., occlusion lenses, filter lenses, or cosmetic lenses) were not included in the lens selection algorithm, as these types of lenses are directly related to their specific indications. Medical refractive indications, including high refractive error (i.e., refractive error that exceeded +/-10 diopters [D]), aphakia, and anisometropia, were tailored to the individual patient's needs. The best-fitting CL material and design was prescribed to each individual patient based on the practitioner's judgment using trial lenses.

A detailed description of the scleral lens fitting protocol has been described previously.[13,15,25] In brief, fitting was based on the landing of the scleral lens on the sclera and vaulting of the lens over the cornea and limbus. Ideal scleral lens fitting has



SiHy = silicone hydrogel; RGP = rigid gas-permeable.

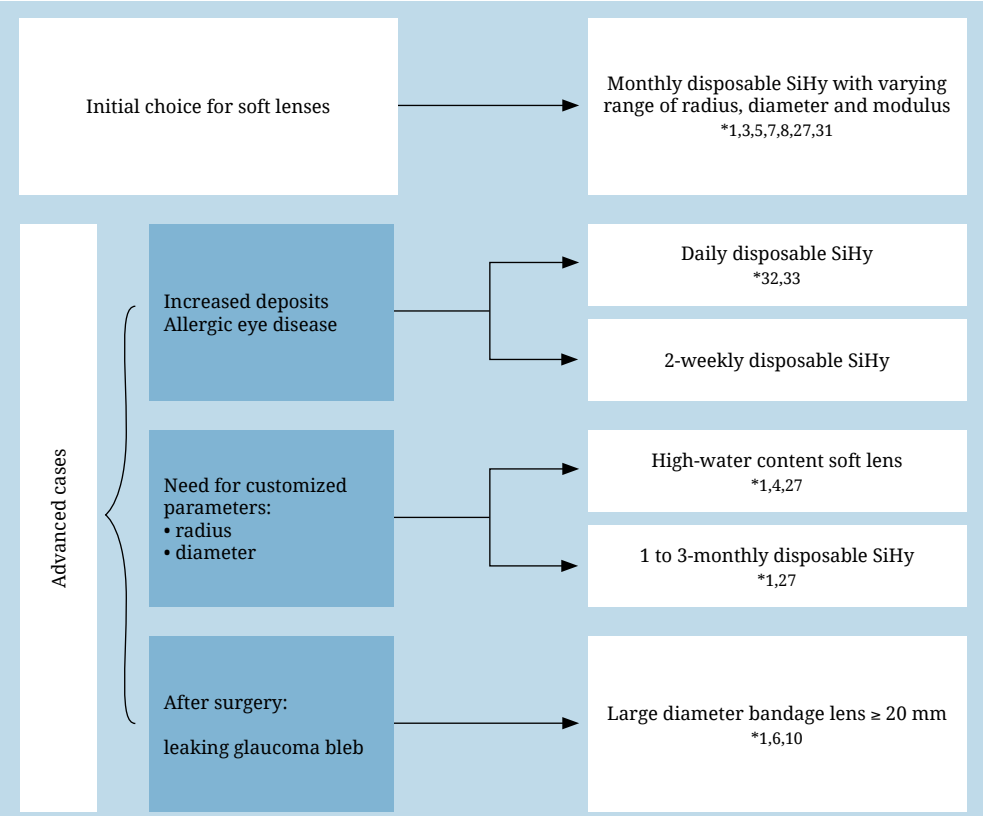
Mild corneal irregularity = acceptable subjective visual quality with SiHy; Moderate corneal irregularity = unacceptable subjective visual quality with SiHy, acceptable lens fit with RGP corneal; Advanced corneal irregularity = unacceptable subjective visual quality with SiHy, no acceptable lens fit with RGP corneal.
Note: The grading of severe dry eye included grade IV and V based on the Oxford Index for staining and tear film break-up time.[30] SiHy or RGP corneal trial lenses were used to determine the grade of "mild", "moderate", or "advanced" corneal irregularity. A grading system for irregular astigmatism based on absolute values measured using corneal topography was not applicable in this study.

FIGURE 1. Contact lens selection algorithm. A selection algorithm for selecting contact lenses for two principal medical uses: irregular astigmatism and bandage.

a well-balanced haptic bearing, gentle movement of the lens with the push-up test, and adequate corneal and limbal clearance. All other lenses were fitted in accordance with the applicable manufacturers' protocols.

Statistics

One eye in each subject was selected at random using an autonomous software tool (nQuery Advisor, version 7.0, Statistical Solutions, Cork, Ireland). All Snellen visual acuity values were converted to logarithm of the minimal angle of resolution (logMAR) values for statistical calculations.



SiHy = silicone hydrogel; RGP = rigid gas-permeable.

FIGURE 2. Initial and advanced selection of soft lenses for medical use. Flowchart for the initial and advanced selection of soft lenses (conventional soft or SiHy lenses) for medical use.

All variables were tested for normal distribution using the Kolmogorov-Smirnov test. The only variable that was found to be distributed normally was patient age. For non-normally distributed paired data, the Wilcoxon signed rank test was used. Differences between groups were analyzed using the non-parametric Kruskal-Wallis test (for continuous outcomes), the Fisher's exact test (for categorical outcomes), or ANOVA (age). With the exception of patient age (which is reported as the mean and standard deviation), all summary data are reported as the median and range. Subgroup analyses were performed on the following stratified data: primary clinical indication (keratoconus, dry eye disease, or post-keratoplasty) and primary CL type (scleral lens or soft lens). Differences with a *P*-value <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY,US).

RESULTS

This study included 281 eyes from 281 patients; 160 patients were female (57%), and 142 eyes were right eyes (51%). The mean age of the patient cohort was 55 ± 17 years (range: 18 to 93 years). Slightly more than half of the patients (n=158) wore CLs in both eyes, whereas 63 and 60 patients wore a single lens in the right or left eye, respectively. Thirty-four percent of patients presented with some form of allergy, and 15% had eczema. Sixty-one percent of patients used topical eye drops; among the patients who used eye drops, 47% used a lubricant, 24% used prophylactic antibiotics, 15% used steroids, 7% used glaucoma eye drops, 5% used anti-allergy eye drops, and 2% did not specify the type of eye drops used.

Clinical indications

The three most common clinical indications in our study cohort were keratoconus (in 25% of cases), dry eye disease (23%), and keratoplasty (20%). The primary clinical indications and the CLs applied are summarized in Table 1. The results of these three main indication groups were further analyzed, and the demographic data are summarized in Table 2.

In total, 26 of the 281 eyes (9%) had a secondary clinical indication for CL fitting; these indications included dry eye disease (n=5), aniridia (n=4), decompensated cornea (n=3), corneal scarring after trauma (n=3), anisometropia (n=2), aphakia (n=2), high refractive error (exceeding +/-10 D; n=1), corneal scarring after infection (n=1), keratoplasty (n=1), corneal dystrophy (n=1), recurrent erosions (n=1), trichiasis (n=1), and white pupil secondary to cataract (n=1).

All corneal transplants, with the exception of one anterior lamellar keratoplasty, were perforating grafting procedures. Indications for transplant surgery included keratoconus (n=24), Fuchs endothelial dystrophy (n=18, all of which were performed in the pre-endothelial keratoplasty era), post-infectious keratitis scar (n=8), cornea decompensation (n=4), and unspecified corneal dystrophy (n=1).

The most common primary clinical reasons for applying CLs were to improve visual acuity (in 63% of cases) and as a bandage (34%). A small number of patients were fitted with CLs for cosmetic purposes (n=4), occlusion (n=3), or for improved contrast vision (n=1).

Contact lens types

The types of CLs used by the study cohort are summarized in Table 1. The most commonly

Indication	N (%)	Contact lens type						
		Scleral	Soft	RGP	Occlusive	Iris	Filter	Othera
Keratoconus	71 (25)	60	4	6	0	0	0	1
Dry eye disease	66 (23)	14	52	0	0	0	0	0
Keratitis sicca	60	10	50	-	-	-	-	-
Keratitis lagophthalmos	6	4	2	-	-	-	-	-
Keratoplasty	55 (20)	51	1	2	0	0	0	1
Corneal scar	25 (9)	17	2	4	1	0	0	1
After herpes simplex keratitis	9	6	2	1	-	-	-	-
After other infectious keratitis	13	9	-	3	1	-	-	-
After trauma	3	2	-	-	-	-	-	1
Refractive	19 (7)	3	11	2	0	1	2	0
High refractive error >+/-10 D	9	3	4	1	-	1	-	-
Aphakia	6	-	4	-	-	-	2	-
Anisometropia	4	-	3	1	-	-		-
Cornea decompensation	17 (6)	0	14	0	0	1	2	0
Corneal erosions	12 (4)	0	12	0	0	0	0	0
Other irregular astigmatism	5 (2)	3	0	2	0	0	0	0
After surgery (other than keratoplasty)	4	3	-	1	-	-	-	-
Unknown cause	1	-	-	1	-	-	-	-
Miscellaneous indications	11 (4)	0	3	1	4	2	0	1
Binocular diplopia	3	-	-	-	3	-	-	-
Trichiasis	2	-	2	-	-	-	-	-
Aniridia	1	-	-	-	-	1	-	-
Entropion	1	-	1	-	-	-	-	-
Bulbus atrophy	1	-	-	-	-	-	-	1
Iris atrophy	1	-	-	-	-	1	-	-
Nystagmus	1	-	-	1	-	-	-	-
White pupil	1	-	-	-	1	-	-	-
Total no. of eyes, n (%)	281 (100)	148 (53)	99 (35)	17 (6)	5 (2)	4 (1)	4 (1)	4 (1)

D = Diopter; RGP = rigid gas-permeable. a Other = a piggyback system for keratoconus (n=1), a hybrid lens for keratoplasty (n=1), a tinted soft keratoconus lens for a corneal scar after trauma (n=1), and a prosthetic scleral lens for bulbous atrophy (n=1).

Table 1. Clinical indications and contact lens type.

Indication group	No. of eyes	Mean age (range)	Gender (% male)	Allergy (%)	Eczema (%)
Keratoconus	71	47 (21-74)	47	33 (46)	19 (27)
Dry eye disease	66	59 (20-87)	24	20 (30)	10 (15)
Keratoplasty	55	63 (27-90)	51	19 (35)	5 (9)
Variation between groups		<0.001[a]	0.004[b]	0.13[b]	0.03[b]

a: Analysis of variance (ANOVA) test. b: Fisher's exact test.

Table 2. Main groups of clinical indications: general data.

Indication / lens type	VA >1 / 300 (%)	Spectacle CDVA	Contact lens CDVA	CDVA difference	*P*-value*
Total group	263 (94)				
LogMAR		0.30 (2.52 – -0.10)	0.10 (2.52 – -0.20)	-0.15 (1.00 – -2.10)	<0.001
Snellen equivalent		20/40	20/25	N/A	N/A
Keratoconus	71 (100)				
LogMAR		0.40 (2.52 – -0.10)	0.10 (1.00 – -0.10)	-0.30 (0.12 – -1.70)	<0.001
Snellen equivalent		20/50	20/25	N/A	N/A
Dry eye disease	64 (97)				
LogMAR		0.10 (1.30 – -0.10)	0.07 (0.80 – -0.20)	0.00 (0.14 – -1.13)	=0.007
Snellen equivalent		20/25	20/24	N/A	N/A
Keratoplasty	55 (100)				
LogMAR		0.42 (2.52 – 0.00)	0.05 (2.22 – -0.10)	-0.32 (0.15 – -2.10)	<0.001
Snellen equivalent		20/53	20/22	N/A	N/A
Scleral lenses	148 (100)				
LogMAR		0.40 (2.52 – -0.10)	0.05 (1.30 – -0.20)	-0.30 (0.15 – -2.10)	<0.001
Snellen equivalent		20/50	20/22	N/A	N/A
Soft lenses	88 (89)				
LogMAR		0.19 (2.52 – -0.10)	0.12 (2.52 – -0.10)	0.00 (0.14 – -1.40)	=0.032
Snellen equivalent		20/31	20/27	N/A	N/A

VA = visual acuity; CDVA = corrected distance visual acuity; LogMAR = logarithm of the minimal angle of resolution; CDVA outcomes are presented as median (range); N/A = not applicable. * Wilcoxon signed ranks test.

Table 3. Spectacle and contact lens CDVA.

Indication / lens type	Daily wear (%)	Wearing time per day (range)	wearing time per week (range)
Total group	216 (77)	15 (5-18)	7 (1-7)
Keratoconus	71 (100)	15 (5-18)	7 (4-7)
Dry eye disease	29 (44)	16 (6-16)	7 (2-7)
Keratoplasty	54 (98)	15 (6-18)	7 (2-7)
Scleral	148 (100)	15 (5-18)	7 (2-7)
Soft	34 (34)	16 (7-17)	7 (4-7)

Table 4. Wearing time per day and per week.

used CLs were scleral lenses (in 53% of cases) and soft lenses (either conventional soft lenses or silicone hydrogel lenses; 35%); the results of these two groups were analyzed further.

The scleral lens group contained patients who used mini-scleral lenses (15-18 mm in diameter; n=20 patients) or regular scleral lenses (18-22 mm in diameter, n=128 patients).

The most popular soft lenses were monthly disposable silicone hydrogels (n=65); the remaining soft lenses were 3-month disposable silicone hydrogels (n=13), daily disposable silicone hydrogels (n=7), daily disposable soft lenses (n=4), large-diameter soft lenses (n=4), 2-week disposable silicone hydrogels (n=2), 3-month disposable soft lenses (n=2), monthly disposable soft lenses (n=1), and aphakia soft lenses (n=1).

The RGP corneal lens designs included a standard corneal design (n=8), a keratoconus design (n=6), and a keratoplasty design (n=3).

Visual acuity outcomes

There was a significant improvement in median logMAR CL CDVA (-0.15; range: 1.00 to -2.10) compared to the median logMAR spectacle CDVA (P<0.001). The visual outcomes for the total cohort, the major clinical indication subgroups, and the lens subgroups are summarized in Table 3. CDVA improvement by CL wear differed significantly between the major indication groups (P<0.001, Kruskal-Wallis test); specifically, CL CDVA improved significantly more in the patients with keratoconus and keratoplasty compared with the patients with dry eye disease. Furthermore, users of scleral lenses had significantly more CDVA improvement than users of soft lenses (P<0.001, Kruskal-Wallis test).

Eighteen of the 281 eyes (6%) had visual acuity that was <1/300 (i.e., <distinguish hand motion).

Indication / lens type	n (%)	Comfort	Visual Quality	Lens Handling	Overall Satisfaction
Total group	281 (100)	84 (14-100)	N/A	N/A	85 (7-100)
Eyes CDVA >1/300[b]	259 (92)	N/A	76 (4-100)	N/A	N/A
Eyes daily wear	216 (77)	N/A	N/A	86 (15-100)	N/A
Keratoconus	71 (100)	85 (24-97)	N/A	N/A	86 (34-98)
Eyes CDVA >1/300	71 (100)	N/A	74 (27-97)	N/A	N/A
Eyes daily wear	71 (100)	N/A	N/A	94 (34-79)	N/A
Dry eye disease	66 (100)	78 (14-100)	N/A	N/A	85 (28-100)
Eyes CDVA >1/300	64 (97)	N/A	75 (15-100)	N/A	N/A
Eyes daily wear	29 (44)	N/A	N/A	85 (15-100)	N/A
Keratoplasty	55 (100)	84 (14-97)	N/A	N/A	85 (15-97)
Eyes CDVA >1/300	55 (100)	N/A	84 (14-96)	N/A	N/A
Eyes daily wear	54 (98)	N/A	N/A	85 (44-96)	N/A
Scleral lenses	148 (100)	84 (14-100)	N/A	N/A	85 (15-100)
Eyes CDVA >1/300	148 (100)	N/A	77.5 (14-100)	N/A	N/A
Eyes daily wear	148 (100)	N/A	N/A	86 (15-100)	N/A
Soft lenses	99 (100)	84 (14-97)	0	0	85 (26-98)
Eyes CDVA >1/300[c]	85 (86)	N/A	75 (4-97)	N/A	N/A
Eyes daily wear	34 (34)	N/A	N/A	91 (55-97)	N/A

CDVA = corrected distance visual acuity; VAS = visual analogue scale, scores 0-100; VAS outcomes are presented as the median (range); N/A = not applicable. b 4 patients didn't complete this question. c 3 patients didn't complete this question.

Table 5. Subjective outcomes measured using a VAS questionnaire

Wearing time and duration of CL use

Daily-wear contact lenses were worn by 77% of patients, with a median of 15 hours per day (range: 5 to 18 hours) and a median of 7 days per week (range: 1 to 7 days). The remaining 23% of patients wore their lenses continuously. The wearing time data in the clinical indication and lens type subgroups are summarized in Table 4.

In our cohort, 96% of patients wore their CLs ≥8 hours per day. Among the patients who wore their CLs <8 hours per day, 5 used scleral lenses, 2 used occlusive lenses, 1 used a soft lens, 1 used a tinted soft keratoconus lens, 1 used a filter lens, and 1 used a prosthetic scleral lens.

The median duration of wearing the current CL type was 6 years (range: 3 months to 39 years), and median CL wear duration in general was 11 years (range: 4 months to 53 years). Fifty-eight percent of patients had used a different CL type prior to the study.

Subjective performance

Median VAS outcome for the entire cohort was 84 for the topic of comfort (range: 14 to 100), 76 for visual quality (range: 4 to 100), 86 for lens handling (range: 15 to 100), and 85 for overall satisfaction (range: 7 to 100). The outcome of the patient questionnaire for all patient subgroups is summarized in Table 5.

The three clinical indication groups did not differ significantly with respect to comfort (P=0.16), visual quality (P=0.14), lens handling (P=0.15), or overall satisfaction (P= 0.43; Kruskal-Wallis test).

Scleral lens users did not differ significantly from soft lens users with respect to comfort (P=0.29), lens handling (P=0.21), or overall satisfaction (P=0.21, Kruskal-Wallis test). However, with respect to subjective visual quality, scleral lens users differed significantly from soft lens users (median VAS scores were 77.5 and 75, respectively; P=0.009, Kruskal-Wallis test).

Five percent of patients scored <50 in the comfort topic; 3 used scleral lenses, 6 used soft lenses, 2 used corneal lenses, 2 used iris lenses, and 1 used a filter lens. Fifteen percent of patients scored <50 for visual quality; 14 used scleral lenses, 18 used soft lenses, 2 used filter lenses, 2 used iris lenses, 2 used corneal lenses, and 1 used a tinted soft keratoconus lens. Five percent of patients scored <50 in for lens handling; 9 used scleral lenses, and 1 used a tinted soft keratoconus lens. Lastly, 5% of patients scored <50 for overall satisfaction; 5 used scleral lenses, 6 used soft lenses, and 2 used iris lenses.

Effectiveness of the lens selection algorithm

We defined good performance of the lens selection algorithm as an overall satisfaction VAS score ≥70 (out of 100); this criterion was achieved in 81% of patients. Moreover, 90% of patients reported an overall satisfaction score ≥60. Importantly, 33% of patients reported an overall satisfaction score ≥90.

DISCUSSION

The primary goal of this study was to evaluate the objective and subjective performance of various contact lens types that were fitted based on a lens selection algorithm and were used for a broad range of clinical indications. Our results show that similar outcome can be achieved with both soft lenses and scleral lenses when applying this algorithm. Importantly, subjective comfort, handling, and overall satisfaction were similar between scleral lens users and soft lens users. In addition to underscoring the clinical value of scleral lenses, our results also highlight the need for practitioners to be familiar with a wide range of lens types and tailored lens selection.

A large number of studies have been published recently regarding the indications for—and the application of—medical CLs. In our study, the most common indications were keratoconus, dry eye disease, and keratoplasty; moreover, the most commonly used lens types were scleral lenses and soft lenses (including conventional soft lenses or silicone hydrogel lenses). The objective performance of scleral lenses in our study cohort is consistent with previous reports by our group[15,25,26] and others.[21,22] Specifically, we observed high outcome with respect to median visual acuity. The improvement in CL CDVA compared to spectacle CDVA was the most pronounced in the patient subgroups with optical indications (i.e., the keratoconus and keratoplasty subgroups). This finding supports the putative optical benefit of CLs and is consistent with other studies that report on the use of lenses (including scleral lenses) for medical indications with irregular astigmatism.[11,21,22] With respect to therapeutic lenses, CL CDVA improved as well, even though the primary objective of the lenses was to protect or promote healing of the compromised cornea.[6] The optical advantage of lenses (including scleral lenses) in dry eye disease due to compensation of optical disturbances that arise from tear instability, punctate epithelial erosions, and/or corneal scars have been described previously.[28,34,35] Thus, scleral lenses may be preferred when soft lenses fail, and scleral lenses may even surpass soft lenses in terms of hydrating the cornea, protecting the cornea, and/or correcting an irregular corneal surface.[28,29]

Subjective lens performance has also been reported previously. Interestingly, although scleral lenses are often considered to be cumbersome to handle, our study cohort reported remarkably high overall satisfaction, regardless of lens type. Studies of CL performance in which different lens types were evaluated simultaneously in a clinical setting and with various indications have not been reported previously. This paucity

of comprehensive studies prevents a comparison of either objective or subjective outcomes, as study design, patient selection, and the types of lenses vary widely. Moreover, the indications for CLs are continuously changing due to developments in ophthalmology.[1] Thus, our study is the first to provide an overarching perspective, and our lens fitting algorithm can support the practitioner in selecting the most appropriate lens type.

Our study has several notable strengths. First, the CL practitioners in this study participate in continuing education, with an emphasis on the specific skills needed to advise patients in a tertiary academic clinical setting. Thus, our standardized protocols for lens selection, lens fitting, and patient instruction are the result of many years of experience with a wide range of CLs. Furthermore, all of the major steps and decisions in the lens selection algorithm are based on peer-reviewed literature. In addition, it is important to fit CLs individually when applying bandage CLs to complicated eyes,[10,27] which is reflected in our flow chart for soft lenses and silicone hydrogel lenses. Thus, the appropriate material, parameters,[10] modulus,[31] and replacement strategy are all essential for achieving an optimal lens fit. Importantly, our contact lens service is not affiliated with any CL manufacturer, and health insurance companies reimburse patients for CLs prescribed due to medical indications. Therefore, lens selection was not guided by any factors other than the individual patients' needs and preferences. Another strength of this study was our random selection of unilateral eyes; this step was important, given the high degree of correlation between eyes with respect to lens performance. Lastly, subjective performance was analyzed solely in the eye under study, thus further avoiding any possible undue effects due to the performance of the other eye.

This study also had some considerations that merit mention, the most important of which is patient selection. Our contact lens service is in a tertiary academic center, and this may have resulted in a disproportionate selection of more severe clinical indications. Because of its excellent cornea unit, our ophthalmology department has a relatively large population of patients with severe dry eye and—at the other end of the clinical spectrum—a relatively large proportion of post-graft and keratoconus patients. Thus, our clinic is an interregional referral center for patients with keratoconus, and the most severe cases are referred to our contact lens service for evaluation and—if needed—revision of their current CLs. The stage of the disease limited the available lens types to more advanced solutions; thus, a relatively higher proportion of scleral lenses were prescribed, whereas other lens types (for example, RGP corneal lenses)

were underreported. Wu et al.[24] illustrated this phenomenon by reporting that RGP corneal lenses do not ensure improved quality of life for patients with severe keratoconus; thus, Wu et al. stressed the importance of prescribing the appropriate CL type for each grade of keratoconus. Moreover, patients may require refitting as their disease stages change,[6] and the optimal CL type for an irregular cornea should not be determined solely by the degree of irregularity. Secondary features such as tear film deficiency and elevated corneal scars can also play an important role, as summarized in our lens selection algorithm.

Interestingly, we found that 58% of patients previously wore different lenses, and the new lens type yielded a high level of overall satisfaction. This result suggests that the majority of patients wore lenses that were not optimally fitting prior to changing their lens type. Expanding this prospective study to include a more general population will likely reveal important information regarding various CL types in patients in earlier stages of disease.

A limitation of our study was the fact that the cross-sectional observational design did not allow us to study complications associated with the lenses. Thus, we were unable to evaluate the safety, durability, or refractive stability of the lenses. Interestingly, however, four of the 281 patients in our study cohort needed (relatively minor) revision in their lenses (all four of which were scleral lenses); these revisions were based on either suboptimal fitting or altered corneal refraction. This finding is consistent with our previous finding that updating scleral lenses with relatively minor changes every 1.5-2 years is common practice and is recommended in order to ensure the lens material's quality and oxygen permeability.[26] A detailed analysis of these four cases did not provide additional insight (data not shown). In their recent review of scleral lenses, Van der Worp et al. [21] concluded that adverse events are rare in these modalities. In addition, other studies found that the therapeutic use of CLs does not appear to affect the incidence of CL-related complications.[3-6,9] The availability of silicone hydrogel materials with high oxygen permeability has opened new opportunities for patients with hypoxia-related corneal complications. Indeed, several studies reported that silicone hydrogels are both safe and efficacious when worn continuously for therapeutic purposes.[3,7,8] Nevertheless, it is obvious that the wearing of CLs involves some risk, and care should be exercised when fitting a compromised eye. Patients must be educated regarding proper lens care and to identify signs of potential complications before they begin using medical CLs.

The high subjective performance of all CL types was reflected by the fact that patients reported wearing their CLs many hours per day and many days per week; likewise, the VAS scores were relatively high with respect to comfort, visual quality, lens handling, and overall satisfaction. Thus, the lens selection algorithm was found to be effective in terms of subjective overall satisfaction. On the other hand, relatively low subjective performance was reported by a small group of patients, which was expressed by lower VAS scores (i.e., <50) and shorter daily use (<8 hours per day). The lack of longitudinal follow-up in these lower-performing patients precludes our ability to draw any conclusions regarding whether the lower scores are related to CL performance and/or the underlying disease. In general, good wearing time results[21,22,23,35] and good general subjective outcomes have been reported among patients who use scleral lenses,[15,25] although poor outcome has been reported for some patients.[21] Wu et al. [24] reported good vision-related quality of life among patients with a non-severe stage of keratoconus who used appropriate corneal CLs. Interestingly, the results of the Collaborative Longitudinal Evaluation of Keratoconus (CLEK) studies[36,37] support this finding, although the CLEK study found slightly more ocular discomfort among RGP corneal lens wearers,[36] and patients with keratoconus generally grow increasingly less tolerant to wearing rigid contact lenses.[37] Lastly, Erdurmus et al. [18] reported that patients with keratoconus experience similar CL impact on quality of life, regardless of whether they use RGP corneal lenses, hybrid lenses, or soft toric CLs.

With respect to subjective performance and lens handling, scleral lenses were similar to soft lenses when applying the lens-selection algorithm. This finding is somewhat remarkable, given the initial psychological resistance that patients often express in response to scleral lenses. Nevertheless, other studies have reported similar patient satisfaction results among patients who use scleral lenses.[14,15,29]

In conclusion, we comprehensively evaluated the objective and subjective performance of a broad range of contact lens types used for a variety of clinical indications. Our results revealed that high outcome can be achieved when applying the lens-selection algorithm in terms of visual acuity and overall patient satisfaction. Our results also underscore the role of scleral lenses in modern contact lens practice, and they emphasize the need for the availability of several CL types in order to fit the CL to each patient's needs and preferences. Thus, our lens selection algorithm is effective and can help practitioners select the appropriate CL type.

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5

Transepithelial versus epithelium-off corneal crosslinking for the treatment of progressive keratoconus: a randomized controlled trial.

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ABSTRACT

Purpose

To compare the clinical effects and safety of transepithelial corneal crosslinking (CXL) to epithelium-off (epi-off) CXL in progressive keratoconus.

Design

Randomized controlled trial

Methods

Patients received either transepithelial CXL with Ricrolin TE (n=35) or epi-off CXL with isotonic riboflavin (n=26) in 1 academic treatment center, using a simple unrestricted randomization procedure. The main outcome measure was clinical stabilisation of keratoconus after 1 year, defined as a maximal keratometry (Kmax) increase < 1 diopter (D).

Results

Average Kmax was stable at all visits in the transepithelial group, while after epi-off CXL a significant flattening of 1.2 to 1.5 D was demonstrated from the 3 months follow-up onwards. The trend over time in Kmax flattening was significantly different between the groups (*P*=0.022). Eight eyes (23%) in the transepithelial group showed a Kmax increase of > 1 D after 1 year (range 1.3 to 5.4 D) versus none in the epi-off group (*P*=0.017). There was significant different trend in corrected distance visual acuity (CDVA), with a more favorable outcome in the transepithelial group (*P*=0.023). In the transepithelial group, no complications occurred and in the epi-off group, 4 eyes (15%) developed complications due to healing problems (sterile infiltrate, herpes keratitis, central haze and stromal scar).

Conclusion

This study showed that although transepithelial CXL was a safe procedure without epithelial healing problems, 23% of cases showed a continued keratoconus progression after 1 year. Therefore, at this time, we do not recommend replacing epi-off CXL by transepithelial CXL for treatment of progressive keratoconus.

