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DOTTORATO DI RICERCA IN BIOTECNOLOGIE MEDICHE

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Ciclo XXXVII

*Shot file-assisted ex vivo eccentric keratoconic eye model
recipient for stromal lenticule addition keratoplasty (SLAK)*

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Index

Chapter 1: Preliminary notions

Keratoplasty	p. 7
Definition	p. 7
Penetrating keratoplasty	p. 8
Deep anterior lamellar keratoplasty	p. 9
Keratoconus	p. 11
Definition	p. 11
Epidemiology	p. 12
Aetiology	p. 13
Histology	p. 14
Diagnosis	p. 15
Classification	p. 17
Keratoconus management	p. 20
Medical and parasurgical	p. 20
Surgical	p. 21
Stromal keratophakia	p. 24
History	p. 24

Femtosecond laser in ophthalmology	p. 27
Lenticule implantation	p. 29
Stromal lenticule addition keratoplasty (SLAK)	p. 33
Surgical technique	p. 35
Results	p. 37
Corneal tomography	p. 38
In vivo confocal microscopy	p. 39
Epithelial and stromal remodelling	p. 40
Excimer laser lenticule shape customization	p. 41
Surgical technique	p. 42
Results	p. 47
Limitations	p. 53

Chapter 2: The experimental study

Introduction	p. 55
Materials and methods	p. 57
Surgical technique	p. 58
Statistical analysis	p. 63
Results	p. 64
Discussion	p. 70

Conclusions p. 75

References p. 76

List of figures

Figure 1. Schematic representation of tissue replacement in PK vs. DALK	p. 9
Figure 2. Keratoconus tomography	p. 16
Figure 3. Femtosecond laser mechanism of action: photodisruption	p. 27
Figure 4. Corneal modifications induced by SLAK surgery	p. 34
Figure 5. SLAK donor preparation – FLEEx procedure	p. 36
Figure 6. SLAK surgery	p. 37
Figure 7. SLAK surgery results	p. 39
Figure 8. Keratoconus models creation	p. 44
Figure 9. Non-customized lenticules	p. 45
Figure 10. Planar and negative customized lenticules	p. 46
Figure 11. Planar and negative lenticule customization process	p. 47
Figure 12. Pre-implantation topographies of keratoconus models	p. 49
Figure 13. Anterior segment OCT scan of recipient cornea (keratoconus model 2) after stromal pocket creation and lenticule implantation	p. 51
Figure 14. Topographies before and after lenticule implantations	p. 53
Figure 15. Ablation patterns applied to create keratoconus model with eccentricity of 1.5 mm	p. 60
Figure 16. Marking and orientation of the cornea	p. 62

Figure 17. Keratoconus model with custom asymmetric excimer laser

shot file ablations

p. 65

Figure 18. Keratoconus model anterior segment OCT

p. 66

List of tables

Table 1. Amsler-Krumeich classification of keratoconus	p. 18
Table 2. ABCD classification of keratoconus	p. 19
Table 3. Mean outcome values between preoperative and postoperative times in keratoconus model corneas	p. 68-69

Chapter 1: Preliminary notions

Keratoplasty

Definition

The term keratoplasty define a group of procedures that are performed to substitute diseased corneal tissue and restore visual function. Usually, the main pathological characteristics that lead to corneal transplantation are the presence of an irregular curvature, a loss of transparency, or tissue thinning.

Corneal ectasia is still one of the main reasons for corneal transplantation in developed country (2022 Eye Banking Statistical Report). For many corneal diseases, medical therapy does not exist at all and surgery is the only option available when visual quality is unacceptable. Other conditions are amenable to conservative treatment but when this approach fails, surgery is the last option. If indication for optical keratoplasty exists, the aim of the procedure is to remove opacities and irregularities of shape that affect the visual axis. Tectonic keratoplasty aim is to restore normal thickness when severe thinning or even perforation are present. Therapeutic keratoplasty must be considered in situations in which severe infections are unresponsive to medical treatment and tend to spread inside the globe, leading to irreversible eye damage risk. Corneal tissue is normally avascular and this unique privilege makes it suitable for transplantation, usually without the need of long-term immune suppression therapy.