INTRODUCTION

Progressive keratoconic corneas can be stabilized and strengthened by corneal crosslinking (CXL).[1] The standard technique of CXL was first applied in 1998 and consists of an epithelial removal, after which riboflavin eye drops and ultraviolet-A (UVA) light are applied.[1,2] The rationale for the removal of the epithelium was described as allowing adequate penetration of riboflavin into the stromal tissue, where it absorbs the UVA light and produces the actual crosslinking between collagen fibrils in the corneal stroma.[3]

The downside of epithelial removal is that it causes significant pain and discomfort during the first postoperative days, in addition to the 3-8% chance of epithelial healing problems.[4–6] To circumvent these downsides of epithelium removal, a transepithelial CXL technique was developed. Transepithelial CXL avoids the need for epithelial removal. Wollensak et al. investigated the biomechanical effect in rabbit eyes and estimated that transepithelial CXL with benzalkonium chloride would create one fifth of the corneal biomechanical rigidity compared with epithelium-off (epi-off) CXL in human eyes.[7] Transepithelial CXL with the use of sodium ethylenediaminetetraacetic acid (EDTA) in riboflavin (Ricrolin TE) has been investigated in ex-vivo rabbit eyes as well, showing minimal riboflavin uptake in the group with intact epithelium receiving Ricrolin TE solution.[8]

The clinical effects of transepithelial CXL with Ricrolin have been reported in case series and non-randomized comparative trials. Filippo et al. reported clinical outcomes after 18 months in 20 eyes treated by Ricrolin assisted transepithelial CXL, compared with their untreated fellow eye.[9–11] A significant improvement in visual acuity (0.35 to 0.24 logMAR) and decreased central keratometry values (steepest keratometry (Ksteep): 51.0 to 48.1 diopter (D)) were seen in the transepithelial CXL eyes, not in the untreated group. A stromal demarcation line at 60 µm depth was measured, indicative of an effective treatment. Caporossi et al. performed Ricrolin assisted transepithelial CXL in 26 eyes, age 11 to 26 years, and reported unchanged visual acuities, but significantly increased maximal keratometry (Kmax) values (48.6 to 50.1 D) after two years of follow-up.[12] Leccisotti et al. reported the one year results on transepithelial CXL with Ricrolin TE in 51 eyes with the untreated fellow eye serving as control and found some stabilizing effect in the transepithelial CXL group (Kmax changed from 54.3 to 54.8 D, compared to 51.7 to 53.3 in the control group).[13] A prospective case series by De Bernando et al. in 36 eyes treated by Ricrolin assisted transepithelial CXL showed an increased visual acuity and stable keratometry after 6 months of follow-up.[14]

The natural course of keratoconus can be long-lasting, with years of apparent stable keratometry readings after a period of latent progression.[15] Furthermore, the clinical effects of epi-off CXL have been well described in randomized controlled trials with adequate follow-up.[16–18] To address these two considerations and adequately describe the clinical effects of transepithelial CXL, a non-inferiority randomized study design is mandatory.

In this randomized controlled study, we investigated the clinical effects and safety of transepithelial CXL with Ricrolin compared to epi-off CXL in progressive cases of keratoconus and tested the hypothesis that transepithelial CXL is equally effective.

MATERIALS AND METHODS

Study group & protocol

This non-inferiority randomized controlled trial included patients diagnosed with progressive keratoconus who were found eligible for a CXL procedure at a tertiary academic centre (University Medical Center Utrecht, The Netherlands), from May 2011 through September 2013 with a follow-up of 1 year. The study was prospectively approved by the University Medical Center Utrecht Ethics Review Board (REF number NL29961) and registered at ClinicalTrials.gov (identification number NCT02349165). All procedures complied with the Declaration of Helsinki and local laws regarding research on human subjects. Written informed consent was obtained from all patients prior to their participation.

Inclusion criteria were age ≥ 18 years, a clear central cornea, and a documented progression as defined by an increase in Kmax, Ksteep, mean keratometry and/or topographic cylinder value by ≥ 0.5 D over the previous 6 to 12 months. Exclusion criteria were a minimal pachymetry of less than 400 μm prior to UVA irradiation, pregnancy or breastfeeding, and a history of previous ocular infection.

Keratoconus diagnosis and study eligibility were determined by one corneal specialist (NT). Progression of keratoconus was documented by minimally 2 topography measurements in all patients. Patients were randomized using a simple unrestricted randomization procedure to either transepithelial CXL or epi-off CXL.

Measurements and Devices

Patients were examined at baseline and at 1, 3, 6 and 12 months post-CXL. Manifest refraction, visual acuity, Goldmann applanation tonometry, slit lamp examination and Scheimpflug topography (Pentacam HR, Oculus, Germany) measurements were performed at each follow-up. Endothelial cell density (Topcon, SP3000P, Tokyo, Japan) was measured at baseline and at the 6 and 12 month follow-up. Demarcation line depth was measured at the 1, 3 and 6 month follow-up using high resolution corneal imaging (Visante Optical Coherence Tomography, Carl Zeiss, Germany). All contact lens wearers were instructed to discontinue contact lens wear at least 1 week for scleral and soft contact lenses or 2 weeks for hybrid and rigid permeable lenses prior to all evaluations. During CXL, pachymetry measurements were performed with a handheld ultrasound (US) pachymeter (Handy Pachymeter, SP-3000, Tomey, Japan). The CXL device was used at a working distance of 5 cm with an irradiance of 3 mW/cm^2 (UV-X, Peschke Meditrade, Switzerland). Before every treatment session, a calibration was performed to confirm

the correct UVA emission level. Throughout the whole study, the same devices and time points were applied.

Surgical Technique

In the transepithelial CXL group, local anaesthetic eye drops (oxybuprocaïne 0.4% and tetracaïne 1%) were applied 3 times during 5 minutes, and Ricrolin TE solution (consisting of riboflavin 0.1% eye drops with Dextran T500 15 mg and EDTA, SOOFT Italia) were instilled every 2 minutes for 15 minutes. Next, an eyelid speculum was placed and a silicone ring was positioned between the eyelids, which was filled with Ricrolin TE and used to remain a Ricrolin 'pool' on the cornea. After 15 minutes, the silicone ring was removed, the cornea was rinsed with balanced salt solution, and pachymetry was performed. UVA irradiation was performed during 30 minutes, while Ricrolin TE solution was re-applied to the cornea every 5 minutes.

The epi-off CXL technique was performed following the Dresden protocol, adjusted with the avoidance of the eyelid speculum during riboflavin instillation.[19,20] Epithelial removal (9-mm) was performed using a blunt knife. After pachymetry measurements, isotonic riboflavin 0.1% solution with 20% Dextran (Medio Cross™) was applied every 3 minutes during 30 minutes, with no eye lid speculum in place. When pachymetry was < 400 µm, hypoosmolar riboflavin was additionally applied every 20 seconds during 5 minutes and repeated up to 2 times until the required pachymetry value of ≥400 µm was achieved.[21] With an eye lid speculum in place, UVA irradiation was performed during 30 minutes, during which isotonic riboflavin drops was given every 5 minutes.

In both groups, the post-CXL medication consisted of antibiotic eyedrops (Vigamox® 5mg/ml Alcon Nederland BV) and preservative-free artificial tears (Duratears Free® 2% Alcon Nederland BV) and were used for 4 weeks, while non-steroidal anti-inflammatory drops (Nevanac® 0.1% Alcon Nederland BV) were used during the first week. Starting 1 week after CXL, topical steroids (Fluorometholone 0.1% drops Allergan BV) were applied twice a day for two weeks. In the epi-off group only, oral pain medication (Tramadol 50 mg 1-2 a day; diclofenac 25 mg 1-2 a day) were prescribed on the treatment day and the day after. A bandage lens (Purevision, Bausch & Lomb) was placed in the epi-off group, and was removed after 1 week if the epithelial healing was complete.

Statistical analysis and power calculation

Baseline measurements between the treatment groups were compared using an independent samples t-test. Primary outcome was pre-defined in the study protocol as

clinical stabilisation of keratoconus one year after CXL, defined as a Kmax increase of no more than 1 D over the preoperative Kmax value. Fischer's exact test (two tailed) was used to determine the relation between treatment and stabilisation.

Decimal visual acuity was converted to the logarithm of the minimal angle of resolution (logMAR).

We analyzed all outcome measures at all follow-up visits using a linear mixed model with a generalized estimating equations correction.[22] The outcomes over time were corrected for baseline values. Normality and homoscedasticity of the residuals were tested visually, and in a Q-Q plot and scatterplot, respectively. A *P*-value <0.05 was considered statistically significant. Data are recorded as mean ± standard deviation. All tests were performed in SPSS version 20.0 for Windows.

Power of this study was calculated based on a non-inferiority design, which was determined by the expected average Kmax change after treatment (Raiskup et al.: -1.46 D[23]) minus the acceptable average Kmax change after treatment (Koller et al: Kmax + 1 D[5]). The standard deviation reported by Raiskup et al. was 3.76. Using alpha 0.05, beta 0.2, and a non-inferiority margin of -2.46, we calculated a sample size of 29 for each group.[24]

RESULTS

Of the 105 patients eligible for this study, 61 patients were willing to participate and provided informed consent. This study included 61 eyes from 61 patients (47 males and 14 females) with progressive keratoconus, who were randomly assigned to either epi-off (n=26) or transepithelial CXL (n=35). One eye in the epi-off group received hypoosmolar riboflavin, since the corneal thickness was <400 µm after 30-minutes of isotonic riboflavin instillation.

Four patients (6%), two in each group, did not complete the one year-follow-up; two patients were lost to follow up due to a move abroad, one patient scheduled the follow-up visits in another hospital closer by, and one patient was re-treated by epi-off CXL after 10 months (see the complication section for details).

Both groups were comparable at baseline, apart from a lower spherical equivalent and logMAR UDVA in the transepithelial CXL group. Mean keratoconus progression before treatment was not significantly different between the groups. Baseline characteristics are listed in Table 1. All variables, except for age, were normally distributed.

Baseline parameter	Transepithelial CXL	Epithelium-off CXL
Median age (years, range)	24 (18-48)	24 (18-44)
Male / Female (n)	28 / 7	19 / 7
Right / left (n)	19 / 16	13 / 13
Spherical Equivalent (D)	-1.5 ± 2.5	-3.0 ± 3.0
Uncorrected distance visual acuity (logMAR)	0.8 ± 0.5	1.1 ± 0.6
Corrected distance visual acuity (logMAR)	0.3 ± 0.3	0.3 ± 0.3
Pachymetry thinnest point (µm)	457 ± 27	467 ± 29
Maximal keratometry (D)	56.4 ± 5.0	57.8 ± 7.1
Intraocular pressure (mmHg)	10 ± 2	11 ± 3
Endothelium (cells/mm^2)	2627 ± 363	2764 ± 252

CXL = corneal crosslinking; D = diopter; mean ± SD

Table 1. Transepithelial versus epithelium-off corneal crosslinking for keratoconus, baseline characteristics (n=61).

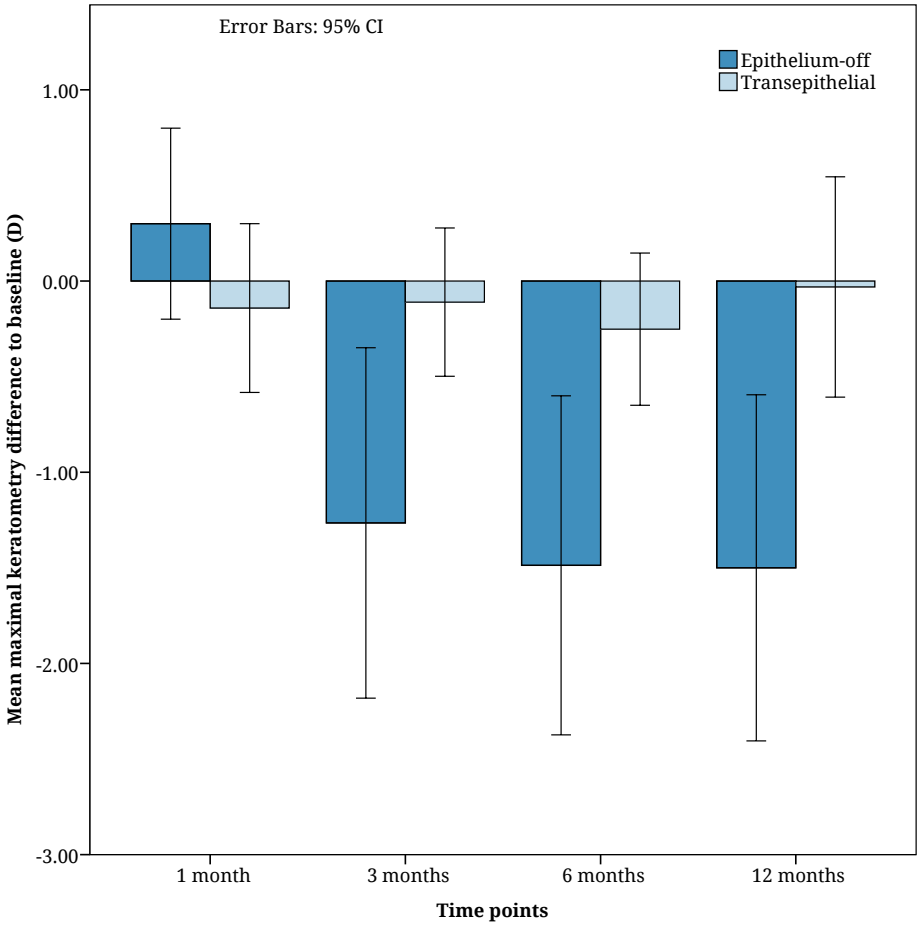

FIGURE 1. Difference in maximal keratometry over time compared to baseline in transepithelial vs epithelium-off corneal cross-linking for keratoconus.

Keratometry

Transepithelial CXL showed less potent effects on keratoconus stabilization and regression compared to epi-off CXL; in the transepithelial CXL group, Kmax remained virtually stable at all follow-up visits, while in the epi-off group, Kmax demonstrated flattening from 3 months post-treatment onwards (Figure 1). The trend over time in Kmax flattening was significantly different between both groups (P=0.022).

The steep and flat central keratometry values (Ksteep and Kflat) increased slightly over time in the transepithelial CXL group, and decreased slightly in the epi-off group (supplementary data).

Parameter	Group	1 month	3 months	6 months	12 months	*P*-value
ΔKmax (D)	Transepithelial	-0.1 ± 1.1	0.0 ± 1.0	-0.1 ± 1.2	0.3 ± 1.8	0.022*
	Epithelium-off	0.3 ± 1.1	-1.2 ± 2.0	-1.4 ± 2.0	-1.5 ± 2.0	
ΔCDVA (logMAR)	Transepithelial	-0.05 ± 0.24	-0.10 ± 0.21	-0.12 ± 0.22	-0.14 ± 0.21	0.023*
	Epithelium-off	0.09 ± 0.18	-0.04 ± 0.18	-0.09 ± 0.23	-0.07 ± 0.21	
ΔUDVA (logMAR)	Transepithelial	-0.06 ± 0.25	-0.08 ± 0.29	-0.02 ± 0.31	-0.06 ± 0.37	0.591
	Epithelium-off	-0.10 ± 0.36	-0.18 ± 0.31	-0.16 ± 0.35	-0.15 ± 0.43	
ΔSE (D)	Transepithelial	0.4 ± 1.1	0.3 ± 1.1	0.3 ± 1.6	0.3 ± 1.6	0.436
	Epithelium-off	0.6 ± 1.4	0.5 ± 1.6	0.9 ± 1.8	0.4 ± 3.0	
ΔCorneal thickness (μm)	Transepithelial	0 ± 7	2 ± 9	-3 ± 8	0 ± 12	<0.001*
	Epithelium-off	-18 ± 10	-14 ± 15	-9 ± 11	-4 ± 8	

Δ= differences post-pre crosslinking; UDVA = uncorrected distance visual acuity; CDVA = corrected distance visual acuity; SE = spherical equivalent; Corneal thickness = corneal thickness on thinnest point; D = diopter; *P*-value from Generalized Estimating Equations corrected for baseline; * = statistically significant

Table 2. Transepithelial versus epithelium-off corneal crosslinking for keratoconus. Outcome after 1, 3, 6 and 12 months compared to baseline.

Patient #	Maximal keratometry (Kmax) increase	Time after initial treatment	Result after retreatment
1	4.7 diopter	10 months	Kmax decreased 1.6 diopter after 1 year
2	1.8 diopter	27 months	Kmax decreased 1.1 diopter after 1 year
3	2.9 diopter	15 months	Kmax decreased 0.2 diopter after 1 year
4	5.4 diopter	13 months	Kmax decreased 0.4 diopter after 1 month
5	4.6 diopter	33 months	No data available after retreatment

Table 3. Overview of patients from the transepithelial group, re-treated by epithelium-off corneal crosslinking for keratoconus.

Group	Kmax (D)	Range	CDVA (logMAR)	Range	CCThin (µm)	Range
Transepithelial CXL entire group	56.4	46.2 to 68.1	0.30	-0.08 to 1.00	457	410 to 516
Transepithelial CXL stable/regression	56.4	46.2 to 68.1	0.32	-0.08 to 1.00	456	410 to 516
Transepithelial CXL progression	55.8	50.7 to 59.6	0.21	0.00 to 0.52	460	424 to 495
Epithelium-off CXL entire group	57.8	47.2 to 73.8	0.26	-0.08 to 1.00	467	412 to 546

CXL= corneal crosslinking; Kmax = maximal keratometry; CDVA = corrected distance visual acuity (logMAR); D = diopter ; CCThin = pachymetry at the thinnest point

Table 4. Transepithelial versus epithelium-off corneal crosslinking for keratoconus: baseline characteristics and outcome after 1 year (stabilization, regression or progression)

Primary outcome: treatment failure, as pre-defined in the study protocol

In the transepithelial CXL group, 8 eyes (23%) showed continued progression of the disease (range 1.3 to 5.4 D). One eye showed a 4.7 D increase in Kmax after 10 months and was retreated by epi-off CXL, seven other eyes showed a Kmax increase after 1 year, of which currently four eyes are retreated by epi-off CXL, Table 3. In the epi-off group, all eyes demonstrated clinical stabilisation after one year.This difference in clinical stabilization between the two treatments was statistically significant (*P*=0.016).

The number of patients with a continued progression was considered too small for subgroup analysis to detect predictors for the transepithelial CXL outcome. The baseline characteristics of the eyes that presented with continued progression after transepithelial CXL compared to the transepithelial CXL group in general, or the total study population were shown in Table 4.

Visual acuity and refraction

There was a statistically significant different trend in corrected distance visual acuity (CDVA) between both groups, with a more favorable outcome in the transepithelial CXL group (*P*=0.023). Figure 2 shows the largest difference in CDVA at the one month follow-up. When analyzing the data without the one month results, there is no significant difference between the two groups (*P*=0.088). No difference in the trend over time in uncorrected visual outcomes was observed between the groups (*P*=0.591).

Refractive cylinder values increased in both groups after treatment by ± 1.5 D, with

no difference in trend over time between the groups (*P*=0.720). Spherical refraction increased in both groups by ± 1 D, with no difference in trend over time between the groups (*P*=0.281). Trend over time in spherical equivalent was also unchanged between treatment groups (*P*=0.436).

Pachymetry, intra ocular pressure and endothelium cell counts

Corneal thickness remained stable in the transepithelial CXL group. The epi-off group showed an expected lowered optical pachymetry after treatment, which normalized at the 12 month time point. No difference in IOP over time was measured between the groups, the endothelial cell counts were unremarkable (supplemental table).

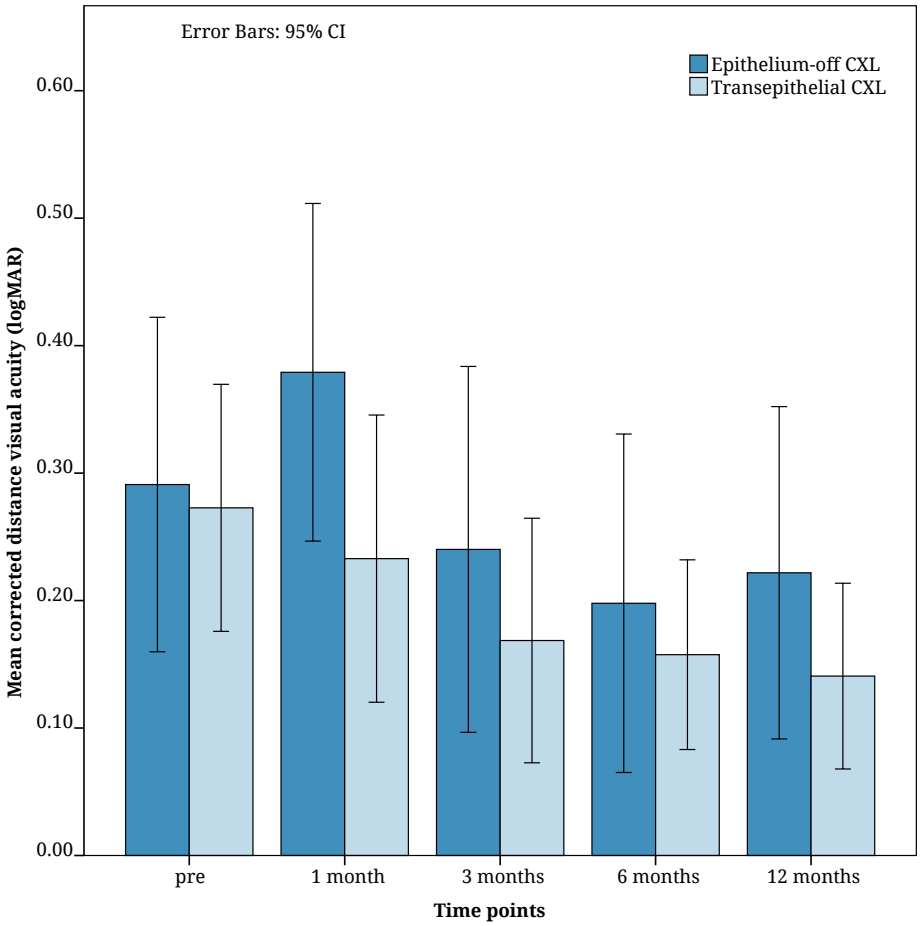

FIGURE 2. Corrected distance visual acuity at different time points in transepithelial vs epithelium-off corneal crosslinking for keratoconus.

Demarcation line

In none of the transepithelial CXL cases, a demarcation line was visible after 1 month (Table 5). The average demarcation line depth the epi-off group was 266 µm ± 64 after 1 month, measured in 22 of 26 eyes; data of 4 eyes was missing by equipment failure at location.

Adverse events

In all eyes in the transepithelial CXL group, the epithelium remained intact after one week and no adverse events were recorded.

Adverse events occurred in four eyes (15%) in the epi-off group. One eye developed a herpes simplex keratitis one week post-CXL, which was adequately treated and did not result in visual acuity loss (pre- and post-CXL decimal CDVA was 0.8) or scarring. One eye developed a sterile infiltrate, though a clear cornea was seen at the 1 month follow-up. One eye had epithelial healing problems and a small central haze spot in the anterior stroma one week post-CXL, possibly associated with his peri-ocular eczema (pre-CXL decimal CDVA was 0.6, after 1 year 0.8). Finally, one eye also showed delayed epithelial healing leading to a “cloudy stroma” at the 3 month follow-up and a deep stromal haze at the 6 month follow-up (pre- and post-CXL decimal CDVA was 0.1).

DISCUSSION

This non-inferiority randomized controlled trial showed that transepithelial crosslinking with EDTA riboflavin (Ricrolin TE), although showing no adverse events, was less effective to halt keratoconus progression after 1 year compared to epithelium-off crosslinking; 8 eyes (23%) showed an increase of maximal keratometry of more than 1 diopter compared to none of the eyes in the epi-off group.

A major strength of this study was the adequately powered design and the very low percentage of cases who were lost to follow-up (approximately 4%). The interventions were standardized and did not change throughout the course of study. All diagnoses were made by a corneal specialist (NT) and all refractions were measured by a trained optometrist (NS). The unequal sample size in this (non-double blinded) study can be considered a limitation, however, some discrepancy would be expected since a simple unrestricted randomization procedure was followed instead of a block randomization.[25]

The rigidity of the cornea and the Ricrolin TE concentration in the stroma have not been investigated in this study, since our main focus was to show the clinical effects. Unfortunately, confocal microscopy to analyze changes in corneal structures after CXL was not available in our setting. We were therefore unable to assess and compare potential keratocyte apoptosis, as was reported by Fillipello et al.[10]

The general indicators for a CXL effect (with stabilization being the main purpose) are a visible demarcation line, a flattened keratometry and reduced pachymetry.[26,27] Recent developments of transepithelial CXL in another manner, for instance by iontophoresis, showed increased uptake of riboflavin into the stroma, and resulted in stable and decreased keratometry and improved UDVA or CDVA after 1 year in small groups of patients (20 to 22 eyes).[28,29] In our study, no demarcation line was found in the transepithelial CXL group and the average central keratometry, maximal keratometry, and pachymetry were unchanged after treatment. The fact that these indicators were absent in the transepithelial CXL group suggests this treatment was not sufficiently effective in halting progressive keratoconus. However, if we compare the mean Kmax value after 1 year in our transepithelial CXL group (+ 0.3 D) to the untreated control groups of three randomized controlled trials (+1.2D[16] , -0.1 D (18 months)[30] and +0.3D [18]), the effect is debatable. Another notable finding in our transepithelial CXL group was a significant CDVA increase, in addition to a significantly increased cylinder. This indicates that there might be something going on in transepithelial CXL with Ricrolin TE after all.

The average Kmax flattening after one year in the epi-off group in our study was

more pronounced (-1.5 D) compared to the results of Wittig-Silva et al. (-0.7 D), which could be explained by the steeper corneas at baseline in our study (52 D versus 58 D) which are known to flatten more after CXL.[19] The statistically different trend in CDVA over time between the groups can be explained by the haze formation at the 1 month follow-up in the epi-off group which was noted after epithelium removal.[31] Another explanation for CDVA and keratometry changes at the 1 month follow-up could be the remodeling of epithelium (in keratoconus, the epithelium layer is thinnest at the cone, and the epithelium thickness profile can re-establish a smoother surface). [32]

In our transepithelial CXL group, eventually 5 eyes (14%) were retreated, which is in line with the study of Caporossi et al. who decided to retreat 19% of the Ricrolin-assisted transepithelial CXL patients after 2 years.[12] In contrast to Filippello et al. who reported flattening of Kmax 18 months after transepithelial CXL, average Kmax remained stable in our transepithelial CXL group after 1 year.[9,11] Furthermore, the authors measured a demarcation line in the transepithelial CXL group which was more superficial than generally reported in eyes undergoing epi-off CXL, while the demarcation line was absent in our transepithelial CXL group. The most apparent difference in surgical technique was the use of the silicone ring throughout the entire procedure (including UVA irradiation) by Filippo et al. We adhered to the manufacturer's instruction and removed the ring prior to UVA irradiation

Endothelial damage has been described when thin corneas were irradiated with UVA without containing sufficient riboflavin to absorb the UVA light.[20,33] In transepithelial CXL, both the Ricrolin TE solution and corneal epithelium absorb UVA.[34] ECD count remained stable in both treatment groups, suggesting sufficient UV absorption to avoid endothelium damage.

In general, keratoconus progression does not follow a linear trend over time. In contrast, periods of progression intersperse with periods of stability. Keeping this in mind, the patients who underwent transepithelial CXL and were classified as 'stable' after 1 year, could also have passed a physiologic stable period of their condition. Some patients showed stable keratometry values at the 1 year follow-up, and progression 2 years after treatment (unpublished data), suggesting either a less effective treatment or an ineffective treatment with physiologic stable period. Considering whether transepithelial CXL is unsuitable for any patient group, is difficult. Perhaps transepithelial CXL would be advisable for patients with a corneal thickness < 350 μm who are excluded for epi-off CXL, or patients whose lack of compliance increases the risk of post-operative adverse events.

In this study, we showed the clinical results of a randomized controlled trial with transepithelial CXL with Ricrolin TE versus epi-off CXL. transepithelial CXL showed a

poor potential for halting keratoconus progression when compared to regular epi-off CXL: a demarcation line could not be identified and a considerable percentage of transepithelial CXL treated eyes needed retreatment due to an increased maximum keratometry.

Therefore, although epithelial removal is a painful procedure and associated with considerably more adverse events, we would recommend epi-off CXL for patients who present with a progressive keratoconus.

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6

A multivariate analysis and statistical model for predicting visual acuity and keratometry one year after cross-linking for keratoconus.

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ABSTRACT

Purpose

To investigate putative prognostic factors for predicting visual acuity and keratometry 1 year following corneal cross-linking (CXL) for treating keratoconus.

Design

Prospective cohort study.

Methods

This study included all consecutively treated keratoconus patients (102 eyes) in 1 academic treatment center, with minimal 1-year follow-up following CXL. Primary treatment outcomes were corrected distance visual acuity (logMAR CDVA) and maximum keratometry (K(max)). Univariable analyses were performed to determine correlations between baseline parameters and follow-up measurements. Correlating factors ($P \leq .20$) were then entered into a multivariable linear regression analysis, and a model for predicting CDVA and K(max) was created.

Results

Atopic constitution, positive family history, and smoking were not independent factors affecting CXL outcomes. Multivariable analysis identified cone eccentricity as a major factor for predicting K(max) outcome (ß coefficient = 0.709, $P = .02$), whereas age, sex, and baseline keratometry were not independent contributors. Posttreatment visual acuity could be predicted based on pretreatment visual acuity (ß coefficient = -0.621, $P < .01$, $R^2 = 0.45$). Specifically, a low visual acuity predicts visual improvement. A prediction model for K(max) did not accurately estimate treatment outcomes ($R^2 = 0.15$).

Conclusions

Our results confirm the role of cone eccentricity with respect to the improvement of corneal curvature following CXL. Visual acuity outcome can be predicted accurately based on pretreatment visual acuity. Age, sex, and K(max) are debated as independent factors for predicting the outcome of treating keratoconus with CXL.

INTRODUCTION

Keratoconus is a progressive non inflammatory disease, wherein the cornea becomes thinner, inducing irregular astigmatism and reducing quality of vision. Corneal crosslinking (CXL) is a relatively new treatment to increase the mechanical and biochemical strength of the stromal tissue, subjecting the ectatic cornea to riboflavin and ultraviolet-A light. When successful, CXL prevents keratoconus progression, even potentially inducing regression of the ectatic cornea.[1] This cone stabilization might prevent the need for future corneal grafting.[2] Outcome of CXL regarding visual acuity is generally good, although loss of visual acuity is a known complication.[3] Another safety concern is the effect of CXL on the healthy endothelium, for which treatment safety guidelines have been proposed.[4]
Not all patients benefit equally from CXL treatment though. Every clinician working in this field encounters patients whose keratoconus proceeds seemingly unhampered. A reliable pre-operative prediction of treatment effect could help managing patient expectations and prevent exposure to potential side-effects.

Etiological factors of keratoconus are extensively studied. Factors associated with keratoconus are a positive family history,[5] an atopic constitution,[6] eye rubbing, [7] contact lens wear[8] and a myriad of syndromes (i.e. M. Down,[9] chromosome 7,11 translocation[10] and chromosome 13 ring abnormality[11]). It is not yet unequivocal established whether these factors also play a role in CXL treatment effectiveness.

The understanding of factors related to CXL treatment success is currently emerging, Treatment success is a combination of different characteristics like post-operative visual acuity, improvement in keratometry and the absence of adverse events. A comprehensive literature search was conducted to define potential prognostic factors for CXL treatment effectiveness and safety. Previously described prognostic factors are the pre-operative visual acuity, eccentricity of the cone, maximum keratometry (Kmax) pre-treatment, age above 35 years and gender.[12-13]
Greenstein et al. demonstrated that males and patients with central cone location seem to benefit more from CXL treatment in terms of Kmax regression. Whether a high Kmax prior to -treatment influences Kmax regression remained controversial.[14] Lamy et al. addressed visual acuity outcomes after CXL. Central cone location, a visual acuity <20/25 and age under 35 predicted a better corrected distance visual acuity (CDVA) one year after treatment.[15]

Spoerl et al. demonstrated a negative association between smoking and keratoconus.[16] Hafezi et al. suggested that this could be explained by the biomechanical changes in the cornea through smoking.[17] Furthermore, Altinors et al. demonstrated that smoking has a deteriorating effect on the lipid layer of the pre-corneal tear film.[18] Especially when CXL is accompanied with a corneal abrasion, smoking could hypothetically affect treatment results.

We investigated the value of aforementioned predictors for CXL treatment effectiveness in keratoconus and additionally assessed the potential prognostic factors such as family history, atopic constitution and smoking. By combining abovementioned factors, we aimed to create a prediction model, aiding clinicians in their therapeutic decisions.

MATERIAL AND METHODS

Dataset and study design

Data were derived from an ongoing prospective treatment cohort on CXL treatment in our institution for patients with progressive keratoconus. All patients were consecutively treated at the University Medical Center Utrecht, between January 2010 and December 2011, with a one year follow-up. Inclusion criteria were a progression of Kmax>1.0 D within 6-12 months, and a corneal thickness >400µm (thinnest point). Exclusion criteria were corneal scarring, presence of concurrent infection and pregnancy or lactation. Treatment effects were assessed at one year follow-up. This study for predictor research was approved by the UMC Utrecht Ethical Review Board and the requirement for informed consent was waived. The procedures of the treatment cohort complied with the Declaration of Helsinki and local laws involving research on human subjects.

Surgical procedure

Surgical procedure was performed as described by Spoerl and by Raiskup.[4,19] An 9mm corneal abrasion was made using a blunt knife, after which riboflavin 0.1% (Peschke Meditrade GmbH) solution was applied every 3 minutes for 30 minutes. When pachymetry was less than 400 µm, hypo-osmolar riboflavin was additionally instilled every 20 seconds for 5 minutes and repeated up to 2 times until adequate thickness (ie, ≥400 µm) was reached. The cornea was exposed to a UV light source (UV-X, Peschke Meditrade GmbH, using a perpendicular emission plane) with a wavelength of 365 ± 10 nm for a total time of 30 minutes. After the treatment, a bandage lens was placed (PureVision®, Bausch + Lomb Nederland BV). Post-operative medication consisted of nepafenac 0.1% (Nevanac®, Alcon Nederland BV) drops TID for one week, moxifloxacine 0.5% (Vigamox®, Alcon Nederland BV) drops TID for one month, and dextran/hypromellose (Duratears®, Alcon Nederland BV) drops TID for one month. When the epithelium was healed the bandage contact lens was removed and fluormetholon 0.1% (FML Liquifilm®, Allergan BV) drops BID were prescribed.

Data collection

Standardized pre-operative assessment yielded a series of potential predicting factors. Measurements included uncorrected distance visual acuity (UDVA), corrected

distance visual acuity (CDVA), corneal tomography (Pentacam HR type 70900, Oculus), endothelial cell count (SP-3000P, Topcon), and automated tonometry (CT-80, Topcon). CDVA was obtained via manifest refraction. Measurements were repeated post-operatively at one, three, six and twelve month's intervals. All patients were requested to discontinue contact lens wear 2 weeks before each evaluation.
Patients were questioned using standardized forms on their family history, atopic constitution and smoking status. Family history for keratoconus was taken up to the fourth degree (i.e. nephews/nieces), and was defined positive in case of a first or second degree relative with keratoconus. An atopic constitution was defined as either having asthma, hay fever, eczema, a food allergy and/or taking anti-allergic medication. Smoking status was defined whether currently smoking or with smoking in the personal history. Pack-years were noted. Missing data in the medical files were completed by consulting patients via phone or mail.

Statistical analysis

Visual acuity was converted to the logMAR of visual acuity. Two primary outcomes were defined as follows: differences between visual acuity (logMAR CDVA) and differences between keratometry (Kmax) at baseline and at one year follow-up. Paired-samples T tests were used to determine significance of the difference between Kmax and logMAR CDVA at baseline and one year after treatment. Missings were excluded pair wise from analysis.

Linearity of baseline data and outcome measurements was determined visually in a histogram. Normality was tested based on skewness and kurtosis with a cut-off point of 3.29 (P<0.001) and showed no deviations. The pre-treatment measurements and potential prognostic factors atopic constitution, family history, smoking habits, factors derived from literature study, and pre-operative measurements were included in univariable analysis. Pearson's correlation coefficients were determined between the potential prognostic variables and the primary outcomes. The ß-coefficient represents how a dependent variable will change, per unit increase in the predictor variable, it has a size and a positive or negative direction. For instance: a ß-coefficient of +2 for age implicates that for every year a subject is older, the dependent variable will increase with 2 units.
To determine the independent relationship between potential prognostic variables and the outcome a multivariable linear regression model was built. Initially this model including all variables that had a P-value of <0.20 in the univariable analysis. This analysis was performed with generalized estimating equations, correcting for patients

included with two eyes in the dataset. A prediction model was created by performing stepwise backward selection of least contributing variables. Variables were removed until quasi-likelihood ratio began to deteriorate. Internal model validity was tested by plotting the predicted value of linear predictor against the measured differences after one year, and by calculating the coefficient determination between predicted and measured outcome values (R^2). A likelihood ratio test was performed after a squared term was included to the regression model. It did not show an increased model fit. Data collection and analysis were performed in SPSS 20.0 (IBM SPSS statistics).

RESULTS

Dataset characteristics

One-hundred-and-two eyes of 79 patients were consecutively treated. Six eyes of four patients were excluded from analysis due to loss to follow-up (=5%). Drop-out baseline characteristics did not differ significantly from the main group. Details of overall baseline characteristics are displayed in table 1. At one year follow-up Kmax decreased or stabilized in 85/96 of eyes (88.5%). Eleven eyes showed progression of keratoconus of >1.0D (11.5%), with a mean increase in Kmax of 2.6D (range 1.3-5.2).

Clinical outcomes

Both primary outcomes improved significantly compared to baseline. Mean Kmax

	Mean / n	Range / %	Missing
Age (years)	23	12 to 50	0
Male	56	71%	0
Right eye	43	42%	0
Kmax (D)	59.5	44.8 to 82.2	0
Snellen CDVA	20/32	20/400 to 20/16	0
logMAR CDVA	0.31	-0.08 to 1.30	0
ECD (cells/mm²)	2744	1900 to 3347	32*
Positive family history	8	10%	2
First degree	3	4%	2
Second degree	7	9%	2
Third degree	0	0%	2
Fourth degree	2	3%	2
Atopic constitution	34	43%	2
Asthma	14	18%	2
Eczema	20	20%	2
Hay fever	28	35%	2
Food allergy	10	13%	2
Anti-allergic medication	25	32%	2
Smokers	11	14%	3
Average pack-years	0.5	0.25 to 7	3

Kmax: maximum keratometry. CDVA: Corrected Distance Visual Acuity. logMAR: log of minimal angle of resolution. ECD: endothelial cell density. Lost to follow-up: 6 eyes (6%) in 4 patients (5%). *: in severe keratoconus endothelial densities were not attainable

Table 1. Baseline table. Characteristics of 102 eyes of 79 keratoconus patients

	Changes in CDVA (logMAR)			Changes in maximum keratometry (D)		
	ß-coefficient	95% CI	*P*-value	ß-coefficient	95% CI	*P*-value
Age (years)	0.00	-0.01 to 0.01	0.77	0.04	-0.13 to 0.10	0.13
Male gender	0.04	-0.08 to 0.16	0.50	1.22	0.27 to 2.17	0.01*
Positive family history	0.00	-0.17 to 0.17	0.97	0.69	-0.69 to 2.08	0.32
Atopic constitution	0.12	0.01 to 0.23	0.03*	0.25	-0.68 to 1.17	0.60
Smoking	-0.05	-0.20 to 0.11	0.54	-0.41	-1.69 to 0.85	0.52
Spherical equivalent (D)	-0.00	-0.02 to 0.01	0.99	0.10	-0.02 to 0.23	0.12
LogMAR UDVA pre-treatment	-0.18	-0.29 to -0.07	<0.01*	-0.87	-1.79 to 0.05	0.06
LogMAR CDVA pre-treatment	-0.52	-0.64 to -0.41	<0.01*	-0.77	-2.06 to 0.52	0.24
Kmax pre-treatment (D)	-0.01	-0.02 to 0.00	<0.01*	-0.04	-0.09 to 0.01	0.14
Eccentricity (mm)	0.09	0.03 to 0.17	<0.01*	0.96	0.40 to 1.15	<0.01*
Central corneal thickness (µm)	0.00	0.00 to 0.00	0.04*	0.01	0.00 to 0.02	0.04*

Statistical analysis using univariate linear regression. LogMAR: log of minimal angle of resolution. CDVA: corrected distance visual acuity. UDVA: uncorrected distance visual acuity. Kmax: maximum keratometry. 95% CI: 95% Confidence Interval. ß-coefficient: value referring to how a dependent variable will change, per unit increase in the predictor variable. *: significant *P*-values, significance set at < 0.05. *P*-values <0.20 were included in multivariate analysis.

Table 2. Univariate factor analysis of baseline characteristics for corneal crosslinking effects at one year follow-up in keratoconus eyes

decreased by 1.3 D from 60.1 to 58.7 ($P<0.01$) and mean logMAR CDVA decreased by 0.13 from 0.33 to 0.19 ($P<0.01$). These values are based on the 96 included eyes. Endothelial cell densities remained stable with a mean density at follow-up of 2831 (±309) cells/mm^2, without cases showing a remarkable decline. A mild post-operative haze at one month occurred in twenty-two cases, which mostly resolved. At one year three eyes experienced a slight persistent haze. The epithelium was healed after one week in 80.5% of eyes, between one and two weeks in 16.1% and after two weeks in 3.4%. No cases of infectious keratitis were encountered.

Univariable analysis

All candidate predictors were univariable correlated with both primary outcomes. An overview of assessed predictors is given in table 2. Notable are the greater improvement in Kmax in males then in females (ß-coefficient 1.22, $CI_{95\%}$0.27;2.17, $P=0.01$) and a slightly lesser improvement in visual acuity in atopic patients (ß-coefficient 0.12, $CI_{95\%}$ 0.01;0.23,

P=0.03). Neither family history of keratoconus nor smoking influenced treatment outcomes. Significant univariate associations were entered in a multivariable analysis.

Multivariable regression analysis and prognostic models

Multivariable linear regression was performed for both primary outcomes. Results are displayed in table 3. With regards to visual acuity outcome, only pre-treatment logMAR CDVA appeared an independent factor (ß-coefficient -0.62, $CI_{95\%}$-1.00;-0.62, *P*<0.01); a higher logMAR at baseline is associated with a lower logMAR at one-year follow-up The same applies for cone eccentricity in respect of Kmax outcome (ß- coefficient 0.71, $CI_{95\%}$ 0.12;1.30, *P*=0.02); a more eccentric cone is associated with less flattening of Kmax at follow-up. All other parameters assessed in this multivariable analysis, including atopic constitution, do not seem to pertain an individual effect on treatment outcome.

For both primary outcome parameters prediction models were created. Visual acuity

Changes in CDVA (logMAR)

	ß-coefficient	95% CI	*P*-value
Atopic constitution	-0.05	-0.12 to -0.05	0.14
logMAR UDVA	0.07	-0.08 to 0.07	0.38
logMAR CDVA	-0.62	-1.00 to -0.62	<0.01*
Kmax (D)	0.01	0.00 to 0.01	0.14
Eccentricty (mm)	0.02	-0.05 to 0.02	0.58
Central corneal thickness (µm)	0.00	0.00 to 0.00	0.61

Changes in maximum keratometry (D)

	ß-coefficient	95% CI	*P*-value
Male gender	0.82	-1.92 to 0.28	0.14
Spherical equivalent (D)	0.10	-1.90 to 0.28	0.14
logMAR UDVA	-0.02	-1.11 to 1.07	0.98
Kmax (D)	-0.01	-0.07 to 0.50	0.77
Eccentricty (mm)	0.71	0.12 to 1.30	0.02*
Central corneal thickness (µm)	0.00	-0.07 to 0.50	0.84

Statistical analysis using multivariable linear regression. LogMAR: log of minimal angle of resolution. 95% CI: 95% Confidence Interval. ß-coefficient: value referring to how a dependent variable will change, per unit increase in the predictor variable. CDVA: corrected distance visual acuity. Kmax: maximum keratometry. UDVA: uncorrected distance visual acuity. *: significant *P*-values, significance set at < 0.05.

Table 3. Multivariable predictor analysis of selected baseline characteristics for corneal crosslinking effects at one year follow-up in keratoconus eyes

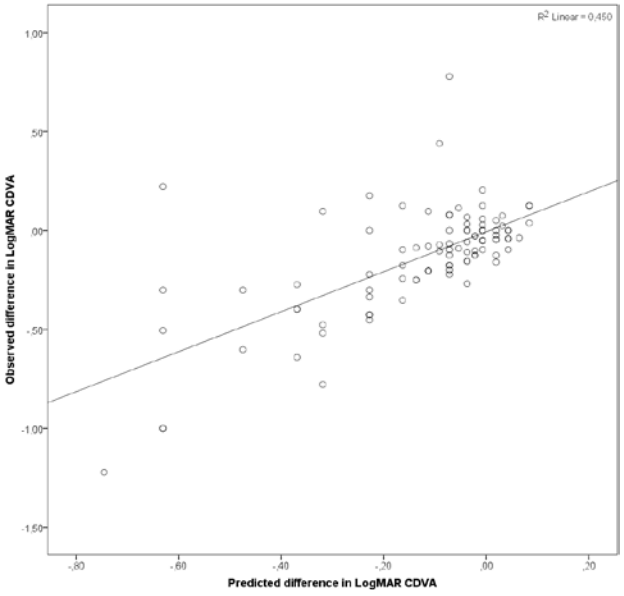

FIGURE. The observed logMAR corrected distance visual acuity values are plotted against the predicted outcomes after corneal cross-linking for keratoconus. The solid line is a linear fit of the data.

at one year follow-up was most strongly predicted by pre-operative (logMAR) CDVA alone. Correlation between predicted and measured outcomes was very high with a R^2 for model fit of 0.45, indicating excellent predictive value. The statistical prediction model for visual acuity led to the following equation: Change in logMAR CDVA = -0.518 x Baseline logMAR CDVA + 0.043. A scatter plot showing observed vs. predicted values is given in figure 1. A low pre-treatment CDVA predicts for visual improvement, though a high pre-treatment CDVA is expected to decline after treatment. Details on this relationship are given in table 4.

Keratometry at one year follow-up was predicted most strongly by cone-eccentricity, pre-operative spherical equivalent and gender. Correlation between predicted and measured outcomes however was poor with a R^2 for model fit of 0.15, indicating a low predictive value.

Baseline Snellen CDVA	Baseline logMAR CDVA	Calculated change in log-MAR CDVA*	Predicted logMAR CDVA	Predicted Snellen CDVA
20/16	-0.097	0.084	0.005	20/20
20/20	0.000	0.043	0.043	20/22
20/25	0.097	-0.007	0.090	20/25
20/32	0.222	-0.072	0.150	20/28
20/40	0.301	-0.113	0.188	20/31
20/50	0.398	-0.163	0.235	20/34
20/100	0.699	-0.319	0.380	20/48
20/125	0.796	-0.369	0.427	20/53
20/400	1.301	-0.631	0.670	20/94

CDVA: corrected distance visual acuity. LogMAR: log of minimal angle of resolution. *The statistical prediction model for visual acuity led to the following equation: Change in logMAR CDVA = -0.518 x Baseline logMAR CDVA + 0.043

Table 4. Predicted visual acuity after crosslinking treatment at one year follow-up in keratoconus eyes, based on baseline visual acuity

DISCUSSION

Principal target of this study is to investigate atopic constitution, family history and smoking as potential prognostic factors for visual acuity and keratometry one year after CXL. Patients with an atopic constitution improved less regarding visual acuity after CXL (P=0.03). Smoking habits and a family history did not seem to influence treatment outcomes. These data are not previously reported and provide potential new insights in the pathogenesis of corneal crosslinking. However, these outcomes were primarily assessed in a univariable manner and should be interpreted with caution since many potential predictors are interrelated since a potential interrelation exists between atopic constitution and visual acuity.

This study adds a multivariable analysis of predictors for CXL treatment effects in keratoconus patients. Due to a high interrelationship between many prognostic factors, only a limited number of distinct predicting factors remain. The investigated factors such as atopic constitution, family history and smoking were not significantly correlated with treatment outcomes in a multivariable analysis. Especially for smoking this was an unexpected outcome, since Hafezi et al. proved that smoking stiffens the cornea, likely to affect CXL outcomes.[22] However, the patients in our study were much younger (23 vs. 44 years), and did not smoke the many pack years Hafezi obliged for his trial inclusion (0.5 vs. 10 pack years).

Regarding visual acuity at one year follow-up, the only independent predictor found is the pre-treatment visual acuity in logMAR (ß-coefficient -0.62, $CI_{95\%}$-1.00;-0.62, P<0.01). This means that a higher logMAR at baseline is associated with an improvement in visual acuity. Cone eccentricity was associated with visual acuity at follow-up in the univariable analysis, however this effect disappeared in the multivariable analysis. This could be explained by the fact that the eccentric cones had a better VA at baseline (data not shown), indicating an interrelationship between cone location and VA.

Keratometry outcomes at one year follow-up are predicted solely by the eccentricity of the cone (ß-coefficient 0.71, $CI_{95\%}$ 0.12;1.30, P=0.03). A more eccentric cone is associated with a higher keratometry at one-year follow-up. No other factors remained significant in a multivariable analysis, for either visual acuity or keratometry. The role of cone eccentricity is confirmed in literature. A possible explanation for eccentricity of the cone being the only predicting factor for Kmax outcome could be attributed to the employed UV-light-source, using a flat, perpendicular emission plane. The peripheral cornea is exposed to less intense UV light compared to the central part, owing to an oblique

incident angle of UV light rays.[21] Another explanation could be that the biomechanical effect of cross-linking tends to make the cornea more symmetric, causing a peripheral cone to migrate more centrally. These considerations were previously addressed by Greenstein et al.[22]
Higher age is regarded as a prognostic factor for the development of keratoconus. The chances of progression are lower for an older individual, but ectatic progression at higher age has been reported. The role of age as predictors for crosslinking effectiveness are debated in our study since our multivariate analyses do not indicate them as independent factors. All our patients demonstrated a topographic progression of >1D prior to treatment, independent of their age. A subgroup analysis of our patients above 35 years old showed that crosslinking effectiveness did not deviate from the entire cohort (data not shown).

The final target of this study was to create prediction models to aid the ophthalmologist with clinical decision making. We succeeded to do so for visual acuity (VA), with a R^2 of 0.45. This model showed that CDVA one year after treatment can be predicted reliably, based on pre-treatment CDVA. Patients with a low CDVA are likely to improve in corrected distance visual acuity. For a Snellen CDVA of 20/25 and better, this effect diminishes and even seems to reverse. The proposed prediction model for keratometry did not lead to accurate estimates, based on pre-treatment parameters.

A strength of our study is in its data completion and standardized examinations, gathered almost exclusively by one trained optometrist (NS). Other strengths of our study are the degree of data completion and the low percentage of lost to follow-up, making our outcomes unlikely susceptible for attrition bias. Treatment outcomes after CXL are in line with other studies[2-4], supporting the generalisability of our analyses. We experience little CXL safety concerns in our treatment cohort, with an absence of infectious complications, stable endothelial cell densities, and only rarely a persisting haze.

A consideration is that model outcomes are statistical by nature and do not necessarily reflect a pathophysiological process. One hypothesis regarding the improvement in vision could be that on average the crosslinked cornea has a more regular shape, with subsequent better VA. Another is that some baseline characteristics, like cone eccentricity, are interrelated with visual acuity at baseline. The independent predictive effect is not just caused by mere visual acuity, but also by the improvement of its interrelated components. The reversal of this effect at high pre-treatment VA might be

due to regression to the mean. By chance only, a very good VA pre-treatment is likely to have a more average VA at follow-up measurement. This is supported by our clinical experience that these patients do not report a loss in VA. Another consideration is that the choice of included predictors remains arbitrary. We ensured all currently regarded predicting factors and pre-treatment measurements were included in univariable analysis. But a presently unknown though important and interrelating factor could be overlooked.

In conclusion, multivariable predictor analysis of crosslinking effects for keratoconus elucidate the large interrelation of previously identified predictors. Cone eccentricity and corrected distance visual acuity are identified as individual predictors for crosslinking treatment effects at one year follow-up in keratoconus. Cone eccentricity is negatively associated with corneal flattening and visual acuity after treatment can be predicted by using its baseline value. The proof of these prediction models would be to subject a new crosslinking dataset to our methods and compare predicted with measured outcomes.

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Contributions of Authors

Design of the study (AL,RW); Conduct of the study and collection of the data (NS,DG); Management, analysis and interpretation of the data (RW,DG); Preparation, review and approval of the manuscript (RW, DG, NS, AL, SI)

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7

The independent effects of higher-order aberrations one year after corneal crosslinking for keratoconus.

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Submitted

ABSTRACT

Purpose

To investigate the effect of corneal crosslinking (CXL) in progressive keratoconus patients on higher-order aberrations (HOAs) and the effect of change in HOAs on visual acuity between baseline and one year after CXL.

Methods

This study included 187 consecutive keratoconus patients in one academic treatment center who were treated with epithelium-off CXL and followed for a minimum of one year. The following corneal HOAs were reported as measured with Scheimpflug tomography: coma, trefoil, spherical aberration and total corneal HOAs. A T-test was used to compare baseline and postoperative aberrations. A multivariable linear regression was applied to assess the independent effects of HOA subtypes on changes in uncorrected and corrected distance visual acuity (UDVA/CDVA).

Results

Overall, the average degree of corneal HOA in the patient cohort was relatively unchanged after CXL, with an average change of -1.34% (P=0.272). Horizontal coma contributed most to the total amount of HOA, but was virtually unchanged on average. The HOA subtype spherical aberrations did decrease significantly (-15,68%, P<0.001). There was no effect of the change in HOAs on the change in CDVA, but there was a significant effect of the change in horizontal coma on the change in UDVA (P= 0.003; B -0.475).

Conclusions

Corneal HOAs in general were relatively unchanged from baseline one year after crosslinking to treat progressive keratoconus. Change in horizontal coma has a strong and independent effect on uncorrected visual acuity.

INTRODUCTION

Keratoconus is a progressive corneal disease in which an ongoing loss of stromal tissue leads to irregular astigmatism and reduced quality of vision.[1] In recent years, corneal crosslinking (CXL) has become an established treatment modality designed to increase the mechanical and biochemical strength of the stromal tissue.[2] The effectiveness of CXL stems from its potency to stabilize keratoconus and its effects on corneal curvature.[3] Specifically, crosslinking flattens the cone, which in turn increases both uncorrected and corrected visual acuity.[4–6] This flattening can persist for several years or longer.[7] Factors that can potentially predict treatment outcomes following CXL have been studied extensively.[5] CXL is considered to be a safe, effective, and predictable treatment for the prevention of keratoconus progression. With respect to safety, adverse events occur in a minority of cases, with only a small risk of severe keratitis.[8] A transient demarcation line or subepithelial haze has been reported following CXL, although these are rarely observed one year after treatment. High visual acuity (CDVA >20/25) is not generally regarded as an exclusion criterion for performing corneal crosslinking.[2] This fact—combined with the favorable safety profile and increased availability of crosslinking—has led to an increase in the number of patients with high visual acuity who receive this treatment. The archetypal corneal curvature in keratoconus contributes to an increase in higher order aberrations (HOAs) and subsequent decreased spectacle-corrected visual acuity. Overall, visual acuity is reported to increase after crosslinking.[5] But from a clinical perspective, assessing whether acceptable levels of HOAs can be retained after crosslinking is important.

Here, we examined the relationship between visual acuity, manifest refraction and changes in HOAs one year after crosslinking for keratoconus in a large treatment cohort of consecutively treated patients. We also assessed whether HOA subtypes contribute independently to visual acuity outcomes or manifest refraction using multivariable modeling.

METHODS

Dataset and study design

Data were derived from an ongoing prospective treatment cohort of patients at our institution who underwent CXL for treating progressive keratoconus. We included all consecutive patients who were treated at the University Medical Center Utrecht from January 2010 through April 2013, with one-year of follow-up. The following inclusion criteria were applied: a progression of maximum keratometry (Kmax) >1.0 D within 6-12 months and corneal thickness >400 µm (at the thinnest point). The exclusion criteria included corneal scarring, the presence of a concurrent infection, and pregnancy and/or lactation. Treatment effects were assessed at the one-year follow-up visit. The detailed data collection and surgical procedure were reported previously and were adapted for this study.[7] This study of HOAs in this treatment cohort was approved by our institution's Ethics Review Board, and the requirement for informed consent was waived.

Surgical procedure

An epithelium-off procedure was performed following the Dresden protocol.9 The cornea was exposed to a 3 mW/cm2 UV light source (UV-X, Peschke Meditrade GmbH, Germany, equipped with a perpendicular emission plane) with a wavelength of 365 ± 10 nm for a total exposure time of 30 minutes.

Data collection

Measurements included uncorrected distance visual acuity (UDVA), corrected distance visual acuity (CDVA), corneal tomography (Pentacam HR type 70900, Oculus GmbH), endothelial cell count (SP-3000P, Topcon), and automated tonometry (CT-80, Topcon). If the tomogram failed to reach the 90% quality criterion it was repeated up to three times and the best scan was used for HOA calculation. CDVA was measured using manifest refraction, taken by one optometrist (NS). The measurements were repeated one, three, six, and twelve months after crosslinking. All patients were requested to stop using their contact lenses two weeks before each evaluation.

Assessment of corneal optical aberrations

Corneal optical aberrations were calculated using the Pentacam software program, based on the central 6.0 mm as determined by the corneal apex, of anterior and posterior elevation maps obtained using Scheimpflug imaging. The software program

reports corneal optical aberrations for the anterior and posterior surfaces, as well as for the total cornea. Total cornea optical aberrations were chosen as the outcome parameter. The Pentacam software then subdivides this outcome into the following two composite values: total corneal lower-order aberrations, and total corneal higher-order aberrations. Normalized coefficients were used, expressed in microns of wavefront error (RMS) and labeled with ISO standardized double index Zernike symbols. HOAs were reported with their Zernike weight coefficient since the polynomial coefficient is considered invariant. Total corneal HOAs were calculated based on the 3rd to 8th order aberrations. The following HOA subtypes were reported in detail: horizontal and vertical coma (Z_3^1 and Z_3^{-1},respectively), horizontal trefoil and vertical trefoil(Z_3^3 and $Z_{3^-}^3$, respectively), and spherical aberration (Z_4^0).