Penetrating Keratoplasty

Penetrating keratoplasty (PK) is, nowadays, the second most performed corneal surgical procedure in the world, being overtaken by endothelial keratoplasty (2022 Eye Banking Statistical Report). All corneal layers are substituted with a cadaveric donor cornea button by means of a full-thickness circular trephination. Donor tissue is secured to the recipient corneal rim through Nylon stitches with a running or interrupted suture. Usually, corneal buttons vary in diameter from 7.50 to 8.50 mm depending on the recipient cornea diameter.

The major indications for penetrating keratoplasty are keratoconus, bullous keratopathy, re-grafts, corneal scars, and corneal dystrophies¹⁻³. The order of frequency varies depending on the considered population and from study to study^{1,2}. Other but less frequent indications are corneal ulcers, degenerations, and trauma³.

The main disadvantages of this technique are immunologic rejection and open-globe surgery related complications². It is now well known that large diameter grafts are susceptible of higher risk of rejection but, on the other side, they give better visual results with lower astigmatism. Therapeutic penetrating keratoplasty add the risk of infection spreading inside the eye causing endophthalmitis². Graft survival at 5 years and 20 years from surgery has been reported to be 73% and 23.9% respectively⁴. The main reason of this high failure rate is related to endothelial cell population that may drop up to 50% in density at 5 years after PK⁵.

Deep Anterior Lamellar Keratoplasty

Deep anterior lamellar keratoplasty (DALK) is a lamellar corneal transplantation technique attempted since the first experiments in 1800s. The idea of substituting only diseased corneal layers to reduce complications has been the driving concept since the beginning of corneal transplantation. Several authors have proposed intriguing techniques of stromal removal but excellent results were achieved only recently when Anwar and Teichmann in 2002 described a method to facilitate the separation of Descemet membrane by forcefully injecting air into the corneal stroma⁶. They named it the “Big Bubble” technique and probably they did not recognized at first that different type of bubbles could be produced, a concept that later on was clarified by H.S. Dua⁷.

Deep Anterior Lamellar Keratoplasty (DALK) is now considered the gold standard procedure for surgical treatment of all corneal pathologies that do not affect the endothelium, despite its difficult execution⁸. Corneal stroma is trephined at partial depth and then, depending on the technique used, the anterior portion is separated from the posterior layers with a planar dissection and substituted with a donor stromal button devoid of the endothelial layer (Figure 1).

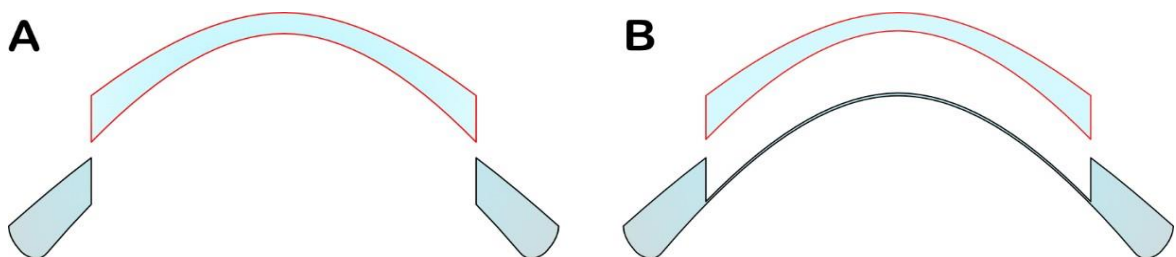


Figure 1. Schematic representation of tissue replacement in PK (A) vs. DALK (B).