Statistical anzalysis

Visual acuity was converted to the logMAR of visual acuity (VA). UDVA and CDVA were both used as outcome parameters. A paired-sample Student's t-test was used to determine significance between HOAs at baseline and one year after CXL. In cases with missing data at the one-year follow-up time point, the 6-month follow-up data were entered, if available (i.e., the last measurement was carried forward). We compared the baseline characteristics of the cases lost to follow-up with all other cases in the cohort. Linearity of the baseline data and outcome measurements was determined visually in a scatter plot, normality was tested based on skewness, and kurtosis was based on a cut-off value of 3.29 (P<0.001). Mutual correlations between the different HOA subsets were calculated.

Univariate analyses with changes in UDVA and CDVA as dependent variables were performed for all baseline parameters to aid in identifying potential confounders for the relationship between changes in HOAs and changes in UDVA/CDVA. The following factors were determined to be potential confounders: VA at baseline and the lower order aberrations (LOAs: defocus (Z_2^0), horizontal astigmatism and vertical astigmatism (Z_2^2 and Z_2^{-2}, respectively). These factors were entered into the multivariable analysis. This analysis was performed using generalized estimating equations to correct for patients for whom both eyes were included in the dataset. Data collection and analyses were performed using SPSS version 21.0 (IBM). Patients who developed post-operative scarring and/or haze formation were excluded from the HOA analysis, as this this might reflect a pathophysiological mechanism other than a change in corneal curvature that affected VA.

RESULTS

Dataset characteristics

One-hundred-and-eighty-seven eyes of 162 patients were treated consecutively at our institution. Five of these 187 eyes (2.6%; 3.1% of patients) were excluded from analysis due to a loss to follow-up, and eight eyes (4.3%; 4.9% of patients) had the last follow-up measurement carried forward (see methods). The baseline characteristics of these 13 patients did not differ significantly from the main group; however, only patients with an affected right eye were lost to follow-up. The baseline characteristics of the participants are summarized in Table 1.

Clinical outcomes

At the one-year follow-up visit, Kmax either decreased or was unchanged in 164 of 187 eyes. In 16 eyes, the keratoconus progressed by >1.0 D, with a mean increase in Kmax of 2.6 SD± 2.0 D (range: 1-9.40 D). Visual acuity improved significantly at the one-year follow-up compared to baseline for both logMAR UDVA (from 0.81 to 0.71, P=0.002) and logMAR CDVA (from 0.33 to 0.20; P<0.001). The cylinder value obtained using manifest refraction increased significantly with 0.62D (P< 0.001) on average, where the corneal astigmatism obtained using tomography remained virtually stable (-0.06D; P=0.493). Endothelial cell density was unchanged from baseline; mean cell density at follow-up was 2526 SD±366 cells/mm^2, with no apparent clinical signs of endothelial dysfunction. At the one-year follow-up, 16 eyes had a slight—albeit persistent—haze. The baseline

	Baseline		Post-CXL		P value	Corr.	LTFU
	mean	SD	mean	SD			
logMAR UDVA	0.81	0.51	0.71	0.52	0.002	.692	9%
logMAR CDVA	0.33	0.35	0.20	0.29	< 0.001	.562	3%
Manifest refraction (D)							
Sphere	-0.75	3.28	-0.11	3.58	0.002	.689	3%
Cylinder	-3.15	2.20	-3.77	2.41	< 0.001	.516	3%
Maximum keratometry (D)	58.6	8.2	57.4	8.1	< 0.001	.950	3%
Thinnest pachymetry (µm)	456	42	448	47	< 0.001	.895	3%
Astigmatism* (D)	4.12	2.65	4.06	2.59	0.493	.903	3%

CXL: crosslinking. UDVA: uncorrected distance visual acuity. CDVA: corrected distance visual acuity. P: paired-samples t-test with missings excluded pair wise. Corr.: correlation coefficient. LTFU: lost to follow-up. *: corneal astigmatism power as reported by scheimpflug tomography

Table 1. Characteristics of 187 eyes of 162 patients at baseline and at one-year follow-up

	Baseline		Post-CXL		%	P value	Corr.
	Mean	SD	Mean	SD			
Compound variables (RMS)							
Total corneal aberrations	5.751	2.994	5.378	2.886	-6.49	< 0.001	0.934
Corneal LOAs	5.601	2.939	5.221	2.824	-6.78	< 0.001	0.932
Corneal HOAs	1.268	0.647	1.251	0.665	-1.34	0.272	0.955
HOA subtypes							
Horizontal coma	-0.888	0.562	-0.888	0.621	0.00	0.995	0.950
Vertical coma	-0.082	0.488	0.001	0.483	-101.22	0.465	0.950
Horizontal trefoil	0.048	0.176	0.061	0.166	27.08	0.374	0.444
Vertical trefoil	0.017	0.148	-0.007	0.159	-141.18	0.101	0.291
Spherical aberration	-0.370	0.416	-0.312	0.418	-15.68	< 0.001	0.911

CXL: crosslinking. RMS: Root Mean Square. HOA: higher order aberration. LOA: lower order aberration. P= paired samples T-test. *: eyes with post-operative haze excluded from analysis

Table 2. Changes in corneal optical aberrations one year after crosslinking (n = 166*)

characteristics of this subgroup did not differ significantly from the main group, with the exception of poorer mean logMAR CDVA (0.52 vs 0.31, respectively; *P*=0.026); in 14 of these 16 eyes, pre-existing striae were noted. Mean logMAR CDVA at follow-up was also significantly worse in this subgroup (0.37 vs 0.17, respectively; *P*=0.011). These 16 eyes were excluded from further HOA analysis. None of the patients in the cohort developed infectious keratitis.

Change in higher-order aberrations

The absolute values for optical aberration at baseline and one year after CXL, as well as the percentage of change, are summarized in Table 2. Total lower order aberrations significantly decreased after crosslinking treatment (*P*<0.001). However, total higher order aberrations did not (with a mean change of only -1.34%; *P*=0.272), although the HOA subtype spherical aberration did significantly decrease (*P*<0.001). The effect size of this decrease was relatively small. Vertical coma HOAs contributed the most to the total corneal HOAs, but this subtype did not change significantly after treatment. Univariate confounder analysis for CDVA identified baseline spherical refraction (*P*=0.037; B 0.12), Kmax (*P*=0.004; B -0.009), baseline logMAR UDVA (*P*=0.034; B -0.102), and baseline logMAR CDVA (*P*<0.000; B 0.748) significantly associated with the dependent variable. Based on the effect size only pre-treatment CDVA was considered a relevant confounder. Next for UDVA, only baseline logMAR UDVA was significantly associated (*P*=0.003; B 0.257) and considered relevant. An analysis of mutual correlations for each

HOA subtype revealed a significant correlation for horizontal (Z_3^1) and vertical (Z_3^{-1}) coma (*P*=0.009; ρ: -0.204), horizontal (Z_3^3) and vertical (Z_3^{-3}) trefoil (*P*=0.006; ρ:0.213), and vertical coma and vertical trefoil (*P*=0.001; ρ: 0.264).

Multivariable analysis

The results of the multivariable analysis for both CDVA and UDVA are displayed in Table 3. Here the calculated effects of the potential confounders (visual acuity and LOAs at baseline) and the HOA subtypes are given for both determinants. No independent relationship between any HOA variable and change in CDVA was observed. The putative confounder CDVA at baseline was indeed strongly related to the change in CDVA. Interestingly, an independent effect of the change in horizontal coma was observed on the change of UDVA (*P*= 0.003; B -0.475), and again UDVA at baseline was strongly related to this changes.

	B coefficient	95% CI	P value
CDVA			
Confounding factors at baseline			
CDVA	-0.575	-0.724 to -0.426	<0.001*
Defocus	0.000	-0.050 to 0.049	0.993
Horizontal Astigmatism	-0.091	-0.223 to 0.042	0.180
Vertical Astigmatism	0.051	-0.068 to 0.171	0.398
HOA subtypes			
Δ Horizontal coma	0.032	-0.195 to 0.130	0.698
Δ Vertical coma	-0.095	-0.378 to 0.188	0.511
Δ Horizontal trefoil	0.068	-0.215 to 0.351	0.638
Δ Verical trefoil	-0.093	-0.318 to 0.132	0.416
Δ Spherical aberration	-0.084	-0.442 to 0.275	0.647
UDVA			
Confounding factors at baseline			
UDVA	-0.315	-0.432 to -0.198	<0.001*
Defocus	0.062	0.010 to 0.115	0.020
Horizontal Astigmatism	-0.081	-0.217 to 0.056	0.247
Vertical Astigmatism	-0.011	-0.278 to 0.257	0.937
HOA subtypes			
Δ Horizontal coma	-0.475	-0.787 to -0.163	0.003*
Δ Vertical coma	0.205	-0.273 to 0.684	0.400
Δ Horizontal trefoil	0.044	-0.230 to 0.318	0.753
Δ Verical trefoil	-0.060	-0.423 to 0.303	0.746
Δ Spherical aberration	-0.346	-0.909 to 0.217	0.228

CDVA: corrected distance visual acuity. UDVA: uncorrected distance visual acuity. LOA: lower order aberrations. HOA: higher order aberrations. 95% CI: 95% Confidence Interval. RMS: Root Mean Square. Δ = changes in variable post cross-linking. *: Significant P values

Table 3: Multivariable analysis of the effect of a change in optical aberrations on CDVA and UDVA one year after crosslinking (n=166)

DISCUSSION

The principal aim of this study was to report on higher-order aberrations one year after performing corneal crosslinking to treat keratoconus, and to investigate whether variations in HOA are independently associated with a change in (corrected) distance visual acuities. On average, with the exception of spherical aberration HOAs, the higher-order aberrations were largely unchanged following treatment. A multivariable analysis revealed no independent effect of any higher-order aberration subtype on change in CDVA after crosslinking. However, changes in horizontal coma were significantly and strongly associated with the post-operative change in UDVA. Strikingly, the measured corneal astigmatism did not change on average (4.12 vs. 4.06D), but the manifest refraction did increase and became more in agreement with the topographical cylinder (-3.15 vs. -3.77D; $P < 0.001$).

A major strength of this prospective study is the inclusion of a relatively large treatment cohort (187 eyes from 162 patients), with very few cases lost to follow-up (approximately 3% of patients). The intervention was standardized, in accordance with current protocols, and did not change throughout the course of study. All patients underwent epithelium-off crosslinking with non-accelerated UVA irradiation, and all refractions were measured by an optometrist experienced in keratoconus care (NS). Moreover, the treatment outcomes (i.e., improvement in keratometry, UDVA, and CDVA) are consistent with recent published literature.[7,10,11] Furthermore, we focused on the HOA subtypes that are most relevant to clinical practice (i.e., coma, trefoil, and spherical aberration), and the effect of more complex forms of optical aberrations were assessed via the compound HOA variable. The Pentacam software program calculates the total corneal HOA based on anterior and posterior elevation maps. We therefore chose to measure these composite HOAs, as the individual anterior and posterior outcomes are less relevant from a patient-oriented perspective.

On the other hand, several features of our study and analysis may have affected our results. First, we used the Pentacam software program, which calculates/expands optical aberrations, rather than using an aberrometer, which measures optical aberrations. A wavefront device was not used in this study and we are unable to determine whole eye HOAs. Furthermore, internal optical aberrations can potentially compensate for aberrations that are attributable to the anterior segment; however, a previous study reported that these internal optical aberrations are relatively unchanged following

after corneal treatment.[12] Our study design could be considered suitable to detect changes in corneal HOAs after treatment, rather than measuring whole eye HOAs. The Pentacam is considered a reliable instrument for assessing corneal shape with good repeatability and reproducability[13–16], although recent papers debate its reproducibility with regards to the HOA assessment. A second consideration is that we excluded cases with an apparent corneal haze from our analysis. Corneal haze can—at least in principle—affect optical aberrations without changing the corneal curvature (or the resulting elevation maps). Although the Pentacam can perform densitometry measurements, these measurements are not used to calculate corneal HOAs.[17] A corneal haze may have influenced the edge detection software; however, this likely had little effect, as all of the Scheimpflug images used in this study were of sufficient quality.

Previous reports of post-CXL HOAs point towards a general decrease in ocular HOAs. For example, Greenstein et al. reported a significant decrease in corneal coma HOAs based on anterior and posterior elevation maps.[18] The authors also found no significant correlation between HOA and the change in visual acuity, although their analysis was based on only 31 keratoconus eyes. In 2009, Vinciguerra et al. reported a significant decrease in total ocular HOAs, coma HOAs, and spherical aberrations (n=28 eyes).[19] In a more recent study using a larger cohort (n=92), the same group reported a decrease in total HOAs and coma HOAs, but not in spherical aberrations.[6] They did not, however, examine the correlation between HOAs and treatment outcome. The authors used absolute values to calculation the change in HOA, thus accounting for shifts from negative to positive HOAs. Here, we chose to report the outcomes as they were supplied by the Pentacam. Analyses were performed based on absolute values and did not materially alter our findings (data not shown). Ghanem et al. reported a significant decrease in total corneal aberrations two years after CXL (n=42), calculated based on topographies, but the contribution of HOAs herein is not convincing. Coma, trefoil and spherical aberration are attenuated significantly, but the statistical analysis did not account for multiple testing and the absolute differences are rather low.[20] Baumeister et al. reported no significant change in HOAs at the 6-month follow-up visit (n=20).[21] This finding is more consistent with our finding that—on average—no relevant change in corneal HOAs was observed. A recent study by Buzzonetti confirmed a stable amount of HOAs 15 months after iontophoretic transepithelial CXL in children (n=14), also calculated from Scheimpflug corneal imaging. By using ISO standard double indexed Zernike polynomials, we put effort to present our findings unambiguously.[24]
Previous experimental research showed that the individual Zernike polynomials have

a different impact on visual function; spherical aberration RMS error contributes more than coma, which in turn contributes more than trefoil.[23,24] Our results do not repeat that finding, since horizontal coma had the strongest relationship with changes in UDVA in our multivariable analysis. Naturally, keratoconus eyes have a different distribution of HOAs than healthy eyes, and especially decentered cones may induce high amounts of coma.[25]

The inconsistency in our data of changes in astigmatism as obtained using manifest refraction and corneal tomography deserves attention. On average, manifest cylinder measurements increased where topographic derived corneal astigmatism did not. This effect could partly be attributable to the inability to correct for HOAs using spectacles. A wrong amount of astigmatic correction can be measured when the cylinder axis is placed on top of the coma, since then the patients perceives a slight improvement. We hypothesize that an increased visual acuity leads to an improved quality of manifest refraction, where the better perception of coma partly translates to a higher manifest refraction. The discrepancy of the independent effect of horizontal coma in UDVA vs. CDVA might reflect this. Without spectacle correction horizontal coma is a strong independent factor for visual acuity, however after a manifest refraction this effect diminishes (on average). Is the horizontal coma accidentally corrected by increasing cylinder power, meaning that it lost its independent effect on visual acuity? On the other hand, one can debate whether the Pentacam is the best tool to detect these subtleties in corneal tomography.

Determining the true effects of crosslinking requires disentangling many interrelated variables.5 The continuous flattening of the cone is a structural parameter that can affect HOA, and the possible migration of the cone apex can result in reduced cone eccentricity.[7,25] Changes in corneal collagen fibril composition and/or the development of corneal haze can exert effects on both contrast sensitivity and HOA.[17] We therefore used a structured approach to identify potential confounders regarding the role of measured HOAs on changes in visual acuity, and we assessed the independent contribution of each HOA subtype on treatment outcome.

In conclusion, we report that on average, higher-order aberrations are essentially unchanged one year after performing corneal crosslinking to treat progressive keratoconus when assessed using Scheimpflug imaging. Only changes in horizontal coma had a strong and independent effect on uncorrected visual acuity.

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Potential conflict of interest

None of the authors has any commercial or proprietary interest in the products mentioned in the manuscript. The manuscript has been presented as a poster on the annual Dutch Ophthalmic Society meeting, Groningen, The Netherlands, March 2015.

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8

A comparison of the reliability of the Diaton transpalpebral tonometer with Goldmann applanation tonometry for the assessment of intraocular pressure in keratoconus patients.

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ABSTRACT

Aim

To investigate the added value of using a Diaton transpalpebral tonometer (DT) to measure IOP in keratoconus. Most type of tonometers use corneal applanation or biomechanical resistance to measure intraocular pressure (IOP); however, these factors can be altered by keratoconus. Specifically, we examined whether DT can detect false-negative low Goldmann applanation tonometry (AT) measurements.

Methods

Patients with keratoconus were recruited from our tertiary academic treatment center. Measurements included AT and DT (in random order) and Scheimpflug imaging. An age- and gender-matched group of control subjects with no history of corneal disease or glaucoma was also recruited.

Results

In total, 130 eyes from 66 participants were assessed. In the keratoconus group, mean AT was 11.0 ± 2.6, mean DT 11.2 ± 5.5 (P=0.729), and the two measures were correlated significantly (P=0.006, R=0.323). However, a Bland-Altman plot revealed a wide distribution and poor agreement between both measurements. Previous corneal crosslinking, corneal pachymetry, and Krumeich classification had no effect on measured IOP.

Conclusions

Measurements obtained using a Diaton tonometer are not affected by corneal biomechanics; however, its poor agreement with Goldmann AT values calls into question the added value of using a Diaton tonometer to measure IOP in keratoconus.

Keywords

Diaton, Goldmann applanation tonometry, transpalpebral tonometry, keratoconus, Bland-Altman plot

INTRODUCTION

The presence of corneal pathology can potentially affect measurements of intraocular pressure (IOP) and several methods for measuring IOP in corneal pathology have been described[1–3]. For example, Rosentreter et al. compared rebound tonometry, applanation tonometry, and dynamic contour tonometry in pathological corneas.[1] However, all of these devices depend upon corneal applanation and/or biomechanical resistance. Both factors can be altered by keratoconus, a progressive condition with thinning of the cornea, irregular astigmatism, and decreased biomechanical resistance.[4–7] In particular, the thinning of the cornea can be extremely severe; applanation of such a thin cornea potentially requires much less pressure and can therefore result in an underestimation of the actual IOP.[8] This effect has been observed when measuring IOP in healthy corneas with varying corneal pachymetry measurements,[9] and this phenomenon was proposed as a factor in normal-tension glaucoma.[10] Specifically, the irregular shape of the cornea might prevent the Goldmann applanation tip from aligning properly, thus preventing uniform contact; this problem is not an issue with other methods (for example, rebound tonometry). Corneal rigidity can further be altered by corneal crosslinking, a widely used procedure for preventing the progression of keratoconus.[11] The effect of crosslinking on various IOP measuring methods has been studied, and these studies revealed increased IOP readings after crosslinking. It is important to note that all devices depend on corneal rigidity for their accuracy.

To circumvent this problem, the Diaton tonometer (DT, manufactured by Ryazan State Instrumental-Making Enterprise, Ryazan, Russia, http://www.diaton-tonometer.com) uses an alternate method to measure IOP; the movement pattern of a small rod falling freely onto the eyelid surface is measured and individual measurements are displayed digitally. The DT is a portable, hand-held device that measures transpalpebral IOP through the patient's upper eyelid while the patient is in a reclined or supine position. The DT has been promoted as a suitable alternative method of tonometry for patients with conjunctivitis and/or corneal disease, or following corneal surgery.[12] Previous research found that the DT is reliable in patients without corneal disease and provides measurements that are similar to Goldmann applanation tonometry (AT); however, DT yields results with wider variation and lower correlation with repeated measurements.[13–15] Thus, the value of using DT for glaucoma screening has been questioned.

Because applanation IOP measurements in keratoconus patients can underestimate

the actual IOP, and because of the claims made by the manufacturer, we investigated the added value of measuring transpalpebral IOP using DT compared to Goldmann applanation tonometry in patients with keratoconus. Specifically, we examined whether false-negative (i.e., low) AT measurements in keratoconus can be detected using DT.

MATERIALS AND METHODS

Patients were recruited from the cornea outpatient clinic in our tertiary academic center from October 2013 through January 2014. The inclusion criteria included a current diagnosis of keratoconus and no gross anatomical eyelid abnormalities hampering DT measurement; patients of all ages were eligible for inclusion. Corneal scarring and/or previous crosslinking treatment did not preclude patients from participating. An age- and gender-matched control group was recruited and consisted of healthy volunteers with no history of corneal disease, ocular hypertension, or glaucoma.

All measurements were collected by one examiner (NP) under standardized conditions; DT measurements were taken in the supine position in accordance with the manufacturer's instructions. The Diaton tonometer indicates the number of measurements necessary for each eye and provides a single reading. AT was measured using standard procedures. The order of IOP measurements (i.e., DT followed by AT versus AT followed by DT) was randomized. All patients underwent a slit-lamp evaluation and Scheimpflug corneal imaging (Pentacam HR type 70900, Oculus GmbH) prior to the IOP measurements. All keratoconus eyes were diagnosed and graded using the Krumeich classification system by one corneal specialist (RW).[16]

Statistical analyses were performed using SPSS version 20.0 (IBM). Box plots, scatter plots, and Bland-Altman plots were used to visualize the outcomes.[17] Differences in AT and DT readings were analyzed using the Student's t-test. A linear regression model using a generalized estimating equation (correcting for patients with two affected eyes) was used to assess the relationship between the difference in IOP and pachymetry and Krumeich classification. Normality was tested based on skewness and kurtosis, with a cut-off value of 3.29 (P<0.001).

This study was approved by our institution's Ethics Review Board and was performed in accordance with the Declaration of Helsinki. None of the eligible participants refused to participate, and all subjects provided informed consent.

RESULTS

One-hundred-and-thirty eyes from 66 participants were initially enrolled; 36 keratoconus patients had 70 eyes with keratoconus. Two eyes from one patient in the keratoconus group were excluded from the analysis due to missing AT measurements. The mean age (±SD) of the subjects in the keratoconus and control groups was 25.8 ±9.3 and 33.1 ±9.8 years, respectively; 62% and 56% of the subjects were male in the keratoconus and control groups, respectively. Baseline characteristics did not differ significantly between the two groups. Among the eyes with keratoconus, 40 (63%) previously underwent corneal crosslinking (CXL). The grading of the keratoconus eyes (based on the Krumeich classification system[16]) was as follows: 23% were grade I, 56% were grade II, 10% were grade III, and 11% were grade IV. The mean value for thinnest corneal pachymetry was 451 ± 57 µm. None of the patients had a history of glaucoma or ocular hypertension.

Applanation vs. Diaton iop measurements

In the keratoconus group, mean IOP measured using AT and DT was 11.0 ± 2.6 mmHg and 11.2 ± 5.5 mmHg, respectively (P=0.729). In the healthy control group, mean IOP measured using AT and DT was 12.7 ± 2.7 mmHg and 7.3 ± 2.5 mmHg, respectively (P<0.001). Thus, the mean difference between the AT and DT measurements in the keratoconus and control groups was -0.2 ±5.2 mmHg and 5.5 ± 3.5 mmHg, respectively (P<0.001). The IOP measurements of keratoconus eyes that received CXL did not differ significantly from their untreated counterparts: AT measurements were 10.8 mmHg vs. 11.5 mmHg (P=0.295), and DT measurements 11.9 mmHg vs. 10.2 mmHg (P=0.194). The mean difference between AT and DT measurements after CXL changed from 1.3 ± 5.4 mmHg to -1.1 ± 4.8 mmHg (P=0.057). Similar results were obtained regardless of whether the AT or DT measurement was taken first (data not shown). The AT and DT measurements in the two groups are summarized in Figure 1.

Correlation between DT and AT IOP measurements

The correlation between the DT and AT measurements was low but significant in the keratoconus group (R^2=0.104 P=0.006), but not in the healthy control group (R^2=0.017, P=0.316). The measurements and their correlation coefficients are shown in Figure 2; R^2 is given for absolute IOP measurements. Trend lines are added to highlight the lack of agreement; perfect agreement would result in a trend with a 45° slope through the origin.

Figure 3 shows a Bland-Altman plot of the AT and DT measurements in the keratoconus

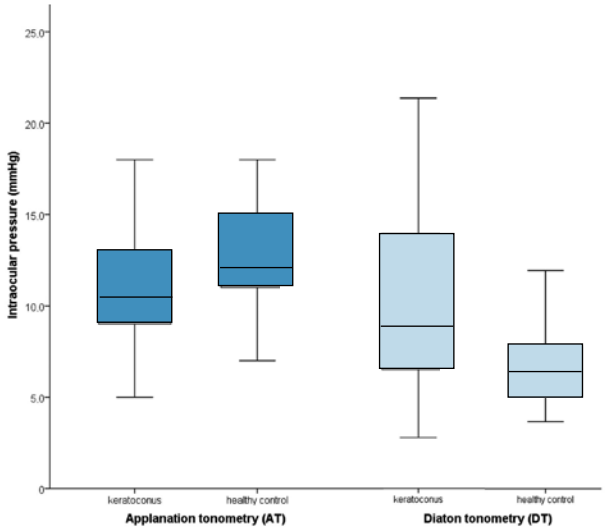

Figure 1: IOP measurements with applanation tonometry (AT) vs. Diaton tonometry (DT) in keratoconus and healthy controls. The mean IOP was comparable in the AT-group (*P*=0.729), and significantly lower for healthy controls in the DT-group (*P*<0.001)

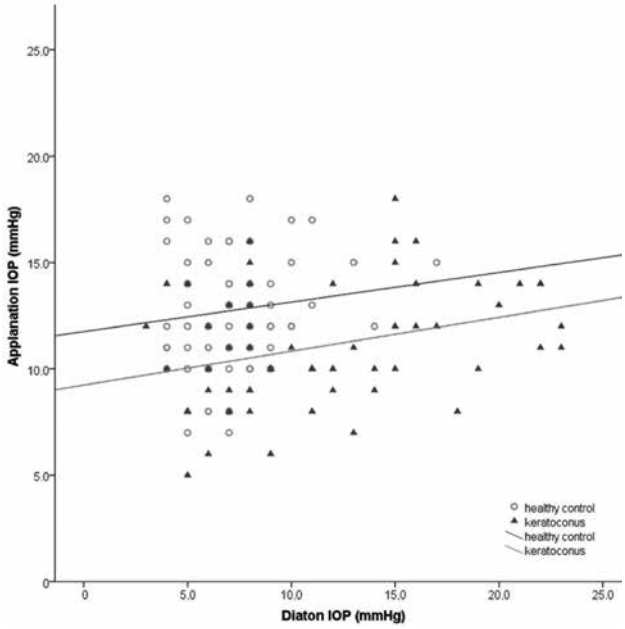

Figure 2: Correlation of applanation tonometry (AT) vs. Diaton tonometry (DT) IOP measurements for the keratoconus group (R2=0.104 *P*=0.006) and healthy controls (R2=0.017, *P*=0.316). Trend lines are given for both groups.

group. Although the mean difference is extremely small (-0.21 mmHg), a big variation of measurements is visualized. This variation exists at low mean IOP levels (left side of the plot) as well as at higher mean IOP levels (right side of the plot). The SD of the difference between the AT and DT measurements is 5.2 mmHg, which means that 27% of the DT measurements differed from their corresponding AT measurement by >1 SD. Only 16% of the measurements are within 2 mmHg range of agreement.

Effect of pachymetry and keratoconus staging on outcomes

Linear regression analysis revealed a small, non-significant effect of pachymetry on the difference between the AT and DT measurements (B: -0.011; $CI_{95\%}$: -0.032 to 0.010; $\chi 2$: 1.022; *P*=0.312), which means that a difference in pachymetry of 100µm estimates a lower difference between AT and DT of 1.1mmHg Krumeich classification had no effect on the difference between the AT and DT measurements ($\chi 2$: 1.331; *P*=0.722).

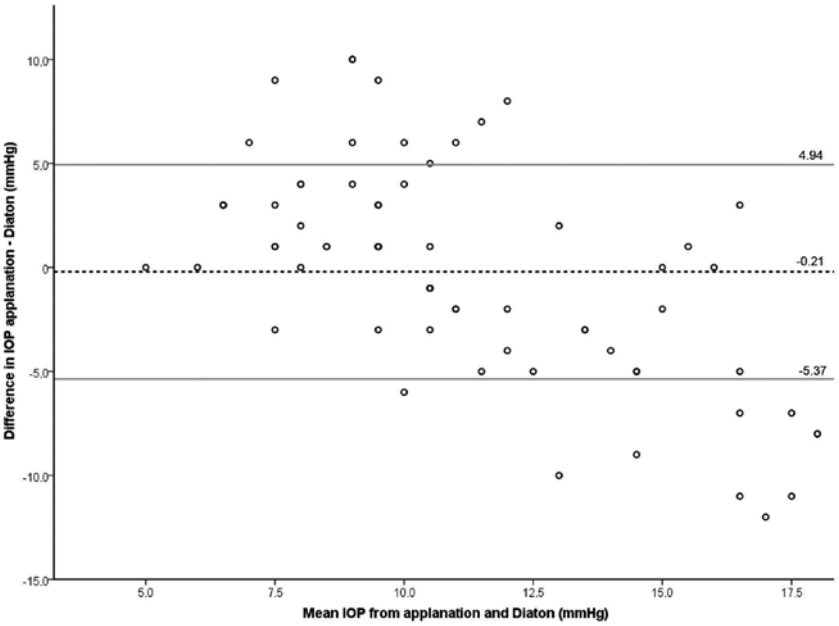

Figure 3: Bland-Altman plot of the agreement of applanation tonometry (AT) vs. Diaton tonometry (DT) in keratoconus patients (n=70). The dashed line represents the mean difference (-0.21 mmHg). The solid lines represent the ±1SD of the mean difference (±5.2mmHg). Note the high spread number of measurements; 16% of measurements are within a 2mmHg range of agreement.

DISCUSSION

In this study, we investigated the added value of performing transpalpebral tonometry versus Goldmann applanation tonometry to measure intraocular pressure in keratoconus. The small mean difference of IOP measurements in keratoconus between both instruments suggest that DT could be an alternative for AT. However, the wider variability of DT measurements and their poor correlation to AT renders the use of the Diaton tonometer in keratoconus debatable.

These findings are consistent with two large studies in which Diaton tonometry was used to measure IOP in eyes without corneal disease.[13,14] Both studies reported remarkably poor agreement between DT and AT measurements and concluded that DT is not a feasible substitute for AT in routine clinical practice. However, patients generally favor DT over AT, particularly young patients.[13,14] Nevertheless, Goldmann applanation tonometry remains the gold standard for measuring IOP, although other devices have been studied extensively and are considered suitable alternatives.[2,18–20] The ocular response analyzer in particular combines IOP measurements with information on CCT and corneal hysteresis.[20]

It is important to note that all IOP measurements were within the normal range; the highest recorded IOP was 23 mmHg. We cannot draw conclusions for higher IOP ranges. In our measurements, we did not account for eyelid abnormalities due to allergic papillary conjunctivitis, which is a potential confounding factor for transpalpebral tonometry in keratoconus. All patients were treated for concomitant ocular allergy; however, eyelid eversion was not performed routinely. Another consideration regarding Diaton tonometry is that the measurements are rather cumbersome to perform, as the patient must be in a supine or reclined position. In addition, the Diaton device has a steep learning curve; however, this was not likely to have affected the outcome, as the examiner in this study (NP) had extensive experience performing Diaton tonometry prior to the start of the study. The significant difference between DT measurements in keratoconus and healthy eyes (with a mean difference of -5.5 ± 3.5 mmHg) could not be explained and is not consistent with previous studies.[13] A quarter of the DT measurements in healthy eyes were < 5mmHg, which is not compatible with the distribution of IOPs in a normal population.[20] The initial patient records and the study database were checked for erroneous data entries, but these were not found. We can only hypothesize on the origin of this difference; statistical chance is highly

unlikely based on the solid significance. A calibration deficit might have clouded the measurements, though the apparatus was calibrated before every measurement according to the manufacturers instruction. Regardless of the origin of this deficit we state that these data do not support our hypothesis that DT can potentially identify false-negative IOP measurements in keratoconus eyes.

The prevalence of glaucoma increases in eyes following penetrating keratoplasty (PK), and applanation tonometry can be difficult to perform in these cases.[22] Although no post-PK eyes were included in this study, we recommend using a device that has been shown to be reliable for measuring IOP in keratoconus and/or post-PK eyes.

The Diaton device is specifically advertised for use in patients with corneal disease; however, although the device is portable, is tolerated well by patients, and is not influenced by corneal biomechanics, our results suggest that it does not measure IOP reliably in patients with keratoconus.

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9

Cytokine expression in keratoconus and its corneal micro-environment, a systematic review.

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ABSTRACT

Keratoconus (KC) is a progressive corneal ecstasia characterized by thinning and weakening of the cornea that leads to a cone-like appearance, scarring, and decreased vision.

Despite the well-described clinical signs, the cause of KC is unknown. Nevertheless, various genes, proteinases, and environmental factors (eye-rubbing, contact lens wear, tear film composition) have been implicated in its etiology. Although classically defined as a predominantly degenerative disease, with mechanically induced trauma accelerating its course, accumulating evidence suggests a pivotal role for inflammation in the pathophysiology of KC.

Several reports have linked various inflammatory mediators (cytokines) with KC, but with contradictory findings. The methods and materials used in these studies vary considerably and warrant critical evaluation to decipher the role of inflammatory mediators in KC. We performed a systematic review of current literature on cytokine expression studies in KC and discuss critical soluble and cellular inflammatory mediators that are implicated in its pathogenesis.

Key words

Cellular inflammatory mediators, cytokine, cytokine receptor, keratoconus, soluble inflammatory mediators, tear film

INTRODUCTION

Keratoconus (KC) is an idiopathic condition of the cornea that can lead to irregular astigmatism, refractive myopia, corneal thinning, and a poorly restorable loss of visual acuity due to corneal scarring and the hallmark 'cone-like' shape of the cornea. KC can be accompanied by iron depositions in the corneal epithelium and rupture of the Descemet's membrane in advanced cases, although overt signs of ocular inflammation like redness, corneal edema, intraocular inflammation, or pain are not typical signs of keratoconus.[1] The disease usually affects both eyes and frequently develops during puberty until its arrest in the third or fourth decade.[2]

Although classically defined as a degenerative disease, with mechanically induced trauma accelerating its course, the pathophysiology of KC remains poorly understood. Currently, KC is considered to be a multifactorial corneal disorder caused by the sophisticated interaction of several environmental (eye-rubbing, contact lens wear) and endogenous factors leading to systemic and corneal oxidative stress by hypersensitive response to oxidative stressors that involves mitochondrial dysfunction and mtDNA damage in genetically susceptible individuals.[3-6]

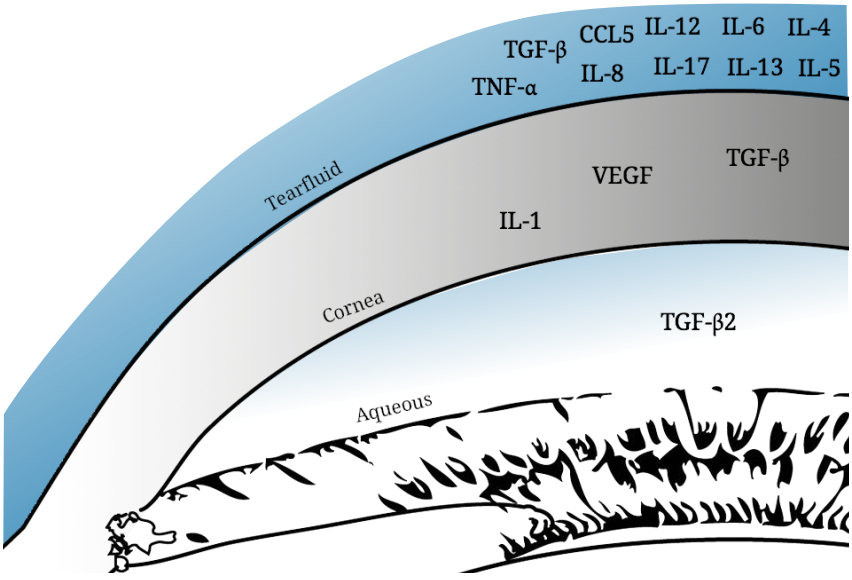

FIGURE 1. Representation of the localization of soluble immune mediators in tear fluid, corneal tissue, and aqueous humor of keratoconus patients. Most soluble immune mediator studies have used the relatively more accessible and less invasive tear fluid samples, contributing to a greater variety of reported cytokines such as IL-6, TNF-α and IL-17. Consequently, the number of studies on soluble immune mediators in corneal and aqueous humor samples is much fewer, but have already revealed altered levels in several factors, including VEGF, IL-1 and TGF-β.

Prolonged eye-rubbing alone is reported as an independent risk factor for the development of KC, with abundant clinical evidence that vigorous eye-rubbing can lead to de novo development of KC.[7,8] However, eye-rubbing without overt KC development will not be clinically recognized if the patient does not seek medical attention, and not all patients with KC will exhibit a history of eye-rubbing.[9]

The role of contact lens wear on the development of KC remains controversial. A majority of KC patients need (rigid gas permeable) contact lenses for adequate visual functioning, and all contact lenses alter the corneal shape by compression to some extent (corneal warpage).[10] Progression of KC is often concurrent with contact lens wear,11 and local tear film alterations (see Section III) could be related to contact lens wear rather than KC alone.[12] In contrast, contact lens wear was not associated with progressive KC in a longitudinal study, and a cause-effect relationship cannot be drawn on cross-sectional data.[11,13]

Proteomic studies revealed dysfunctional levels of proteinases, immunoglobulins, epithelial proteins, and factors associated with collagen homeostasis.[14-16] Currently, a disturbed regulation of the corneal microenvironment that favors an imbalance of enzyme activity is considered to be critical for connective tissue homeostasis. These enzymes include lysyl oxidases and matrix metalloproteinases (MMPs) and have been directly linked to oxidative stress and degradation of the corneal collagen.[17] However, the exact underlying molecular mechanisms remain to be elucidated.[18] It is possible that abnormal susceptibility to apoptosis and enhanced cell death in response to corneal trauma via various apoptotic signaling pathways underlie the loss of keratocytes in in the corneas of KC patients.[19] Curiously, keratocytes in KC corneal tissues also display metabolic and growth impairment.[20]

The reported clustering of cases throughout several families suggests the need for genetic factors that confer risk for developing KC.[21] However, genetic studies have not yet deciphered its complex genetic architecture. Early attempts to correlate HLA alleles with KC predisposition have been inconclusive.[22,23] Nevertheless, there is an increasing number of genomic susceptibility loci associated with KC in Caucasians and Asians that point to multiple underlying pathways predominantly linked to central corneal thickness, corneal curvature, collagen, and oxidative stress.[24-28] However, only a few loci have so far been consistently replicated by multiple groups, perhaps in part due to differential distribution of the risk loci among ethnic populations or the relatively low contribution of genetic variants to developing KC.[27]

The hallmarks that characterize KC are the progressive thinning and decline of the corneal architecture, and KC is therefore classically defined as a mainly degenerative noninflammatory corneal disorder. This is understandable, as KC eyes do not typically

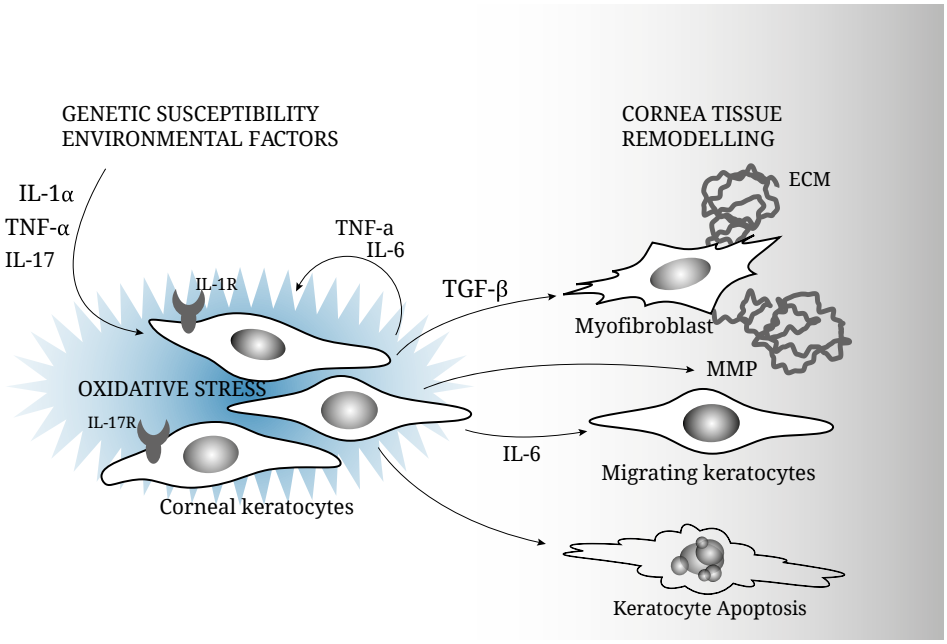

FIGURE 2. Model of the interaction of several soluble inflammatory mediators with corneal stromal keratocytes, leading to the archetypical corneal tissue remodeling. Various genetic loci, including cytokine genes, in combination with environmental factors such as eye-rubbing and contact lens wear contribute to the increased expression of soluble immune mediators such as IL-1α, TNF-α and IL-17 that bind to their receptors expressed by keratocytes of the corneal stroma. In combination with numerous other provocative factors (proteases), these keratocytes suffer from oxidative stress that leads to keratocytes apoptosis, IL-6 mediated migration of keratocytes into the corneal epithelium, and TGF-β-induced differentiation of keratocytes into α-SMA expressing myofibroblast that secreted components of extra cellular matrix (ECM). Together these keratocyte changes contribute to corneal tissue remodeling affecting collagen distribution and corneal architecture in keratoconus.

show signs of inflammation such as redness, corneal edema, or marked vascularization, and KC is not associated with other classical inflammatory diseases.1 However, emerging evidence has linked several inflammatory molecules to KC and challenges the conventional paradigms by putting forward a role for various inflammatory pathways in the pathophysiology (Table 1): eyerubbing, contact lens wear, and an atopic constitution are all associated with specific changes in the immunological corneal microenvironment. Various studies of tear film and sera of KC patients have identified altered cytokine, chemokine, and immune mediator levels in KC patients compared to unaffected individuals.[15,29-38]

In this review, we summarize several studies that investigated cytokine levels in tear samples, cornea samples, and aqueous humor, and discuss the role of interleukin (IL)-1, IL-6, IL-17, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and others in the pathogenesis of KC. Their localization and interactions are illustrated in Figures 1 and 2.

Authors	Outcome	Materials used[$]	Methods used	N cases vs controls	Mean age in years	Male Gender (%)	KC Gradingi (%)			Atopy (%)	Contact lens worn (%)	Eye rubbing (%)
					Cases vs controls	Cases vs controls	Mild	Moderate	Severe	Cases vs controls		
Fabre EJ et al (1991) †	Elevated number of binding sites L-1	Cornea	Cell growth and the binding associations with specific cytokines	N= 8 vs 8	13-35 vs 17-75	NR	NR, corneas obtained during corneal transplantation			50 vs NR	NR	NR
Zhou L et al (1996)	Enhanced expression of TGF-β and IL-1	Cornea	Immunofluorescence and immunoperoxidase staining	N= 17 vs 20	38±15 vs 39±29	NR	NR, corneas obtained after transplantation.			NR	NR	NR
Saghizadeh M et al (2001) †	Decreased expression VGEF	Cornea	PT-PCR and immunofluorescence	N=20 vs 12	34.8± 2.9 vs 33.8±4.1	NR	NR, corneas obtained from surgery.			NR	NR	NR
Lema I et al(2005)†	Increased expression IL-6 and TNF-α	Tear samples	ELISA	N= 28 vs 20	22.4±6.5 vs 22.6±6.6	52 vs 48	21.4	50	28.6	57.1 vs 5	0	64.3 vs 0
Maier P et al (2007)†	Increased expression TGF-β2	Aqueous humor	ELISA	N= 15 vs 38	36.6±13.9 vs 76.3±8.8	60 vs 58	NR, patients were undergoing penetrating keratoplasty (PK).			NR	NR	NR
Lema I et al (2009)	Increased expression IL-6 and TNF-α	Tear samples	ELISA	N=30KC vs 30 SKC vs 20	27.1±8.1 vs 22.6±6.6	70 vs 48	Clinical KCii in one eye, subclinical KC in the other eye.			60 vs 15	47* vs 0	58 vs 0
	Increased expression TNF-α clinical vs subclinical KC						73.3% KC in the right eye.					
Pannebaker C et al (2010) †	No significant difference in cytokines levels	Tear samples	Cytokine antibody array	N = 22GP vs 18KCGP vs 6	35.5±13.4 (KCGP) vs 37.3±11.5 (KC) vs 48.3±12.0	83(KCGP) vs 100(KC) vs 35	NR			NR	67 vs 100	NR

Jun AS et al (2011)	Decreased expression IL-4, IL-12, IL-13, TNF-α and CCL5.	Tear samples and serum samples	Multiplex cytokine analyses, subsequently ELISA for other subjects	N= 18 vs 11 MCA	44±13 vs 33±10	61 vs 45	33	33	56	16.7 vs NR	50 vs 36	NR
	Increased expression IL-17			N= 29 vs 38 ELISA	MCA	MCA	MCA	MCA	MCA	MCA	MCA	
	Increased expression IL-4 contact lens users vs non-contact lens users				38±10 vs 40±12	72 vs 53	6.9	41.4	51.7	10.3 vs 10.5	55 vs 0	
					ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	
Engler C et al (2011)†	Increased expression TGF-β2	Cornea	Immunohistochemistry and RT-PCR	N= 18 vs 9	37±18 vs 43.6±6	38 vs 60	6 mild KCiii			NR	0*	NR
					IH	IH	IH					
					36±15 vs55±23	40 vs 50						
					RT-PCR	RT-PCR						
Balasubramanian SA et al (2012) †	Increased expression of IL-4, IL-5, IL-6, IL-8, TNF-α and TNF-β	Tear samples	Cytokine antibody array	N= 32KC vs 20CXL vs 28	29.5±9.4 (KC) vs 27.3±5.2 (CXL) vs	53(KC) vs 35(CXL) vs 57	NR			0	0*	NR
	Increased expression IL-6 KC compared with CXL				32.6±11.1							
	Increased expression TNF-α CXL											

KC=keratoconus, GP=gas permeable contact lens, KCGP=keratoconus group with gas permeable lens, IH=immunohistochemistry, CXL=subjects who had undergone a corneal collagen cross-linking procedure, MCA=subjects used for multiplex cytokine analysis, ELISA=subjects used for ELISA, SKC=subclinical keratoconus eyes, NR=not reporte. $ tear samples were all obtained using a capillary flow technique and care was taken not to touch the corneal and conjunctival surface, studies report a quantitiy of 15µl. † in these articles one eye per patient was used., *discontinued contactlens wear prior to sampling, i Grading as defined by Zadnik et al in baseline findings in the collaborative longitudinal evaluation of keratoconus(CLEK) study in 1998. Ref: 1,56, ii Grading as defined by Rabinowitz/McDonnell Ref: 1, iii Grading defined as followed: K ≥50D or pachymetry of ≤ 400µm graded as severe and K<50D and a pachymetry reading of >400µm graded as mild.

Table 1: An overview of studies on cytokine alterations in keratoconus

CYTOKINE FAMILIES: CHARACTERISTICS AND ACTIONS

Interleukin-1

The IL-1 family of cytokines consists of over 10 members, including the closely related IL-1α and IL-1β that have strong proinflammatory potential.[39] IL-1α and IL-1β have pleiotropic immune functions and are involved in the promotion of various proinflammatory cytokines, and the regulation of cell growth, differentiation, and motility of inflammatory cells during viral, bacterial, and fungal infections. Genetic susceptibility linked to the IL-1 gene cluster has been reported in several KC populations and spans several genes, including the IL1RN (IL-1 recepter antagonist) gene.[40] Also, the IL1A gene has been linked to KC in Han Chinese patients.[41] In addition, polymorphisms near the IL-1B gene are associated with KC in Korean patients[42] and Japanese patients,[43] but not with Turkish patients.[44] The latter is of interest, given that individuals of Asian descent are more susceptible to developing KC than Caucasians.[45]

Indeed, KC corneas have increased IL-1α and IL-1β expression,[35,46,47] and fibroblasts from KC patients show elevated expression for IL-1α receptors.[48] In general, IL-1α is upregulated during corneal trauma and inflammation.[49] Corneal epithelium secretes IL-1 after injury or tissue damage or apoptosis.[50] KC corneas show a loss of keratocytes, likely due to increased apoptotic cell death.[50] IL-1α and IL-1β both induce apoptosis in corneal endothelium synergistically with cytokines such as TNF-α via the production of reactive nitrogen species.[51]

Interestingly, IL-1α contributes to corneal oxidative damage exclusively in KC, where it was shown to specifically downregulate the synthesis of extracellular-superoxide dismutase (SOD3), a major superoxide scavenger, in cultured KC stromal cells.[52] Alternatively, KC fibroblasts produce ten times more prostaglandin E2 than the normal cornea upon IL-1 stimulation, while collagen production is lower.[48] Also, the thinning and ectasia of the cornea suggests direct degradation of the corneal collagen that could be caused by enzymes such as MMPs.[53,54] In the human cornea, the MMP activity is in part regulated via IL-1β. Therefore, IL-1α and IL-1β have various pathogenic roles in KC that may be endogenously higher expressed in genetically prone individuals and are extensively secreted when the cornea is minimally damaged. After inducing keratocyte apoptosis, they induce production of MMPs, resulting in enhanced tissue damage and alterations of the corneal architecture.

Interleukin-6

IL-6 is a pluripotent factor that drives multiple biological processes and plays a pivotal role in stimulating several immune responses, such as eradication of infection and wound repair.[55] IL-6 can be produced and secreted by many cells, including dendritic cells, endothelial cells, T-cells, and macrophages. The levels of IL-6 are increased in KC corneas.[30,33,56,57]

Keratocytes produce IL-6 upon stimulation with IL-1.[57]

Interestingly, eye rubbing and contact lens wear, which are closely associated with KC, increase the levels of IL-6 in tear fluid in KC.[57,58] This indicates local changes of IL-6 in the cornea and supports the concept of corneal inflammation in KC. How IL-6 interacts with the corneal microenvironment in KC is not well understood. However, the expression of IL-6 in the cornea is influenced by several factors that juxtapose KC, most importantly eye-rubbing,[58] but also atopic constitution[59] and contact lens wear.[60] Curiously, the levels of IL-6 in KC tear fluid are not related to disease severity.[17] IL-6 exerts its effect by binding to the IL-6 receptor on cells or the soluble IL-6 receptor (sIL-6R) that is abundantly expressed in the cornea.[61] Ebihara et al demonstrated that IL-1-activated fibroblasts induce the production of IL-6 and sIL-6R that induce epithelial cell migration.[55] This suggests that IL-6 is directly related to the archetypal corneal thinning induced by predominantly exogenous factors and events associated with KC.

Tumor Necrosis Factor-α

In addition to IL-6 and IL-1β, TNF-α is considered a major pathogenic factor in systemic and corneal inflammation. Evidently, TNF-α is also increased in tear film and corneal samples of KC,[15,30,33,36,37] and fibroblasts of KC patients have increased expression of TNF receptors.[34] In contrast to IL-6 and IL-1β, TNF-α can be detected in very early, subclinical stages of KC.[36] Here, TNF-α contributes to the production of IL-6 by keratocytes.[62] Interestingly, TNF-α induces the expression of MMP-9 in the cornea. The increase of MMP-9 levels is correlated with corneal thinning, probably as a result of degradation of stromal collagen, since MMP-9 activity is also higher in tear fluid of KC patients.[30] In addition, TNF-α disrupts the barrier function of corneal epithelial cells.[63] It is not yet known what cell type is the origin of the production of TNF-α in KC. However, TNF-α can be produced by a variety of cells, including all three major cell types in the cornea: the corneal epithelium, stromal keratocytes, and endothelial cells. Perhaps, corneal damage induced by environmental factors causes the production of TNF-α. For example, eyerubbing and dry eye disease are major risk factors for developing KC and are associated with the induction of TNF-α production by corneal epithelial cells.[58,64,65]

Interleukin-17

IL-17 is a proinflammatory cytokine that is associated with many chronic inflammatory conditions.[66] Interestingly, Jun et al detected elevated levels of IL-17 in tear fluid samples of KC patients. IL-17 has been associated with pathogenic mechanisms in corneal inflammation by stimulating stromal cells to secrete various proinflammatory cytokines,[67] including IL-6, IL-8, and intercellular adhesion molecule 1 (ICAM-1).[68] The IL-17 receptor is constitutively expressed by corneal resident fibroblasts.[67] Stimulation of corneal fibroblasts with IL-17 leads to production of several MMPs. Thus, IL-17 may contribute to corneal damage in KC by activating fibroblast and subsequent metalloproteinase production. Elevated levels of IL-17 may induce tissue damage in KC and could relate to disease severity.[69] Indeed, the levels of IL-17A were shown to be associated with center Keratoconus-Index and Index-of-Surface Variance after CXL.[56]

Although IL-17 is the principle cytokine produced by T helper17 (Th17) cells Th17 cells are upregulated by TGF-ß and IL-6, which are themselves both upregulated in keratoconus cornea[31-33,70,71]), it can be produced by many other cell types.[72,73] Curiously, in herpetic stromal keratitis, elevated corneal IL-17 levels are related to CD4+ T cells, and no IL-17 production is detected in unaffected corneas.[67] This suggests that the primary source of IL-17 in the cornea may be infiltrating IL-17-producing T cells. Interestingly, corneal resident T cells are also found in normal corneas and may therefore also be present in KC tissue.[74] However, the origin of IL-17 in KC is currently unknown and further investigation is needed to clarify the role, if any, of IL-17- producing T cells in the pathogenesis of KC.

Transforming Growth Factor-β2

TGF-β2 controls cell proliferation and differentiation in epithelial and endothelial cells.[75] TGF-β can interact with several collagen types and stimulate the secretion of MMPs that could influence corneal structure and collagen distribution in the cornea.[76] Accumulating evidence supports a role for TGF-β signaling in KC. Keratocytes and corneal fibroblasts are particularly sensitive to TGF-β1, and under its influence differentiate into myofibroblasts.[77] In response to injury to the cornea, TGF-β receptor activation drives myofibroblasts to restore the integrity of the cornea by secreting extracellular matrix. Dysregulation of this pathway has been associated with pathogenic corneal fibrosis.[77] Thus, TGF-β1 may be linked to the scar-formation and tissue repair that is observed in severe keratoconus.[78]

Interestingly, TGF-β and IL-1 have antagonistic effect on corneal myofibroblasts; high levels of TGF-β1 increase myofibroblast viability, while IL-1 induces apoptosis in these cells.[79]

Thus, low levels of TGF-β1 increase the susceptibility of myofibroblasts to apoptotis mediated by IL-1. Indeed, reduced expression of TGF-β1 has been observed in KC.[38] Also, epithelium and stroma proteome analysis reveals downregulation of TGF-β1 in KC patients.[20] In addition, fibroblasts from KC patients are more sensitive to the changes in TGF-β1 levels.[20] Disturbed signaling of TGF-β1 may itself be the result of other pathogenic factors, since cultured corneal stromal fibroblasts from KC patients are able to express significant TGF-β1 mRNA.[80]

Interestingly, Pannebaker et al did not observe differences in TGF-β1, TGF-β2, or TGF-β3 levels in tear fluid of KC patients, which suggests that tear fluid may not be the optimal material for studying this signaling pathway in KC.[15] Nevertheless, recent evidence suggests that the potential imbalance of TGF-β1 and TGF-β3 drives pathological differentiation of corneal keratocytes in KC6, perhaps via the regulation of the glycoprotein prolactin-inducible protein (PIP) that is upregulated in KC and implicated in corneal fibrosis.81 Corneal fibrosis is typically characterized by increased deposition of collagen III.6 In addition to TGF-β1, Engler et al and

Mailer et al found increased levels of TGF-β2 in KC corneas[31] and aqueous humor.[32] Also, immunohistochemistry shows increased TGF-β2 staining in corneal epithelium of KC patients.[20] TGF-β2 is particularly present in the aqueous humor and canonically functions to maintain the immune privileged nature of the eye, but paradoxically induces several (proinflammatory) cytokines under inflammatory conditions.[82]

Although its role in KC is unclear, TGF-β2 could contribute to expression of inflammatory mediators that result in tissue damage. TNF-α induces TGF-β2 expression by corneal epithelial cells, which supports the idea that it is expressed to limit corneal inflammation.[83] However, TGF-β2 signaling can also induce the expression of IL-6 by corneal epithelial cells that contributes to proinflammatory conditions in the cornea,[83] for example, by promoting Th17 differentiation in combination with TGF-β1.[84]

The above-described studies contribute to the notion that TGF-β family members are central to various pathogenic corneal changes such as increased expression of inflammatory mediators, MMPs, and induction of corneal fibrosis in KC patients. This suggests that modulation of TGF-β signaling may be an interesting target for treatment of KC.

Vascular Endothelial Growth Factor and Nerve Growth Factor

Other factors of interest are vascular endothelial growth factor (VEGF) and nerve growth factor (NGF). VEGF is a family of growth factors associated with neovascularization, and it is highly expressed in corneas of healthy subjects.[85] The association of VEGF with KC is not yet conclusive, with some studies reporting lower levels in corneal

samples[38] and others finding no difference in the levels of VEGF in tear fluid samples of KC patients vs controls.[15]

The cornea has a very high density of nerve cells, and denervation alters its epithelial metabolism.[86] VEGF expression is possibly altered by NGF, a cytokine important for corneal innervation.[87] NGF, a normal constituent of the tear film, is increased in KC corneas.[56] The cornea is a naturally avascular and highly innervated organ. NGF is considered to play a role in wound healing of the cornea, and it affects corneal epithelial cell proliferation. NGF mainly affects cells through the specific neurotrophin tyrosine kinase receptor A (TrkA), which is normally found in all corneal cell types.[88,89] TrkA levels were found to be absent in the corneas of KC subjects.[90]

These findings underscore a role for NGF in the pathophysiology of KC. Further investigation is warranted to determine the potential role in KC.[91,92]

INFLAMMATORY MEDIATORS IN TEAR FLUID OF PATIENTS WITH KERATOCONUS

The association of excessive eye-rubbing and sustained contact lens wear with KC suggests that increased mechanical stress at the ocular surface confers risk for developing the condition.[12] Indeed, keratoconic eyes have a damaged ocular surface suffering from the consequences of inadequate tear and mucin production, as well as aberrant antioxidant status and proteome alterations that affect tear fluid stability and quality.[93-97] Thus, the affected ocular surface in KC patients gives rise to symptoms that overlap with those of patients with dry eyes, which is also demonstrated by increased corneal staining in KC.[98]

The tear fluid has a significant role in maintaining a healthy ocular surface and consists of water, electroclytes, and epithelial-derived factors, including lipids, metabolites, and more than 1500 different proteins.[99] Tear fluid is easily accessible and is commonly used to monitor several factors that may indicate the health of the epithelial cell layer covering the ocular surface. However, dynamic changes in the levels of various soluble mediators could also be related to lacrimal gland or conjunctival dysfunction, and levels can vary depending on the methods of obtaining the tear samples and measuring their absolute levels. Also, the yield of tear fluid material varies per condition (i.e., dry eye disease), and the implication of differences in tear osmolality and the exact cytokine concentration on ocular surface epithelium need to be further elucidated.[100,101]Thus, although the tear fluid findings are indeed informative, they need to be carefully considered with regard to their role in keratoconus.

Nevertheless, various proteomic approaches have revealed disease-specific alterations in the tear fluid constituents of KC patients. Although the overall protein levels in tear fluid appear to be lower in KC, higher levels of proteolytic activity and increased levels of MMPs, glycoproteins, and transporter proteins have been observed.[15,102,103] In contrast, other proteins, such as immunoglobulins and the iron binding glycoprotein lactoferrin, which are widely present in healthy tear fluid, are less abundant in KC tear fluid.[102] Curiously, the characteristic high expression of MMPs in tear fluid of KC is also found in patients with other inflammatory diseases, such as rheumatoid arthritis.[30,57,102,104] Evidently, changes in the tear fluid protein composition can ultimately affect tear fluid quality, and this could have significant impact on the health of the ocular surface.

The effect of tear fluid quality on KC is underlined by findings of Hara et al, who

demonstrated that tear film stability had an important role in obtaining a better visual acuity in the early postoperative period after keratoplasty, a currently inevitable intervention for severe keratoconus.[105] A number of studies provide evidence that KC is characterized by a cytokine imbalance in tear fluid and that these inflammatory mediators operate actively at the ocular surface. Multiple inflammatory mediators have been found to be increased in tears of KC patients, including the well-documented IL-1, IL-6, TNF-α, and IL-17, and incidental reports also suggest a role for IL-4, IL5, IL-8, CCL5, NGF and interferon (IFN)-γ.[29,30,33,36,37,56]

As discussed in this review, each of these inflammatory mediators affect the corneal microenvironment and could underlie the above-mentioned changes in tear fluid proteome, stability, and quality. For example, IL-1α, IL-1β, and TNF-α can induce the expression of several proteins in tear fluid of KC patients, including antimicrobial surfactant proteins.[106] Interestingly, the expression of several important mediators (IL-6, TNF-α, ICAM-1, VCAM-1, and others) have been related to contact lens wear and eye-rubbing in KC patients.[58,107,108] Most of these observations are cross-sectional, however, which clouds the determination of a causal relationship between these altered cytokine profiles and KC development. Also, the tear film evidently plays an important role in composing this corneal microenvironment, but we should take into account that the reported changes in tear film cytokine profile may not necessarily reflect intracorneal processes in KC. Although it is tempting to suggest that detrimental inflammation at the ocular surface may largely be mediated via mechanical stress, further research is necessary to elucidate the interactions of these inflammatory pathways at the ocular surface in KC.

CELLULAR INFLAMMATORY MEDIATORS IN KERATOCONUS

The dysregulation of immune pathways in KC can be further appreciated from the unusually strong association of KC with various chronic inflammatory diseases, such as rheumatoid arthritis, ulcerative colitis, and atopic diseases, including asthma.[109] Normal corneal homeostasis exploits active and passive mechanisms that aim to preserve a hypo-immune status; for instance, the cornea lacks blood and lymphatic vessels.[110] The complex interplay of multiple soluble inflammatory mediators (i.e., cytokines) in KC suggest activation of immune pathways via secretion of factors, but it may also indicate the presence of inflammatory infiltrates in corneal epithelium, stroma, or endothelium. First and foremost, KC is characterized by a loss of corneal stromal keratocytes, invasion of fibroblastic keratocytes into the epithelium, and disorganization of collagen fibers in the stroma.

KC keratocytes display a wide variety of disease-specific abnormalities in their secretion and expression of various factors, such as alpha-SMA (myofibroblast marker), Sp1, proteinase inhibitors, and macroglobulins.[111] TNF-α, TGF-β, and IL-1 all activate keratocytes to produce inflammatory mediators (i.e. IL-6, TNF-α) and proteolytic enzymes. These mediators can induce keratocyte apoptosis or differentiation into myofibroblasts.

Clinically apparent corneal infiltrates and concomitant overt signs of inflammation are not associated with KC. Earlier studies have not shown evident infiltration of inflammatory cell subsets into corneal tissues of KC patients.[112] This is also reflected by the low expression of adhesion molecules and integrins in KC corneas.[113] Even in atopic KC patients, cornea buttons display virtually no eosinophils.[114] Significant inflammatory cell infiltrate in KC seems only to be related to the early recovery after epithelium-off collagen cross-linking that is accompanied by keratocyte apoptosis, epithelial regeneration, and appearance of inflammatory cells in the surrounding area of treatment.[115] Attempts to isolate T cell lines from KC corneas have been unsuccessful[74]; this T cell study was performed before IL-17-producing T cells were described and lacked the cytokine-conditioned cultures to set up stable cell lines. In any case, the frequency of corneal resident T cells in normal and keratoconic eyes was very low,[74,112] suggesting that increased IL-17 levels in KC are probably not related to T cells.

KC stroma expresses the hematopoietic progenitor cell antigen CD34, which is decreased in KC due to loss of CD34+ keratocytes that function as multipotent hemopoietic stem cells.[116,117] Most of the CD34+ corneal stromal cells also express the stem cell marker CD133, but a small fraction of the CD133+ cells do not express CD34 and are CD14-positive. A study by Thill et al demonstrated that this intriguing non-hematopoietic cell subset of CD133+ CD34- cells robustly increases in frequency during corneal diseases, including keratoconus, and serve as corneal repair cells that eventually differentiate into keratocytes.[118] The role of these repair cells in corneal homeostasis and disease warrants further investigation.

KC corneas display enhanced expression of CD68, especially around areas of thinning.[119] Although CD68 is frequently used as a lineage marker for macrophages, it actually stains a membrane protein in lysosomes and may be more related to the increased expression of proteases that has extensively been reported in KC. The normal human cornea has resident immune cells, including CD11c+ dendritic cells and CD207+ Langerhans cells and also CD68+ macrophages.[120-122] The contribution of these resident immune cells and the expression of cytokines by these cells in KC is currently unclear. Regardless, KC corneas show an elevated expression of HLA class II molecules.[119] Recent studies revealed that these HLA class II moleculeexpressing cells could very well be corneal resident antigen presenting dendritic cells that particularly express HLA class II molecules.[120] However, corneal epithelial cells are also able to express HLA class II molecules and present antigens directly to CD4+ T cells.[123] Histopathological examination of KC corneas reveals a moderate increase in the number of antigen-presenting cells compared to controls, but this increase is not as apparent as observed in inflamed corneas.[124]

In addition to local activation of inflammatory pathways, there is accumulating evidence that systemic inflammatory changes and systemic oxidative stress may affect the corneal microenvironment in KC. For example, systemic inflammation monitored via the neutrophil-tolymphocyte ratio was recently associated with progressive KC125, and systemic oxidative stress has been reported in KC patients.3 Increased frequency of neutrophils indicates proinflammatory conditions, and neutrophils are associated with the activation of MMPs, which have been found to be elevated in KC.[125]

The sophisticated interaction of corneal and systemic cellular inflammatory mediators that contribute to development of KC is poorly understood, but a central role for keratocytes and corneal epithelial cells is evident. Shetty et al demonstrated that the

administration of cyclosporine A strongly reduced the inflammatory stimulation and expression of MMP-9 in tears of KC patients and decreased the production of IL-6, TNF-α and MMP-9 by corneal epithelial cells, while restoring the expression of collagen. The authors suggest that this anti-inflammatory agent may be a promising new treatment modality for KC.[126]

SUMMARY AND CONCLUSIONS

Keratoconus is a multi-factorial, complex disease of the cornea associated with various genetic and exogenous degenerative factors, such as eye-rubbing, decreased expression of protease inhibitors, and increased protease levels in the corneal micro-environment, ocular surface, and tear fluid.[58] Inflammation is clearly not sufficient to cause KC, as many patients with severe corneal inflammation do not develop KC. Nevertheless, accumulating evidence supports a contribution for several inflammatory pathways that, in part, orchestrate or amplify corneal tissue damage. Most of the evidence points toward local immune responses restricted to the cornea. Interestingly, recent evidence also suggests systemic alterations in KC, such as the increased neutrophil-to-lymphocyte ratio. The list of reported inflammatory mediators associated with KC is not limited to those discussed in this review; other factors include IL-13, IFN-γ, and IL-10.[29,56] We are only beginning to understand the complexity of the interactions of all these mediators in several inflammatory pathways in KC.

We have discussed the role of the tear film in KC. Factors such as atopic constitution, eyerubbing, and contact lens wear, which affect the development of KC, are reflected by alterations in the tear film. It is also evident that cytokine profile alterations in the tear film can be independently linked to KC, regardless of the effect of contact lens wear.[15] However, most of these observations are cross-sectional, which clouds the determination of a causal relationship between altered cytokine profiles and KC development. We postulate that any morphological change associated with KC is accompanied by changes in the cytokine expression.

In addition to recognizing the role of cytokines and corneal cells, we emphasize that there may also be an important role for the conjunctiva and lacrimal glands in KC. Both are wellknown sources of cytokine production and resident immune cells.[98,127-129] As described in Section III, numerous proteases, immunoglobulins, and cytokines have been found in tear fluid of patients with KC, which could reflect changes in lacrimal gland and conjunctiva. The conjunctiva can suffer from severe immune dysregulation with detrimental effects on the ocular surface, as has been shown in diseases such as allergic conjunctivitis.[127-129] It is not hard to imagine that cytokines in the tear fluid associated with KC may be derived in part from the conjunctiva or lacrimal glands, and are not only a consequence of corneal pathology.

Comparability of study results is difficult, due to differences in tissue type (tear fluid, anterior chamber fluid, corneal tissue) and the general lack of stratification for potential confounders of cytokine differences. Tear fluid is relatively easy and safe to obtain, but the expression levels of various mediators may not reflect their actual distribution in the cornea (for example, VEGF). The analysis of tear fluid seems to be more appropriate to address the influence of factors juxtaposing KC, such as related atopy and contact lens wear.

An anterior chamber (AC) tap is an invasive diagnostic procedure, which is rarely medically indicated in KC. Ethically, it is only feasible to perform an AC tap in conjunction with another surgical intervention, so AC fluid is seldom available for investigation. However, AC fluid is very suitable for cytokine expression analysis and has been used in the diagnosis of various inflammatory diseases.

Finally, corneal tissue can be obtained only during transplant surgery or post-mortem. Furthermore, a major indication for surgical treatment is corneal scarring, seen only in the most severe cases of KC. This may bias study outcomes, suggesting association of several inflammatory mediators with KC in general, whereas the underlying mechanisms may be associated only with the more severe cases. Therefore, the evidence for a role of corneal fibroblasts and TGF-β signaling involved in corneal wound repair may be more related to severe cases of KC and mask factors that are linked to its actual cause.

Surprisingly, although the development of KC is inversely related to age, little research has addressed the effect of age on cytokine expression in KC.[32] The different study outcomes may in part be related to differences in the age of the study subjects, since only few studies (partly) stratified their results for potential confounders.[30,36,37] We therefore suggest that the outcomes of cytokine analyses are stratified for subject characteristics like atopic constitution, eye-rubbing, contact lens wear, disease severity, and possibly age. The ideal study design would incorporate several samples from the same subject, ideally obtained at the same time-point; aqueous humor to assess the inflammatory state of the eye, tear fluid to assess KC-associated factors, a corneal sample to identify the cytokine expression at end-organ level, and a peripheral blood sample to assess the general immunological make-up of the subject. Eventually, sampling could be repeated to longitudinally assess the relationship between age, disease severity, and cytokine expression.

In conclusion, various inflammatory mediators have been reported in KC that can modulate the corneal microenvironment and contribute to tissue damage of the cornea. The tear film plays an important role in comprising this corneal microenvironment, but tear film cytokine alterations do not necessarily reflect intracorneal processes. Although KC is not caused by corneal inflammation itself and experimental outcomes are not completely conclusive, data strongly substantiate the emerging concept of underlying inflammatory pathways in the pathogenesis of KC. A thorough understanding of their contribution and interaction with disease progression will facilitate the development of targeted modulatory intervention to preserve vision in KC patients in the near future.

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10
The role of aging processes and the MTOR pathway in keratoconus.

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ABSTRACT

Purpose

Keratoconus (KC) is a disease that can lead to a severe decrease in visual acuity and may warrant a corneal grafting procedure. KC onset and progression is more severe in younger patients. The underlying etiology is multifactorial, but remains enigmatic. Meta-analyses of genome wide association studies have identified loci that confer a relatively large risk for developing keratoconus and all were involved in cellular metabolism. Currently, there is limited evidence of functional roles for the identified KC-associated loci, and the contribution of their related genes in the disease biology is unknown.

Methods

We investigated the gene expression profiles of these confirmed loci and additional genes related to cellular ageing and cell cycle control in corneal tissue of keratoconus patients, healthy controls, and severely failed corneal grafts (DG).

Results

We report, for the first time, the deregulation of a myriad of genes, including FRAP1/MTOR, and other genes involved in the mammalian target of rapamycin complex 1(mTORC1) pathway, in keratoconus. mTORC1 represents a principal pathway for cell cycle control and cellular metabolism. Strikingly, KC corneas show signs of cellular aging far exceeding their healthy, biological older, peers, that are comparable to the severely failed grafts

Conclusions

These functional implications narrow down true causal variants by strengthening previous genetic associations of FRAP1/MTOR identified by genome-wide studies in the development of KC. Selectively targeting the mTOR pathway is a promising concept for the treatment of graft failure and keratoconus.

INTRODUCTION

Keratoconus (KC) is an ocular disease characterized by thinning and a conical ectasia of the cornea that may lead to visual loss due to myopia, irregular astigmatism, or corneal scarring in severe cases.[1] KC typically develops in the first or second decade of life until progress gradually halts.[2] However, onset and progression is generally more severe in younger patients.[3] Although KC has been convincingly associated with atopic constitution and eye rubbing, many cases are considered idiopathic. The underlying etiology is assumed to be multifactorial, but remains enigmatic. Regardless, accumulating evidence supports a complex interplay of enhanced protease activity, corneal tissue remodeling, and activation of several inflammatory pathways.[4] Interestingly, the high degree of concordance in monozygotic twins, and a high prevalence of KC in first degree relatives, suggests a major genetic predisposition or modulation effect.[5,6] Recent progress in genome wide association studies (GWAS) have provided critical insights into potential molecular mechanisms underlying KC, and revealed susceptibility loci linked to central corneal thickness, cell metabolism and cellular ageing.[7] More specifically, meta-analyses of large European and Asian cohorts have revealed that variants near FOXO1, FNDC3B,[7] FRAP1/MTOR,[8] and PDGFRA[9] genes conferred relatively large risks for developing keratoconus.

Since most genetic associations fail to prove a significant functional contribution to disease biology, we investigated the gene expression levels of these confirmed genes, related pathways, and genes associated with cellular aging and cell cycle control, in corneal tissue from KC patients, healthy controls, and diseased controls (decompensated corneal grafts, DG).[10]

METHODS

Acquisition of corneal samples

KC cornea samples were collected from patients receiving a corneal transplant for severe KC or pellucid marginal degeneration. Twelve corneas from 12 patients were included in this group. The group of diseased controls are composed of decompensated grafts (DG). These corneal samples were obtained from patients that underwent a re-grafting procedure, where the primary grafting indication was not keratoconus. Eleven samples from 11 patients were included in this group. All aforementioned cornea buttons were processed using Tissue-Tek (Sakura Finetek U.S.A., Inc.) immediately after resection, cut into five full thickness slices, and stored at -80 °C.

Healthy cornea (HC) controls were obtained from the Euro Cornea Bank (ECB), Beverwijk, The Netherlands, and Department of Anatomy, University Medical Center Utrecht. A total of 10 cornea's were prepared from post-mortem tissue within 24h of death and prepared from eyes from unrelated Caucasian donors who had no history of keratoconus, ocular inflammation, or vitreoretinal disease. Informed consent for the post-mortem donation of ocular tissue was received before the authors acquired the tissue under the auspices of the head of the department of Anatomy, University Medical Center Utrecht, the Netherlands. All tissues were acquired in compliance with Dutch law ("Wet op de lijkbezorging," Art 18, lid 1/ 18–06–2013) and the institutional guidelines of the University Medical Center Utrecht.

The study adhered to the tenants of the declaration of Helsinki and complied with local laws and good clinical practice. The storage of corneal buttons in the University Medical Center Utrecht Biobank was approved by the institution's Ethical Review Board. All patients provided written informed consent.

Clinical data extraction

Patient records were reviewed for additional data collection, such as patient history and preoperative assessment, including slit lamp evaluation, Schirmer's testing, and Scheimpflug corneal tomography (Pentacam HR, Oculus GmbH). Available data for the healthy control group was limited to age, sex and cause of death.

RNA isolation

RNA isolation from corneal buttons was performed using the TRIzol method (Life Technologies, Thermo Fisher Scientific, USA) following the manufacturers protocol. RNA was isolated after dissolving the cornea in TRIzol reagent. After isolation, RNA

levels were detected with the Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific, USA). Synthesis of complementary DNA from the isolated RNA was performed by reverse transcription using random hexamer primers. This complementary DNA was then used for gene expression analysis.

Gene expression analysis

Twenty-nine gene expressions related to cellular ageing and proliferation, including the loci identified by GWAS, were quantified: Aryl Hydrocarbon Receptor (*AHR*), Aryl-Hydrocarbon Receptor Repressor (*AHRR*), V-Akt Murine Thymoma Viral Oncogene Homolog 1 (*AKT1*), Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*), DEP domain containing MTOR-interacting protein (*DEPTOR*), fibronectin type III domain containing 3B (*FNDC3B*), Forkhead Box O1 (*FOXO1*), Forkhead Box O3 (*FOXO3*), Forkhead Box O4 (*FOXO4*), H2A Histone Family, Member X (*H2AFX*), Histone Deacetylase 9 (*HDAC9*), Insulin-Like Growth Factor 1 (*IGF1*), Insulin-Like Growth Factor 1 Receptor (*IGF1R*), Interleukin 6 (IL6), Interleukin 10 (*IL10*), Mouse Double Minute 2 homolog (*MDM2*), Mammalian Target Of Rapamycin (*MTOR*), Nuclear Factor of Activated T-cells, Cytoplasmic 1 (*NFATC1*), Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1 (*NFKB1*), Platelet Derived Growth Factor Receptor Alpha (*PDGFRA*), Phosphatase And Tensin Homolog (*PTEN*), Rapamycin-Insensitive Companion Of MTOR (*RICTOR*), Regulatory Associated Protein Of MTOR (*RAPTOR*), Sirtuin 1 (*SIRT1*), Sirtuin 6 (*SIRT6*), Sirtuin 7 (*SIRT7*), Telomerase Reverse Transcriptase (*TERT*), Tumor Protein P53 (*TP53*), Werner syndrome, RecQ helicase-like (*WRN*).

Gene expression analysis was performed by OpenArray quantitative real-time polymerase chain reaction (qPCR), using *GUSB* and *GAPDH* as housekeeping genes for measuring relative expression levels. All qPCR analyses were performed on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, USA). The qPCR data were interpreted using ExpressionSuite software version 1.0.3 (Life Technologies, Thermo Fisher Scientific, USA), which uses the comparative delta CT (ΔCT) method. Cycle threshold number (CT) is defined as the cycle number at which the SYBR green fluorescence for the target gene crosses a fixed threshold during qPCR, thus a lower CT value means a higher amount of RNA expression. Target gene CT values are normalized to the average of the two housekeeping genes CT values in each sample. Target gene expression levels are thus presented as ΔCT value. Additionally, fold change compared to the average of housekeeping genes was calculated using the formula: Fold change=2^(-ΔCT).

The genes *AHRR, FOXO3, IL6, IL10* and *TERT* did not meet quality control criteria for any KC sample, due to very low or even absent expression in corneal tissue. Six healthy control samples, two keratoconus samples, and four decompensated grafts did not meet qPCR quality control criteria for RNA expression analysis, thus reducing the effective sample size from 34 to 22 corneas. Baseline characteristics of the non-viable KC and DG samples did not differ from the mean group. The non-viable healthy control samples were overrepresented in the Cornea Bank derived samples. All samples derived from the institution's anatomy department were viable for analysis.

Data analysis

Baseline differences were calculated, and checked for normality. Gene expressions were presented as ΔCT values per sample and plotted in grouped scatter plots. The expression fold changes relative to the housekeeping genes were calculated from ΔCT values and graphed in scatter plots. For statistical analysis the ΔCT values were used. Statistical analysis are reported threefold; firstly a comparison of marker levels between KC vs. HC, secondly a comparison of KC vs. both healthy and diseased control groups (HC+DG), and thirdly a comparison of HC vs. both diseased groups (KC+DG). Differences in marker levels were statistically tested using the one-way independent ANOVA for normal distributions or the Kruskall-Wallis Test for non-normal distributions. We used either Tukey's or Dunn's Tests for Post Hoc multiple comparisons. Statistical analyses were performed using SPSS 20.0 (IBM SPSS Statistics, USA). Graphs were made in Prism 6.02 (GraphPad Software Inc., USA).

RESULTS

Study population

The baseline characteristics of the three study groups are depicted in table 1. The mean age of the KC patients was 42.7±18.3 years, 63.5±14.7 years for the DG group, and 80.5±10.5 years for the healthy control (HC) group. Details on baseline characteristics are given in Table 1.

Gene expression profile

Gene expression profiles are indicated in appendix 1 and visually represented in appendix 2. Fifteen genes were significantly different expressed between KC vs. HC, and most of these genes were also affected in the diseased control group (DG). Of the four GWAS previously identified risk loci only *FRAP1/MTOR* showed a significantly altered expression in KC samples (P=0.005). Subsequently, several genes related to the mammalian target of rapamycin (mTORC1) pathway were significantly higher expressed in the KC samples compared to healthy controls: *AKT1* (24.8x higher, P<0.001), *DEPTOR* (4.9x, P=0.006), *FOXO4* (58.8x, P<0.001), *IGF1* (16.5x, P<0.001), *IGF1R* (20.4x, P<0.001), *MTOR* (6.5x, P=0.004), and *RAPTOR* (4.8x, P=0.010). In contrast, the levels of *MDM2* (0.16x, P=0.005) decreased. The expression of *RICTOR* (0.4, P=0.331) was not significantly altered. Strikingly, the aberrant gene expression profile of KC largely overlaps with severely failed corneal grafts (DG), see appendix 2. Finally, the levels of *NFKB1* (13.9x, P<0.001), SIRT7 (52.4x, P<0.001), and *WRN* (16.2x, P<0.001) were

	Keratoconus (KC)		Decompensated grafts (DG)		Healthy controls (HC)	
	Mean/N	Range/%	Mean/N	Range/%	Mean/N	Range/%
Age (years), mean	42,7	22-67	63,5	39-82	80,5	67-94
Male sex, N	7	58,3	3	27,3	6	60
Positive for atopic disease, N	7	58,3	3	27,3		
Contact lens wear <2 weeks before transplantation, N	6	50	1	9,1		
Intra ocular pressure (mmHg), mean	14,1	Jul-20	13,6	0-22		
Schirmer's test outcome (mm), mean	18,7	May-35	17,7	Mar-35		

Table 1. Study population characteristics

Marker	Group	Mean ΔCT ±SD	Median Fold Change	Multiple comparison†	Post Hoc tests				
					Post hoc KC vs. HC‡	Post hoc KC vs. DG‡	Post hoc DG vs HC‡	Post hoc HC vs. KC+DG†	Post hoc KC vs. HC+DG†
FOXO1	HC	43.26 ± 23.00	0.000	0.783	-	-	-	-	-
	DG	60.52 ± 77.74	0.000						
	KC	50.96 ± 34.58	0.000						
FNDC3B	HC	12.79 ± 15.24	0.009	0.021*	0.150	0.220	0.766	0.011*	0.044
	DG	22.75 ± 18.66	0.000						
	KC	39.32 ± 29.19	0.000						
PDGFRA	HC	50.89 ± 76.23	0.000	0.001**	0.304	0.086	0.998	< 0.001**	0.143
	DG	39.64 ± 62.21	0.000						
	KC	336.99 ± 464.68	0.000						
FRAP1/ MTOR	HC	4.299 ± 0.637	0.048	0.005*	0.004*	0.182	0.081	0.005*	0.013*
	DG	2.754 ± 0.970	0.148						
	KC	1.813 ± 0.966	0.313						
AKT1	HC	2.130 ± 0.926	0.159	< 0.001**	< 0.001**	0.843	< 0.001**	< 0.001**	0.047
	DG	-1.713 ± 0.514	3.638						
	KC	-1.901 ± 0.738	3.950						
DEPTOR	HC	5.971 ± 0.732	0.015	< 0.001**	0.006*	0.026	< 0.001**	0.001**	0.815
	DG	2.308 ± 0.883	0.190						
	KC	3.751 ± 0.635	0.074						
FOXO4	HC	4.871 ± 0.372	0.038	< 0.001**	< 0.001**	0.355	< 0.001**	< 0.001**	0.019*
	DG	-0.528 ± 0.810	1.653						
	KC	-1.150 ± 1.034	2.235						

IGF1	HC	9.513 ± 2.257	0.002	< 0.001**	0.005*	0.124	< 0.001**	< 0.001**	0.826
	DG	2.815 ± 1.308	0.084						
	KC	4.887 ± 0.472	0.033						
IGF1R	HC	0.681 ± 1.040	0.621	< 0.001**	< 0.001*	0.837	< 0.001**	< 0.001**	0.049
	DG	-3.704 ± 0.737	14.470						
	KC	-3.952 ± 0.956	12.676						
MDM2	HC	0.647 ± 0.680	0.556	0.005*	0.022*	0.347	0.004*	0.003*	0.726
	DG	4.176 ± 1.306	0.044						
	KC	3.177 ± 1.213	0.093						
RAPTOR	HC	6.565 ± 0.346	0.012	0.010*	0.018*	0.927	0.014*	0.002**	0.306
	DG	3.699 ± 1.729	0.075						
	KC	3.955 ± 0.308	0.058						

GWAS: Genome Wide Association Study, see references 7,8,9. MTORC1: Mammalian Target Of Rapamycin Complex 1. ΔCT: delta cycle threshold. SD: standard deviation. HC: healthy control. DG: decompensated graft/diseased control. KC: keratoconus. *: significance < 0.05, **: significance <0.002 (corrected for multiple testing). †: One-way ANOVA in normal distributions and Kruskal-Wallis for non-normal distribution. ‡: post-hoc Tukey. V-Akt Murine Thymoma Viral Oncogene Homolog 1 (AKT1), DEP domain containing MTOR-interacting protein (DEPTOR), fibronectin type III domain containing 3B (FNDC3B), Forkhead Box O1 (FOXO1), Insulin-Like Growth Factor 1 (IGF1), Insulin-Like Growth Factor 1 Receptor (IGF1R), Forkhead Box O4 (FOXO4), Mouse Double Minute 2 homolog (MDM2), Mammalian Target Of Rapamycin (MTOR), Platelet Derived Growth Factor Receptor Alpha (PDGFRA), Rapamycin-Insensitive Companion Of MTOR (RICTOR), Regulatory Associated Protein Of MTOR (RAPTOR).

Table 2: Gene expressions, median fold change and statistical analyses of GWAS identified genes and MTORC1-associated genes

DISCUSSION

This study identifies the hitherto unknown activation of the *mTORC1* signaling pathway in severe keratoconus corneas. Recent insights by meta-analysis of GWAS data from large European and Asian KC cohorts have revealed susceptibility loci near *FOXO1* and FNDC3B individuals,[7] and MTOR/FRAP1 and PDGFRA[8] in European and Asian cohorts that confer relatively large risk for the development of KC and suggests pathological changes in cellular metabolism and cell cycle control underlying the pathophysiology of KC. We identified several key components of the mTORC1 pathway to be significantly upregulated in KC, including MTOR itself, its accessory gene RAPTOR, the gene coding the major growth factor IGF1, its receptor IGF1R, and the potent stimulator AKT1. These functional implications strengthen the previous genetic association with MTOR/FRAP1 identified by genome-wide studies.

The human mTOR gene encodes a protein of 2549 amino acids and is found in two distinct complexes (mTORC1 & mTORC2) that are characterized by their unique accessory proteins RAPTOR and RICTOR respectively. mTORC1 mainly regulates cell metabolism in response to nutrient availability, while mTORC2 regulates pro-survival mechanisms in response to growth factors. MTORC1 pathway inhibition acts as a protective system against cellular exhaustion.[11] Conversely, the target of rapamycin itself promotes aging in various organisms, including mammals, as shown by the significantly increased lifespan and reduced age-related diseases in mice after inhibition of mTOR by administering rapamycin.[12] Data on the expression of mTOR associated pathways in ocular tissue is currently emerging,[13] but is mostly limited to retinal and neural domains. In perspective of the current knowledge on mTOR, these results indicate that the KC phenotype might in part result from increased upregulation of the mTORC1-pathway in particular. We state that KC corneas show signs of cellular aging far exceeding their healthy, biological older, peers, to a level comparable to severely failed grafts. Thus, this study provides genetic-based translational insights into the underlying biology of this intriguing corneal disease.

Interestingly, the levels of KC risk loci FOXO1, FNDC3B and PDGFRA were not significantly altered between the groups and suggest that the associated SNPs do not alter gene expression. Indeed, publicly available expression quantitative trait locus (eQTL) database Genevar revealed that the previously reported SNPs near FOXO1, FNDC3B or PDGFRA do not function in terms of transcript regulation of these genes.[8,14,15]

In addition to mTORC1 activation we also found evidence for upregulation of NFKB1 (Nuclear factor NF-κ-B), SIRT7 and WRN genes. NFKB1 is a major promoter of various inflammatory pathways, which underlines the emerging concept of inflammatory pathways underlying the pathophysiology of keratoconus.[4] SIRT7 is part of the poorly understood HDAC class III family and is thought to promote cellular senescence after increased genomic stress and, like MTORC1, prevents cellular exhaustion. The higher level of Sirt7 expression in keratoconus therefore point in the similar direction; a state of premature cellular senescence by increased cellular stress.[16] The WRN gene encodes a DNA helicase that is involved in the repair of DNA damage, its upregulation in keratoconus corneas further underscores the presence of genomic stress.[17]

There are a few important considerations with regard to the results of this study. Although the number of included samples is relatively small, the differences were sufficient to reveal distinct gene expression profiles for KC and unaffected controls delineating the robust activation of the mTOR pathway in severe KC. Also, the diseased corneal samples were all obtained during a grafting procedure, and obviously only the more severe cases are indicated to undergo such invasive surgery. This skewed our sample selection, and these outcomes might not necessarily represent all disease stages of keratoconus. For example, corneal scarring is common in the severe cases of keratoconus, while less advanced cases can also show the archetypical conical shape, though with a completely clear cornea. Nevertheless, the sample set in this study also included clear corneas and revealed robust activation for these corneas as well. Finally, the mean age of patients groups differed significantly. In fact, there were no overlapping values for age which made correction for age-effects statistically unsound. Regardless, potential correction for age effects would only strengthen the here described results, since the highest amount of mTORC1 upregulation and associated cellular exhaustion was found in the biologically younger (KC) samples. Since the current cross-sectional data do not permit drawing conclusions on the direction of causality, future research is necessary to reveal the role mTOR in less severe and progressive KC to determine the role of cellular aging as an etiological factor in the development of KC. More importantly, the mTORC1 pathway can specifically be inhibited by Rapamycin (Sirolimus or Rapamune®)[11,18] which is well-tolerated when administrated locally to the eye.[19] This provides potential therapeutic strategies targeting mTORC1 for graft failure, or severe keratoconus alike, and may form an alternative for invasive corneal surgery.

In conclusion, this study identified for the first time, a role for mTORC1 signaling

pathway in severe keratoconus. Further research should confirm the role of the mTORC1-pathway by assessing gene expression in larger populations preferably of several stages of KC. It warrants the evaluation of targeting mTOR signaling as a novel therapeutic alternative to corneal transplantation.

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Marker	Group	Mean ΔCT ±SD	Median Fold Change	Multiple comparisona	Post Hoc tests				
					Post hoc KC vs. HCb	Post hoc KC vs. DGb	Post hoc DG vs HCb	Post hoc HC vs. KC+Dga	Post hoc KC vs. HC+Dga
AHR	HC	-3.219 ± 0.920	107.778	0.08	-	-	-	-	-
	DG	-2.411 ± 0.926	5.393						
	KC	-1.199 ± 1.094	2.639						
AHRR	-	-	-	-	-	-	-	-	-
AKT1	HC	2.130 ± 0.926	0.159	< 0.001**	< 0.001**	0.843	< 0.001**	< 0.001**	0.047
	DG	-1.713 ± 0.514	3.638						
	KC	-1.901 ± 0.738	3.950						
CDKN2A	HC	5.624 ± 0.370	0.023	0.095	-	-	-	-	-
	DG	3.557 ± 0.800	0.085						
	KC	0.055 ± 5.711	0.962						
DEPTOR	HC	5.971 ± 0.732	0.015	< 0.001**	0.006*	0.026	< 0.001**	0.001**	0.815
	DG	2.308 ± 0.883	0.190						
	KC	3.751 ± 0.635	0.074						
FNDC3B	HC	12.79 ± 15.24	0.009	0.021*	0.150	0.220	0.766	0.011*	0.044
	DG	22.75 ± 18.66	0.000						
	KC	39.32 ± 29.19	0.000						
FOXO1	HC	43.26 ± 23.00	0.000	0.783	-	-	-	-	-
	DG	60.52 ± 77.74	0.000						
	KC	50.96 ± 34.58	0.000						
FOXO3	-	-	-	-	-	-	-	-	-
FOXO4	HC	4.871 ± 0.372	0.038	< 0.001**	< 0.001**	0.355	< 0.001**	< 0.001**	0.019*
	DG	-0.528 ± 0.810	1.653						
	KC	-1.150 ± 1.034	2.235						

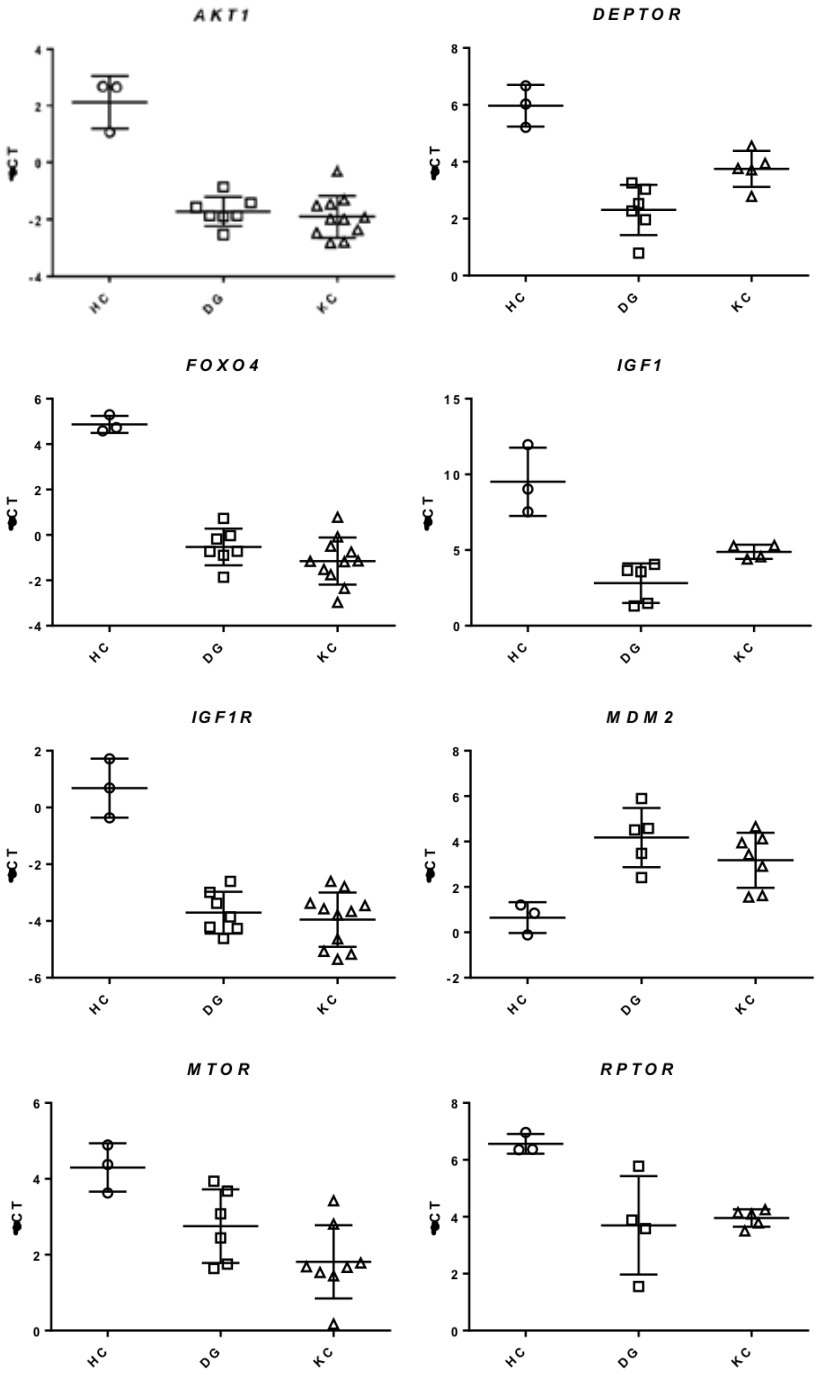
H2AFX	HC	1.490 ± 2.237	0.840	0.021*	0.011*	0.855	0.007*	0.526	0.007*
	DG	-1.764 ± 1.319	3.310						
	KC	-1.415 ± 1.114	2.259						
HDAC9	HC	8.784 ± 1.710	0.002	0.003*	0.003*	0.962	0.007*	< 0.001**	0.129
	DG	3.182 ± 2.034	0.206						
	KC	2.867 ± 2.107	0.088						
IGF1	HC	9.513 ± 2.257	0.002	< 0.001**	0.005*	0.124	< 0.001**	< 0.001**	0.826
	DG	2.815 ± 1.308	0.084						
	KC	4.887 ± 0.472	0.033						
IGF1R	HC	0.681 ± 1.040	0.621	< 0.001**	< 0.001*	0.837	< 0.001**	< 0.001**	0.049
	DG	-3.704 ± 0.737	14.470						
	KC	-3.952 ± 0.956	12.676						
IL6	-	-	-	-	-	-	-	-	-
IL10	-	-	-	-	-	-	-	-	-
MDM2	HC	0.647 ± 0.680	0.556	0.005*	0.022*	0.347	0.004*	0.003*	0.726
	DG	4.176 ± 1.306	0.044						
	KC	3.177 ± 1.213	0.093						
MTOR	HC	4.299 ± 0.637	0.048	0.005*	0.004*	0.182	0.081	0.005*	0.013*
	DG	2.754 ± 0.970	0.148						
	KC	1.813 ± 0.966	0.313						
NFATC1	HC	6.363 ± 0.339	0.011	0.378	-	-	-	-	-
	DG	5.082 ± 1.175	0.023						
	KC	5.180 ± 1.603	0.039						
NFKB1	HC	3.017 ± 0.403	0.133	< 0.001**	< 0.001**	0.885	< 0.001**	< 0.001**	0.058
	DG	-0.521 ± 0.519	1.542						
	KC	-0.716 ± 0.995	1.854						

PDGFRA	HC	50.89 ± 76.23	0.000	0.001**	0.304	0.086	0.998	< 0.001**	0.143
	DG	39.64 ± 62.21	0.000						
	KC	336.99 ± 464.68	0.000						
PTEN	HC	1.868 ± 1.328	0.225	0.300	-	-	-	-	-
	DG	3.093 ± 0.903	0.108						
	KC	2.543 ± 1.219	0.151						
RICTOR	HC	2.755 ± 0.580	0.169	0.109	-	-	-	-	-
	DG	4.363 ± 0.596	0.044						
	KC	3.732 ± 1.241	0.069						
RPTOR	HC	6.565 ± 0.346	0.012	0.010*	0.018*	0.927	0.014*	0.002**	0.306
	DG	3.699 ± 1.729	0.075						
	KC	3.955 ± 0.308	0.058						
SIRT1	HC	2.817 ± 0.223	0.155	0.822	-	-	-	-	-
	DG	3.372 ± 1.551	0.127						
	KC	3.056 ± 1.155	0.125						
SIRT6	HC	7.854 ± 1.332	0.007	0.005*	0.005*	0.783	0.014	0.001*	0.090
	DG	4.075 ± 1.194	0.075						
	KC	3.450 ± 1.520	0.054						
SIRT7	HC	5.774 ± 1.258	0.030	< 0.001**	< 0.001**	0.038	< 0.001**	< 0.001**	0.005*
	DG	0.311 ± 0.863	1.112						
	KC	-0.837 ± 0.801	1.572						
TERT	-	-	-	-	-	-		-	-
TP53	HC	3.751 ± 0.983	0.102	0.680	-	-	0.878	-	-
	DG	4.421 ± 1.497	0.047						
	KC	4.701 ± 1.656	0.042						

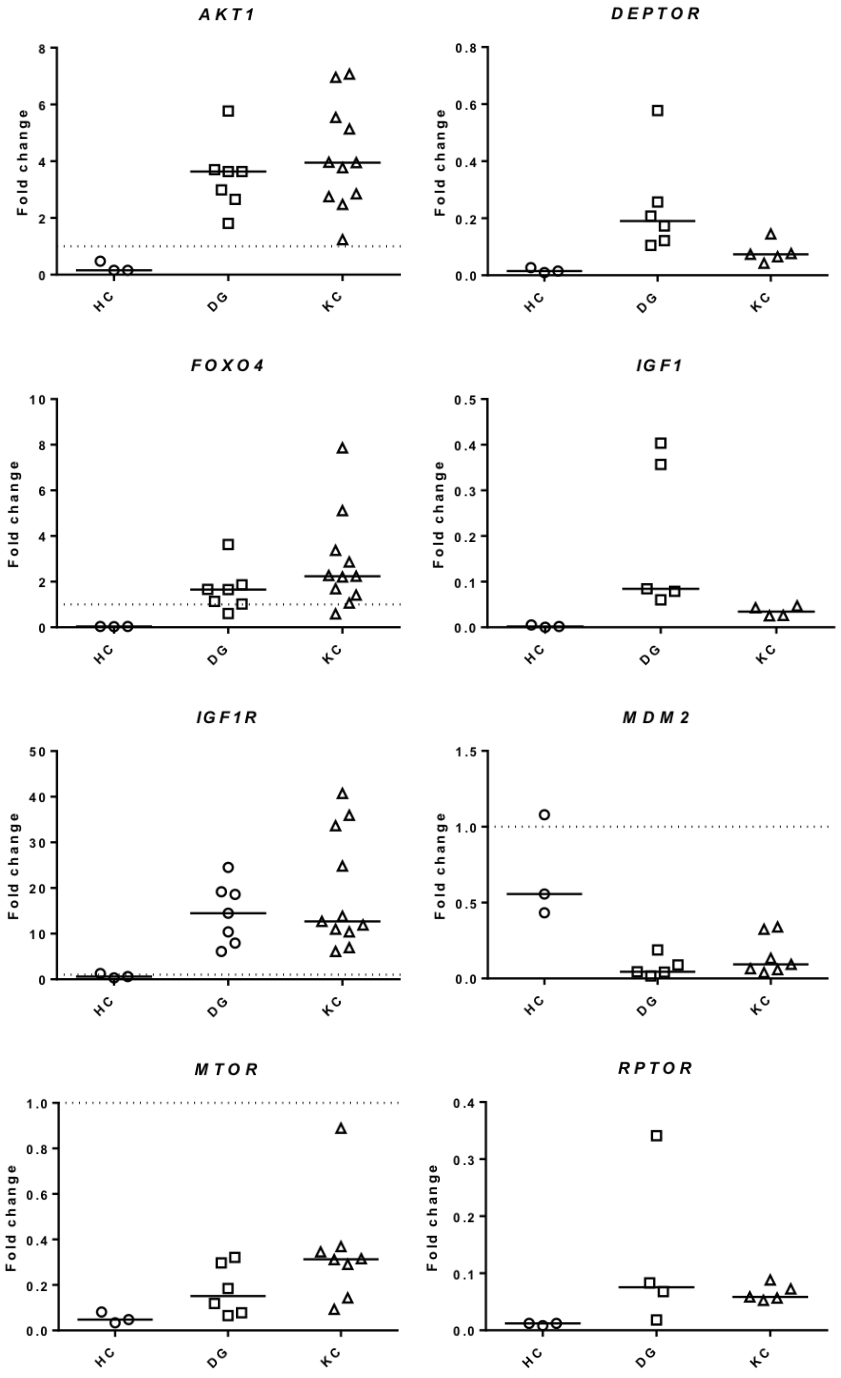
WRN	HC	3.745 ± 0.329	0.067	< 0.001**	< 0.001**	0.994	< 0.001**	< 0.001**	0.110
	DG	-0.300 ± 1.124	1.365						
	KC	-0.356 ± 1.213	1.089						

ΔCT: delta cycle threshold. SD: standard deviation. HC: healthy control. DG: decompensated graft/diseased control. KC: keratoconus. *: significance < 0.05, **: significance <0.002 (corrected for multiple testing). a: One-way ANOVA in normal distributions and Kruskal-Wallis for non-normal distribution. b: post-hoc Tukey. Aryl Hydrocarbon Receptor (AHR), Aryl-Hydrocarbon Receptor Repressor (AHRR), V-Akt Murine Thymoma Viral Oncogene Homolog 1 (AKT1), Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A), DEP domain containing MTOR-interacting protein (DEPTOR), FNDC3B, Forkhead Box O1 (FOXO1), Forkhead Box O3 (FOXO3), Forkhead Box O4 (FOXO4), H2A Histone Family, Member X (H2AFX), Histone Deacetylase 9 (HDAC9), Insulin-Like Growth Factor 1 (IGF1), Insulin-Like Growth Factor 1 Receptor (IGF1R), Interleukin 6 (IL6), Interleukin 10 (IL10), Mouse Double Minute 2 homolog (MDM2), Mammalian Target Of Rapamycin (MTOR), Nuclear Factor of Activated T-cells, Cytoplasmic 1 (NFATC1), Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1 (NFKB1), Platelet Derived Growth Factor Receptor Alpha (PDGFRA), Phosphatase And Tensin Homolog (PTEN), Rapamycin-Insensitive Companion Of MTOR (RICTOR), Regulatory Associated Protein Of MTOR (RPTOR), Sirtuin 1 (SIRT1), Sirtuin 6 (SIRT6), Sirtuin 7 (SIRT7), Telomerase Reverse Transcriptase (TERT), Tumor Protein P53 (TP53), Werner syndrome, RecQ helicase-like (WRN).

Appendix 1: Gene expressions, median fold change and statistical analyses of all studies gene expressions



APPENDIX 2. Visual representation of quantitative real-time PCR analysis of the MTORC1 associated genes: values for relative gene expressions. (ΔCT = delta cycle threshold; HC = healthy control; DG = decompensated graft; KC = keratoconus)



APPENDIX 3. Visual representation of quantitative real-time PCR analysis of the MTORC1 associated genes: fold changes for gene expressions. (HC = healthy control; DG = decompensated graft; KC = keratoconus)

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11
DNA-damage in keratoconus and the mediating role of UV radiation.

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Allegonda van der Lelij, Jasper CA Broen

Submitted

ABSTRACT

Keratoconus (KC) is a disease of the cornea that can lead to a severe decrease in visual acuity and may warrant performing a corneal graft. The pathogenesis of KC is considered to be multifactorial and is associated with oxidative stress. Both oxidative stress and ultraviolet (UV) light can cause DNA damage, and UV light has been implicated in the corneal pathology associated with KC. Therefore, the aim of this study was to investigate DNA damage in corneas with KC and in control corneas. Corneal buttons were obtained from 12 patients with KC who were undergoing corneal transplant surgery, 11 patients with a decompensated graft (DG) not related to KC, and 10 unaffected (healthy) post-mortem donor corneas (HC). Total DNA was extracted from the corneal buttons, and the number of intact Alu elements per genome copy was measured using qPCR and was used quantify intact DNA. Mean (±SD) DNA damage was similar between the KC (0.022 ± 0.030), DG (0.026 ± 0.053), and HC (0.011 ± 0.012) groups (P=0.719). No association was found between DNA damage and patient age (P=0.780), atopic constitution (P=0.495), or contact lens wear (P=0.452). One KC cornea that previously underwent epithelium-off crosslinking had a 100-fold higher level of DNA damage compared to the other samples. In conclusion, corneal DNA damage did not differ between the study groups. Thus, corneal DNA damage does not appear to be a major etiological factor in the pathogenesis of KC.

INTRODUCTION

Keratoconus (KC) is a corneal condition that can lead to refractive myopia, irregular astigmatism, corneal thinning, and poor visual acuity due to the hallmark "cone-like" shape of the cornea (Rabinowitz 1998) and—in advanced cases—corneal scarring. Both environmental (e.g., eye-rubbing, atopic constitution, etc.) and genetic factors have been linked to hypersensitive oxidative stress responses at the ocular surface. (Bawazeer et al. 2000, Jafri et al. 2004, Karamichos et al. 2014) DNA damage induced by ultraviolet (UV) radiation has been suggested as a possible causative factor in the development of KC. (Atilano et al. 2005, Buddi et al. 2002) UV radiation contributes to the formation of reactive oxidative species (ROS) and the subsequent release of reactive aldehydes, nitrotyrosine, and nitric oxides, which can damage DNA, leading to breaks in the strand. Elevated levels of these compounds have been found in corneal tissue of patients with KC. (Buddi et al. 2002, Szabo and Ohshima. 1997)

The cornea is exposed extensively to light and absorbs the majority of UV light that enters the eyes; the UV light that is absorbed is primarily UV-A (i.e., 320-400 nm wavelength) light. Therefore, the corneal epithelium is heavily exposed to the potentially detrimental effects of UV radiation on DNA integrity. (Lombardo et al. 2015) Consequently, the cornea has several robust intrinsic defense systems against UV-induced damage and ROS in particular. Thus, the aberrant expression and/or function of any of these intrinsic corneal anti-oxidant systems could lead to tissue damage and even corneal disease. Indeed, studies have found altered activity of several enzymes in the corneas of patients with KC, including the enzymes superoxide dismutase (Behndig et al. 2001), aldehyde dehydrogenase (Gondhowiardjo et al. 1993), catalase (Kenney et al. 2005), cathepsin (Kenney et al. 2005), glutathione reductase, transferase, and peroxidases (Gondhowiardjo et al. 1993); this altered enzyme activity may therefore contribute to oxidative stress and tissue damage. Moreover, DNA damage induced by oxidative stress secondary to solar UV radiation has been suggested to increase the risk of developing keratoconus. (Atilano et al. 2005)

The relationship between oxidative stress and DNA damage has been studied extensively. (Cooke et al. 2003, Shi et al. 2012) Both ROS and UV light—among other factors—have been associated with DNA damage in several types of cancers. Although various methods are currently available for detecting DNA damage, few methods can accurately quantify DNA damage. Here, we quantified DNA damage by measuring

the number of intact Alu elements, which are short interspersed DNA repeats that were characterized originally by their sensitivity to the restriction endonuclease Alu (Arthrobacter luteus). The human genome contains more than a million Alu repeats, which have specific sequence motifs comprised of long stretches of T (thymine) nucleotides; these motifs are the likely site for the formation of UV-induced lesions and DNA breaks. Englander and Howard measured intact Alu elements as a means to quantify the level of DNA damage in the genome. (Englander and Howard 1997) In, addition, Wang et al. (1999) measured short interspersed DNA elements as a marker of UV-mediated damage and repair. Based on the success of this robust method, we measured the level of DNA damage in corneal samples obtained from patients with KC.

MATERIAL AND METHODS

Corneal samples

Twelve cornea samples were obtained from 12 patients who received a corneal transplant for severe KC or pellucid marginal degeneration (the KC group). A second group of 11 corneal samples was obtained from 11 patients who underwent a re-grafting procedure due to a decompensated corneal graft in which the indication for the primary graft was not KC (the DG group). The corneal buttons in the KC and DG groups were processed using Tissue-Tek (Sakura Finetek USA, Inc., Torrance, CA) immediately after resection, cut into five full-thickness slices, and stored at -80°C.

Corneal samples were also obtained from ten healthy controls (the HC group); these samples were obtained from the Euro Cornea Bank (Beverwijk, the Netherlands) and the Department of Anatomy, University Medical Center Utrecht, Utrecht, the Netherlands. Within 24 hours of death, the corneas were prepared from post-mortem tissue obtained from ten unrelated Caucasian donors, each of whom had no documented history of KC, ocular inflammation, or vitreoretinal disease. All patients provided written informed consent. Informed consent for the post-mortem donation of ocular tissue was provided under the auspices of the head of the Department of Anatomy, University Medical Center Utrecht, the Netherlands. All tissues were acquired in compliance with Dutch law (Wet op de lijkbezorging, Art 18, lid 1/ 18–06–2013) and the institutional guidelines established by the University Medical Center Utrecht.

This study was performed in accordance with the tenants of the Declaration of Helsinki and complied with local laws and good clinical practice. Storage of the corneal buttons in the University Medical Center Utrecht Biobank was approved by the institution's Ethics Review Board.

Clinical data extraction

Additional data was extracted from the patient records and included both the patient history and the preoperative assessment, which included the results of a slit lamp evaluation, Schirmer's test, and Scheimpflug corneal tomography (Pentacam HR, Oculus GmbH, Wetzlar, Germany). The data available for the HC group were limited to age, gender, and cause of death. Each KC cornea was graded as clear, mildly hazy, or clouded based on slit lamp biomicroscopy. All decompensated grafts were considered clouded, and all healthy corneas were considered clear.

Assessment of DNA damage

DNA was isolated from the corneal buttons using TRIzol (Life Technologies, Thermo Fisher Scientific, Grand Island, NY, USA); this approach allows for the isolation of small DNA molecules, which can be lost when using column-based isolation techniques. After dissolving the cornea in TRIzol reagent, the RNA was removed, and the original tubes containing the TRIzol reagent and DNA were stored at -20°C. After isolation, double-stranded DNA was measured using a Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific). The number of intact Alu elements in the DNA was measured using qPCR and was used as a proxy for quantifying intact DNA. (Englander and Howard 1997)

Statistical analysis

The level of DNA damage (measured using the number of amplified Alu elements) was corrected for input DNA and graphed in a box plot; the mean level of DNA damage was calculated for each study group. Statistical analyses were performed using SPSS 21.0 (IBM, Armonk, NY, USA). Outlier analysis was performed by removing samples with DNA damage that exceeded >4 SD; these values were analyzed separately. Differences in DNA damage were tested using the one-way independent ANOVA. Multiple comparisons were tested using the post hoc Tukey's test.

RESULTS

Study population

The characteristics of the three study groups are summarized in Table 1. The mean (±SD) ages of the subjects in the KC, DG, and HC groups were 42.6 ± 15.8, 63.5 ± 14.7, and 80.5 ± 9.9 years, respectively. Concurrent atopic disease was more prevalent in the KC group (in 58.3% of patients) compared to the DG group (27.3%); contact lens wear was also more prevalent in the KC group compared to the DG group (50% vs. 9.1%, respectively). The KG and DG eyes were similar with respect to the Schirmer's test results. Three years before the grafting procedure, one eye in the KC group underwent epithelium-off corneal crosslinking with UV-A irradiation in accordance with the Dresden protocol. (Wollensak et al. 2003) Four samples in the DG group and four samples in the HC group did not yield sufficient DNA for analysis (i.e., were non-viable), thus reducing the effective sample size from 33 corneas to 25. The characteristics of the four DG patients with non-viable samples did not differ from the mean group (data not shown). The donors of the non-viable HC samples were among the oldest samples (the mean age of these four subjects was 87 years). All 12 KC samples were viable for analysis.

DNA damage

The results of the DNA damage analyses are summarized in Table 2. The mean levels of DNA damage in the KC, DG, and HC groups were 0.30, 0.22, and 0.011, respectively (P=0.539; Figure 1A).

The values above represent the total number of breaks in the DNA double strands of all Alu elements per genome copy; thus, considerable variability was observed with

	Keratoconus (N=12)	Decompensated grafts (N=11)	Healthy controls (N=10)
Mean age, years (range)	42.7 (22-67)	63.5 (39-82)	80.5 (67-94)
Male gender, N (%)	7 (58.3%)	3 (27.3%)	6 (60%)
Positive for atopic disease, N (%)	7 (58.3%)	3 (27.3%)	NA
Contact lens wear, N (%)	6 (50%)	1 (9.1%)	NA
Mean IOP, mmHg (range)	14.1 (7-20)	13.6 (0-22)	NA
Mean Schirmer's test outcome, mm (range)	18.7 (5-35)	17.7 (3-35)	NA

IOP = intraocular pressure, NA = not applicable

Table 1: Characteristics of the three study groups

	N	Mean age	Mean ± SD (all samples)	Multiple comparison[1]	Mean ± SD (outliers removed)[2]	Multiple comparison[1]
Keratoconus (KC)	12	41.6	0.30 ± 0.63		0.020 ±0.029	
Decompensated grafts (DG)	7*	71.3	0.22 ± 0.37	*P*=0.539	0.026 ±0.053	*P*=0.719
Healthy controls (HC)	6*	75.0	0.011 ±0.012		0.011 ±0.012	

1 Post hoc Tukey test. 2 Two outliers were removed from the DG group, and two outliers were removed from the HC group. * Four samples in the DG group and four samples in the HC group did not yield sufficient DNA for analysis

Table 2: Summary of DNA damage in the three study groups

respect to the total number of DNA breaks per sample (which ranged from 8.653×10-5 to 2.05 in this study). Therefore, we use outlier analysis to normalize the groups; using this approach, we excluded two KC samples and two DG samples from the analysis. Removing these four outliers reduced the DNA damage in the KC and DG groups to 0.022 ± 0.030 and 0.026 ± 0.053, respectively, but did not affect the statistical analysis (*P*=0.719; Figure 1B and Table 2). Similar results were obtained when the results were adjusted for age (data not shown), and we found no significant correlation between age and DNA damage (Spearman's ρ= - 0.057, *P*=0.780, N=26).

Both atopy and contact lens wear are known risk factors for developing keratoconus. In the KC group, seven patients had atopy and six patients wore contact lenses; in the DG group, three patients had atopy and one patient wore contact lenses (see Table 1).

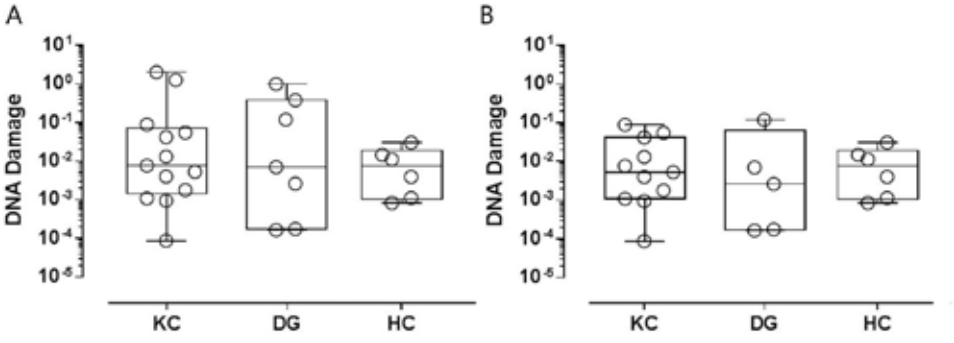

FIGURE 1: Box plots summarizing the DNA damage measured in the three study groups. DNA damage was measured as the number of intact Alu elements per genome copy. Each symbol represents an individual corneal sample. Panel A shows the entire data set. Panel B shows the same data set at A, with two outliers removed from the KC group and two outliers removed from the DG group (for comparison purposes, the HC data are repeated from panel A).

Therefore, we next measured DNA damage in these subgroups. The amount of DNA damage did not differ significantly between the atopic (0.035 ± 0.013) and non-atopic patients (0.121 ± 0.038; *P*=0.495) or between the patients who wore contact lenses prior to surgery and the patients who did not wear contact lenses (*P*=0.452). Interestingly, we found that the level of DNA damage increased slightly from the clear corneas (0.007 ± 0.009) to the mildly hazy corneas (0.021 ± 0.011) to the clouded corneas (0.039 ± 0.018), although this trend was not statistically significant (*P*=0.113). The details regarding all of the cases in this study are summarized in Table 3.

Case	Group	Age	DNA damage	Cloudiness	Atopic constitution	Contact lens wear
1	KC	46	1.79E-03	clear	Yes	No
2	KC	24	2.05E+00*	clear	Yes	Yes
3	KC	29	4.03E-03	clear	Yes	No
4	KC	53	1.08E-03	clear	No	Yes
5	KC	24	5.29E-03	clear	No	No
6	KC	22	8.65E-05	clear	No	No
7	KC	53	7.74E-03	mild haze	Yes	Yes
8	KC	67	1.31E-02	mild haze	No	Yes
9	KC	63	4.20E-02	mild haze	No	No
10	KC	32	5.51E-02	scarred	Yes	Yes
11	KC	50	8.95E-02	scarred	Yes	No
12	KC	49	1.28E+00*	scarred	Yes	Yes
13	DG	77	7.00E-03	scarred	Yes	No
14	DG	49	ND	scarred	Yes	No
15	DG	39	ND	scarred	Yes	No
16	DG	65	ND	scarred	No	No
17	DG	66	3.84E-01*	scarred	No	No
18	DG	69	1.00E+00*	scarred	No	No
19	DG	52	1.75E-04	scarred	No	Yes
20	DG	74	1.64E-04	scarred	No	No
21	DG	46	ND	scarred	No	No
22	DG	79	2.63E-03	scarred	No	No
23	DG	82	1.20E-01	scarred	No	No
24	HC	94	ND	clear	ND	ND
25	HC	94	ND	clear	ND	ND
26	HC	83	4.00E-03	clear	ND	ND
27	HC	67	3.12E-02	clear	ND	ND
28	HC	79	1.15E-03	clear	ND	ND
29	HC	84	8.45E-04	clear	ND	ND
30	HC	68	1.50E-02	clear	ND	ND
31	HC	86	ND	clear	ND	ND
32	HC	69	1.15E-02	clear	ND	ND
33	HC	81	ND	clear	ND	ND

HC = healthy control group, KC = keratoconus group, DG = decompensated graft group, ND = not determined (non-viable sample), NA = not applicable. *: sample was considered an outlier (>4 SD beyond the mean value)

Table 3: Overview of all obtained samples

DISCUSSION

In this study, we found that the level of DNA damage in the corneas of patients with keratoconus was similar to two control groups (patients with a decompensated graft not related to KC and healthy donor subjects). In addition, we found no significant correlation between DNA damage and either age, atopic constitution, or contact lens wear.

Despite our finding that DNA damage was similar between the KC and control eyes, several lines of evidence support the notion that keratoconic eyes have altered anti-oxidant function and/or an inadequate DNA repair system. (Wojcik et al. 2014) In our study, we found no difference in damage to nuclear DNA between the KC and control corneas; in contrast, Atilano et al. (2005) reported increased damage to mitochondrial DNA (mtDNA) in the corneas of patients with KC. However, oxidative stress (i.e., increased ROS production and apoptosis) was not accompanied by increased mtDNA damage in the corneal epithelium. (Atilano et al. 2009) Interestingly, cultured fibroblasts from KC corneal tissue have mitochondrial dysfunction, perhaps via increased mtDNA damage. (Chwa et al. 2006) This finding suggests either that corneal fibroblasts are more susceptible to mtDNA damage than corneal epithelium or that the in vitro cultured cells do not retain the complex anti-oxidant systems found in the intact ocular surface. Nevertheless, our results suggest that nuclear DNA damage is not increased in the corneas of KC patients; moreover, consistent with the mtDNA data reported by Atilano et al. (2009), the corneal epithelium of KC patients does not appear to have increased DNA damage.

Other factors have been associated with the development of KC, including atopic constitution (Bawazeer et al. 2000) and contact lens wear (Moon et al. 2006). However, in our study, we found no correlation between DNA damage and either atopy or contact lens wear. Interestingly, one patient with KC in our study underwent an epithelium-off corneal crosslinking procedure with UV-A irradiation three years prior to the grafting procedure, and this patient had a 100-fold higher level of DNA damage compared with the other samples (Wollensak et al. 2003), thus supporting our hypothesis that our approach can detect UV-mediated DNA damage. This result also indicates that UV crosslinking induces substantial DNA damage in the relatively long-lived keratocytes of the corneal stroma. In support of the putative link between UV-A irradiation and DNA damage, a recent report presented a case in which crosslinking was associated with

intraepithelial neoplasia. (Krumeich et al. 2014)

Although we found an apparent—albeit not statistically significant—correlation between corneal DNA damage and the degree of corneal cloudiness/scarring, it is important to note that patients with more corneal cloudiness/scarring are more likely to undergo a corneal transplant procedure, thereby biasing our sampling method in favor of more severe cases. Nevertheless, the putative link between DNA damage and corneal cloudiness/scarring warrants further investigation.

CONCLUSIONS

In summary, we found no apparent increase in DNA damage in corneal samples obtained from patients with keratoconus compared to healthy controls and patients with decompensated grafts. Thus, the link between DNA repair system dysfunction and/or direct UV radiation in patients with keratoconus and corneal DNA damage remains unclear and should be studied further.

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The authors declare no conflicts of interest.

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12
Summary and Discussion Nederlandse samenvatting

Robert PL Wisse

SUMMARY AND DISCUSSION

The summary is organized in four sections following the contents of this thesis. The title of these chapters can be interpreted as clinical questions relevant from a patient perspective. Firstly, how can we restore visual acuity in patients suffering from keratoconus? This section discusses the here researched treatments that aim to improve the visual acuity in keratoconus patients, namely transplant surgery and fitting contact lenses. Secondly, how can we prevent disease progression? This section is on corneal crosslinking and its stabilizing effect on keratoconus development. Thirdly, which factors contribute to the development of keratoconus? The third section summarizes our findings on the fundamentals of keratoconus and provides insights on the immunological changes associated with keratoconus. Finally, the last section will briefly point out some future perspective of keratoconus research.

RESTORING VISUAL ACUITY IN KERATOCONUS

Many treatment modalities exist that can improve visual acuity in keratoconus; from spectacle and contact lens corrections, toric phakic implant lenses, intra corneal ring segment implantation, and topo-guided refractive surgery, to corneal grafting procedures. Not all these treatments are subject of this thesis. However, the mainstay of restoring visual acuity in keratoconus is the prescription of adequate visual aids and performing corneal transplant surgery in the most severe cases.

Corneal surgery underwent major changes during the last decade, with the popular posterior lamellar graft as its most candid representative. Anterior lamellar surgery saw many innovations as well, and some authors state that (deep) anterior lamellar keratoplasty is the surgery of choice in any eye with a healthy endothelium.[1–3] **Chapter 2** focusses on the actual performed grafting procedures based on the registration database by the Dutch Transplantation Foundation between 2005 and 2010. Here we report that a perforating technique was overall the most performed procedure for grafting keratoconus corneas, even in those with a healthy endothelium. Strikingly, not even in 2010 did lamellar surgery surpass the perforating transplantation rate. It presumes a gap between the inventor-ophthalmologists who report their findings, and the field of corneal surgeons, in terms of acceptance and technical ability, since anterior lamellar techniques are notorious for their technical difficulty. The Dutch transplant registration data are not influenced by patient selection for trial purposes and give insight in the visual acuity and keratometry of transplanted patients. These values could be considered a measure of keratoconus severity. Interesting comparisons can be made with other surgical studies, should the baseline characteristics be adequately reported. Excellent examples exist of national trial registers that provide valuable clinical information.[4–7] Therefore, it would be of considerable interest to investigate how this trend develops over time.

One of the developments in lamellar corneal surgery is described in **Chapter 3**. Here, the results are presented of the PENTACON trial that compared a partial endothelial trepanation (PET) in addition to a lamellar keratoplasty, with a so called big bubble deep anterior lamellar keratoplasty (DALK). The primary outcome parameter was based on the premise that the PET technique would have a far lower rate of surgical complications than the DALK technique; the event of a conversion to a full-thickness penetrating keratoplasty (PKP). Despite the fact that the trial was underpowered and

no solid conclusions can be drawn on the superiority of either technique in terms of surgical safety, some interesting observations can be made. Most importantly, the PET technique was not as save, or easily delivered, as was presumed based on previous experiences.[8] Another observation is on the difficult equilibrium between trial obligations and surgical innovations. Researchers recently debated that the timeframe of a well-conducted trial spans many years; years in which the investigated technique can be adjusted and improved.[9] What then is the value of a trial if it provides evidence based medicine for yesterday's procedures? The latter is of particular relevance in corneal surgery and the here discussed trial, since Busin himself recently published an improved technique for keratoconus surgery which renders the previously reported PET technique obsolete.[10]

Probably the most applied therapy for restoring visual acuity in keratoconus is the prescription of (scleral) contact lenses. **Chapter 4** evaluates the objective and subjective performance of various contact lens types that were fitted based on a lens selection algorithm and were used for a broad range of clinical indications, among which keratoconus. Keratoconus patients in particular are often dependent of contact lenses for an adequate correction of their irregular astigmatism. The presented algorithm aids in the selection of the appropriate lens, based on the amount of corneal irregularity and factors like tear film quality. Similar outcomes can be achieved in terms of visual acuity and subjective lens performance with both soft lenses and scleral lenses when applying this algorithm. Importantly, handling, and overall satisfaction were similar between scleral lens users and soft lens users. In addition to underscoring the clinical value of scleral lenses, our results also highlight the need for practitioners to be familiar with a wide range of lens types and tailored lens selection. It can be debated that the high level of contact lens care in the Netherlands has effects on the selection of keratoconus patients for transplant surgery. In other words, do we transplant patients in a more advanced disease state now scleral contact lenses are so widely applied? An acceptable visual acuity is attainable in almost any clear cornea, regardless of the severity of the keratoconus and the subsequent degree of irregular astigmatism. It would be of interest to quantify the effect on (regional/national) scleral lens availability on corneal surgery rates or indications.

The biomechanical changes in keratoconus eyes affect the measurement of the intra-ocular pressure (IOP), since traditional applanation tonometry depends on a pre-defined corneal contact area and corneal rigidity. The availability of a device that circumvents the cornea for measuring IOP led to **Chapter 5**. Here, two methods

of IOP measurement were compared; the Diaton® device assesses IOP transpalpebrally, and could provide insight in potential false-low IOP outcomes. The comparator was regular Goldman applanation tonometry. The small mean difference of IOP measurements between both devices suggests that the Diaton could be an alternative IOP measurement. However, the wide variability of the Diaton measurements and its poor correlation to applanation tonometry renders the use of the Diaton tonometer in keratoconus debatable.

PREVENTION OF KERATOCONUS PROGRESSION

The advent of corneal crosslinking for keratoconus led to a paradigm shift in the treatment of keratoconus. Now, progressive keratoconus can be treated and stabilized in most cases, which has the potential to prevent corneal transplantations and (scleral) contact lens dependency. Improvements to the initially propagated technique by Seiler and Wollensak are well studied. One drawback of their treatment protocol is the removal of the corneal epithelium to promote penetration of the photo-exciting Riboflavin solution in the corneal stroma. This corneal abrasion is painful, and healing normally takes one to two days. Furthermore, it can be considered a port d'entrée for pathologic micro-organisms increasing the risk of a keratitis. To circumvent these drawbacks, trans epithelial crosslinking (TE-CXL) was conceived. Here, sodium ethylenediaminetetraacetic acid (EDTA) was added to the Riboflavin solution to enable penetration trough an intact corneal epithelium (Ricrolin TE®, SOOFT, Italy). Several studies report on an increased corneal rigidity, keratocytes apoptosis, and a visible stromal demarcation line as signs of TE-CXL effectiveness. Chapter 6 describes the results of a non-inferiorty randomized controlled trial where TE-CXL was compared to the regular epithelium-off treatment for the prevention of keratoconus progression. Although the TE-CXL treatment arm showed no adverse events, it was less effective to halt keratoconus progression after 1 year compared to epithelium-off crosslinking; and almost a quarter of the eyes showed an increase of maximal keratometry of more than 1 diopter compared to none of the eyes in the epithelium-off group. Given the protracted natural course of keratoconus, it appeared that the TE treatment had little effect at all. These outcomes underline the value of a comparative trial with adequate follow-up length to study treatment effectiveness, since outcomes of previously reported case series were unequivocal. Currently, we do not offer the TE CXL treatment in our department, regardless of its appealing safety profile. Alternative solutions to increase the actual crosslinking in the corneal stroma without removing its epithelium, like iontophoresis, a prolonged riboflavin absorption time, or the use of different

photosensitizer agents, are encouraging. Needless to say that these improvements have to be studied in a comparative trial to assess their additional value over the current available treatments.

Chapter 7 focusses on factors that contribute to the effectiveness of epithelium-off CXL, in terms of visual acuity and keratometry at one year follow-up. Baseline parameters like (un)corrected visual acuity (UDVA/CDVA), keratometry measurements, manifest refraction, and the additional parameters of atopic constitution, positive family history, and smoking, where analyzed for both outcomes. Since many potential predictors are interrelated, outcomes of this univariable analysis must be interpreted with caution. Therefore, a multivariable generalized estimations equation analysis was used to assess the independent potential predictors. With respect to visual acuity at the one-year follow-up, the only independent predictive factor was the pre-treatment logMAR CDVA. In short, having a lower visual acuity at baseline leads to improved visual acuity after treatment. Furthermore, cone eccentricity was the sole predictor of keratometry outcomes at the one-year follow-up. Finally, a prediction model was created for both outcomes. Visual acuity could be predicted quite well (R^2 0.45), based on the pre-treatment visual acuity. Validation of these findings in a sequential or extended treatment cohort is mandatory to assess the robustness of this prediction model.

In terms of physical optics, keratoconus is characterized by an increased irregular astigmatism, synonymous for higher order aberrations (HOA). HOAs are associated with a poor quality of vision, resulting in glare, starburst and halos; all symptoms familiar to keratoconus patients. Since CXL alters the keratometry somewhat, a change in the amount of HOAs could be expected. **Chapter 8** reports on our research on the changes in HOAs induced by CXL, at one year follow-up, and any independent effects of HOAs on visual acuity. It should be noted that the different subtypes of HOAs were highly interrelated. We considered coma, trefoil, and spherical aberration the three most relevant subtypes from a clinical perspective. On average, with the exception of spherical aberration, the HOAs were essentially unchanged following treatment for progressive keratoconus. A multivariable analysis revealed no independent effect of any HOA subtype on change in CDVA after crosslinking. However, changes in horizontal coma were significantly and strongly associated with the post-operative change in UDVA. Though HOAs are an important parameter for the quality of vision in keratoconus patients, CXL seem to pertain little changes to the nature and amount of these. The mean increased (un)corrected visual acuity perceived after CXL is therefore

not likely a resultant of the alterations in HOAs, but rather an effect of lowering refractive errors in general (including spherical and cylindrical errors).

UNRAVELING THE IMMUNOLOGICAL BASIS OF KERATOCONUS

Traditionally, textbooks[11-12] and most manuscripts refer to keratoconus as a non-inflammatory ectatic corneal disorder. However, accumulating evidence supports a contribution for several inflammatory pathways that in part orchestrate or amplify the tissue damage observed in keratoconus corneas. A systematic review on soluble and cellular inflammatory changes in keratoconus is found in **Chapter 9**, with the focus on tear film alterations. The cornea is part of a micro-environment where both locally produced and externally derived (tear film, conjunctiva) immune mediators are intertwined. Current literature convincingly shows that pro-inflammatory cytokines and cytokine receptors are upregulated in the tear film of keratoconus patients. Based on current literature it is not yet feasible to discern causal relationships of inflammatory changes on the development of keratoconus. Interestingly however, recent evidence also suggests systemic alterations in keratoconus, and genome wide association studies have identified loci that convey an increased risk for the development of keratoconus. Although keratoconus is not caused by corneal inflammation itself and experimental outcomes are not completely conclusive, these date strongly substantiate the emerging concept of underlying inflammatory pathways in the pathogenesis of keratoconus. It underlines that we are only beginning to understand the complexity of the interactions of all these mediators in several inflammatory pathways in keratoconus. Here again, the peculiar inverted relationship of keratoconus severity and chance of disease progression with age is of particular interest, since this feature might resemble an inflammatory effect mediating or perpetuating the development of keratoconus.

The relationship between age and keratoconus was elaborated upon in **Chapter 10**, were we identified the hitherto unknown potential activation of the mTORC1 signaling pathway in severe keratoconus corneas. Recent insights from meta-analyses of genome wide association studies revealed several susceptibility loci related to (the clinical changes in) keratoconus. These loci (FOXO1, FNDCB3, and MTOR/FRAP1) are involved in cellular metabolism and cell cycle control. In our study, the RNA expression of 28 genes associated with cellular aging was assessed in keratoconus corneas and compared to healthy controls and diseased controls (decompensated grafts). We identified several key components of the mTORC1 pathway to be significantly

upregulated in keratoconus, including MTOR itself, its accessory gene RAPTOR, the gene coding the major growth factor IGF1, its receptor IGF1R, and the potent stimulator AKT1. These functional implications strengthen the previous genetic association with MTOR/FRAP1 identified by genome-wide studies. In perspective of the current knowledge on mTOR, these results indicate that the keratoconus phenotype might in part result from increased upregulation of the mTORC1-pathway in particular. Could it be that keratoconus corneas show signs of cellular aging far exceeding their healthy, biological older, peers, to a level comparable to severely failed grafts? However, these encouraging first findings need to be validated in further studies. Firstly, the number of both healthy and diseased samples should be expanded, and secondly it would be interesting to assess the potential for intervening in this pathway. Finally, it would be very interesting to assess these changes in actual progressive keratoconus, rather than in specimens obtained during grafting surgery.

The availability of novel techniques that quantify UV-mediated DNA damage enabled us to investigate the relationship between UV-radiation and keratoconus. **Chapter 11** shows the results of this study, where we could not convincingly demonstrate different levels of DNA damage for keratoconus corneas, healthy controls, or diseased controls (decompensated grafts). Previous research support a harmful anti-oxidant status of keratoconic eyes and possibly inadequate DNA repair, though a clinical relation relationship between UV exposure and keratoconus development has not been demonstrated. Interestingly, the one case which underwent epithelium-off corneal crosslinking 3 years prior to the grafting procedure revealed a 100x fold higher amount of DNA-damage, confirming the validity of our approach for detecting UV-mediated DNA damage. This also indicates that UV-mediated crosslinking induces substantial DNA damage in the relatively long-living keratocytes of the corneal stroma. The need for a corneal graft after a crosslinking treatment can be considered quite rare, but it would be of great interest to assess the amount of DNA damage in other cross-linked corneas as well. Clinical experience with crosslinking now stretches over a decade, but the actual long term changes on keratocytes functioning are largely unknown.

FUTURE PERSPECTIVES

In addition to the remarks made in the previous sections, several directions for future research can be identified. Firstly, the clinical effectiveness of crosslinking on disease progression has been convincingly shown. Whether CXL actually prevents corneal

grafting procedures is a premise that deserves further attention. Many more patients are now being treated with crosslinking than there were transplanted. Crosslinking evidently harbors short term costs in terms of health care expenditures, temporally decreased quality of life, and loss of productivity. Does this make up for the prevention of a (costly) grafting procedure with evident morbidity and revalidation? Will this be alike for different age groups or disease states? Assessing the cost effectiveness of crosslinking should answer this question.

To assess factors that convey a risk of the serious side effects of crosslinking (keratitis, persistent corneal haze) would clinically be of great interest, since these complications have a detrimental effect on (contact lens corrected) visual acuity. Further improving the safety profile of crosslinking would thus be important. As was mentioned before, new developments should be investigated in a comparative non-inferiority setting, to assess their true additional value in terms of effectiveness. Given to all together low rate of serious adverse events, a proper registration system could help in determining the safety of (new) crosslinking treatment modalities.

The findings with regards to the mTOR pathway activation warrant further research to strengthen our hypothesis on the inflammatory origin of keratoconus. The results should be repeated and validated, and ideally an in vitro inhibition study could provide insights on the expression of mTOR pathway constituents in diseased corneas. Could this lead to novel anti-inflammatory treatments for the treatment of corneal diseases (keratoconus progression? graft decompensation?). Investigating the inflammatory etiology of keratoconus would be of paramount interest as well. A longitudinal design with the acquirement of different samples (corneal, tear film, serum) at different moments in time, ideally capturing the progressive disease stage, could enable the determination of cause and effect relationships in these complex inflammatory cascades.

Finally, the concept that the inflammatory changes might not be restricted to the cornea deserves attention as well. Could a relationship between keratoconus and other inflammatory diseases be identified? To this end, a large epidemiologic study should be undertaken to assess associations and co-occurances with systemic (auto) inflammatory diseases.

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NEDERLANDSE SAMENVATTING

De corneachirurgie heeft grote veranderingen ondergaan in het afgelopen decennium. Een overzicht van deze ontwikkelingen wordt gegeven in het inleidende **hoofdstuk 1**. Opvallend is dat de nieuwere lamellaire operaties toch minder uitgevoerd wordt dan de perforerende transplantatie, gebaseerd op data van de Nederlandse Orgaan Transplantatie Registratie, zie **hoofdstuk 2**. Dit suggereert een kloof tussen de voorlopers die hun bevindingen rapporteren, en het werkveld van cornea chirurgen. **Hoofdstuk 3** beschrijft een trial waar de toegevoegde waarde van twee lamellaire technieken vergeleken werd. De beoogde power werd niet behaald en valide uitspraken over de toegevoegde waarde van enige therapie kunnen niet gedaan worden. Eén waarneming blijft overeind betreffende het moeilijke evenwicht tussen trial verplichtingen en chirurgische innovaties. Wat is nu de waarde van een trial als het evidence-based medicine antwoorden biedt voor de procedure van gisteren? De innovaties betreffende de conservatieve visuele rehabilitatie zijn beschreven in **hoofdstuk 4**. Scleralenzen worden in onze academische praktijk veelvuldig toegepast en de uitkomsten op patiëntniveau zijn goed.

De komst corneal crosslinking heeft geleid tot een paradigmaverschuiving. Trans-epitheliale crosslinking bleek veilig te zijn, echter minder effectief om progressieve keratoconus te stoppen, zie **hoofdstuk 5**. Uitkomsten van crosslinking zijn voorspelbaar in termen van (on) gecorrigeerde gezichtsscherpte en keratometrie. Een predictiemodel wordt beschreven in **hoofdstuk 6**. **Hoofdstuk 7** beschrijft de veranderingen in complexere refractieafwijkingen kenmerkend voor keratoconus; de hoge orde aberraties. Veranderingen in hogere orde aberraties lijkt geen onafhankelijk effect te hebben om de visus één jaar na crosslinking. De biomechanische eigenschappen van de cornea veranderen na crosslinking, wat een oogdrukmeting kan beïnvloeden. Een alternatieve techniek wordt beschreven in **hoofdstuk 8**. Deze transpalpebrale manier om oogdruk af te leiden blijkt echter te onbetrouwbaar om bruikbaar te zijn in de klinische praktijk, specifiek voor keratoconus patiënten.

Er is toenemend bewijs dat verschillende inflammatoire pathways een rol spelen in het ontstaan of verergeren van keratoconus. Een systematic review waarin de bijdrage van celullaire en oplosbare mediatoren (cytokines) in de traanfilm uiteenzet, is weergegeven in **hoofdstuk 9**. Cellulaire veroudering lijkt een rol te spelen in de pathogenese van keratoconus. Wij vonden dat een aantal belangrijke onderdelen

van de mTORC1 pathway aanzienlijk meer tot expressie komt in keratoconus, met inbegrip van mTOR zelf, en genen als RAPTOR, IGF-1, IGF1R, en AKT1, beschreven in **hoofdstuk 10**. We konden niet overtuigend aantonen dat er verschillende niveaus van DNA-schade bestaan voor keratoconus cornea's, gezonde controles, of zieke controles (gedecompenseerde grafts). **Hoofdstuk 11** trekt het concept van mogelijk onvoldoende DNA-reparatie in keratoconus ogen hiermee in twijfel , temeer omdat een klinische relatie tussen blootstelling aan UV licht en keratoconus tot dusver niet is aangetoond. Een overkoepelende conclusie en discussie wordt ten slotte gegeven in **hoofdstuk 12**.

13

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CURRICULUM VITAE

Robert Wisse was born on the 31st of March 1983 in Delft, son of an optician and a teacher. He grew up in Oostburg, Zeeuws-Vlaanderen together with his three younger sisters. After secondary education at 't Zwin College he entered medical school at Utrecht University in 2000.

His interest in ophthalmology in general, and anterior segment surgery in particular, was encouraged after a research elective in refractive surgery (supervisor KTZ dr. A.J.P.Rouwen). He dedicated the final year of his medical study to pursue his dream of becoming an ophthalmologist, where a second research elective was devoted to physical optics and experimental imaging (supervisor prof.dr. Dick van Norren). He graduated in December 2007 and started his residencies in Ophthalmology in the UMC Utrecht (supervisors prof.dr. J.S. Stilma and prof.dr. W.F. Treffers, later followed by prof.dr. S.M. Imhof), after working 6 months for the Central Military Hospital, Ministry of Defense, Utrecht (commander Col J. de Graaf). The peripheral part of his ophthalmologist training was done in Gelre Ziekenhuizen Apeldoorn (supervisor Drs. J. Scheenloop).

During his residencies he completed the basic and clinical examinations issued by the International Council of Ophthalmology. He obtained a grade in medical teaching (BKO), and he was an active board member of the national residents association. His research project commenced with a successful grant application at the Dr. F.P. Fischer Foundation for his work on the PENTACON trial in 2011. The final stages of his residencies were devoted to cornea and anterior segment surgery, which provided the basis for his subsequent fellowship, supervised by Dr. A. van der Lelij. In 2013 he was awarded the P.G. Binkhorst travel bursary and the 18th European Society of Cataract and Refractive Surgeons bursary to aid in his development as a corneal surgeon. He did a course in lamellar corneal surgery hosted by prof.dr. M. Busin (Forlí, Italy) and a clinical observership at dr. G.R.J. Melles (NIIOS, Rotterdam, The Netherlands). His interest in medical education led to participation in the Teaching Scholars Program (hosted by prof. dr. Th.J. ten Cate, prof. dr. J. van Tartwijk and dr. M.S. van der Schaaf) and the acquisition of a virtual reality cataract training platform (Eyesi®).

He is part of the management team of the department of ophthalmology, head of the anterior segment and general ophthalmology unit, and head of the Utrecht Cornea Research Group.

Robert is married to Sanne Nijhof, a passionate pediatrician, and together they have two beautiful daughters, Julia (2011) and Roosmarijn (2014).