The two main drawbacks of DALK are the higher surgical skill needed to perform the procedure and the lower quality of the lamellar interface if proper delamination is not achieved. The surgical conversion rate to PK due to perforation can be as high as 39%⁸. Beyond these disadvantages, all other factors incline toward this technique when recipient endothelium must be preserved. Compared to PK, immunologic rejection is less frequent and limited to the epithelium and stromal tissue (3.6% of epithelial rejection in DALK vs 11% of endothelial rejection in PK)⁹. Stromal rejection is easier to treat and does not reduce endothelial cell density. Borderie et al. analysed graft survival rate and reported that patients who had been treated with DALK showed a graft survival of 97% compared to 73% of PK at 5 years⁴. Endothelial cell loss at 1 year is 2% in DALK and 33% in PK⁵. Given the low risk of rejection, DALK allows the use of large diameter graft (9 mm) with better visual results and complete corneal ectasia removal¹⁰. Because of the lower risk of rejection and the reduced use of steroids, the risk of late onset complications is also lower¹¹. Another advantage of DALK over PK is the increased tectonic strength given by the preservation of the deepest stromal layers¹². This allows to remove sutures earlier in DALK with faster visual rehabilitation¹³.

Above all, best corrected and uncorrected visual acuity were evaluated in a systematic review of the literature and the refractive outcomes DALK and PK proved to be comparable¹³.

Keratoconus

Definition

Keratoconus (from Greek: kerato: cornea and conos: cone) is a non-inflammatory degenerative ectatic disease of the cornea that determines a progressive protrusion of the corneal tissue, making it more similar to a cone rather than a sphere.

This disease progressively affect vision in young adults, and it was the major indication for keratoplasty in the past decades in developed countries. The real cause of keratoconus is still unknown and some disagreement begins even from the definition. Controversy on pathogenesis is determined by the multiple factors proved to play a role in keratoconus development: chronic ocular flogosis is a known risk factor present in atopic patients, and it is in contrast with the “non-inflammatory” definition of this disease, while the description of non-sporadic cases proved that a genetic base is present and makes the term “dystrophy” rather than “degeneration” more appropriate to define it.

The first accurate description of keratoconus was made by Dr. Nottingham in 1854¹⁴, at that time diagnosis used to be based on simple macroscopic evauation, until the development of the first ophthalmic instruments. Signs of keratoconus were visible only in advanced stages when vision was already severely affected. The excessive corneal protrusion produces angulation of the lower lid on downgaze (Munson sign) and apical scarring. On basic ophthalmic examination, a high, irregular myopic astigmatism with a scissoring reflex on retinoscopy can be seen. At the slit lamp, cornea appears thinned at the apex, surrounded by a dark-brown annular superficial opacity circumscribing the area of ectasia, a sign known as Fleischer’s ring. When cornea becomes extremely thin, Descemet membrane folds and

superficial apical stromal scarring develops. Cone apex is usually dislocated infero-temporally relative to the corneal center. Symptoms include progressive visual blurring and/or distortion, photophobia, glare, and monocular diplopia. Visual acuity may be normal even in symptomatic patients. Contrast sensitivity measurement, however, may uncover visual dysfunction before Snellen visual acuity loss can be measured¹⁵. In more advanced cases, the deep layers become involved: the Descemet membrane breaks and acute corneal oedema (hydrops) develops with irreversible scarring leading to severe visual impairment¹⁶. Keratoconus usually develops in the second decade of life but earlier and later cases have been also extensively reported^{17–19}. More aggressive cases develop in young age and increase the chance to undergo surgery during life²⁰.

Epidemiology

Keratoconus is considered a rare disease by the National Institute of Health because of its low incidence, inferior to 1 case per 2000 people per year. This definition is questionable because many studies reported higher incidence and prevalence, probably thanks to the high accuracy of the recently introduced diagnostic tools that can detect less severe cases that may have been missed in the past. Above all, a recent meta-analysis performed on over 7 million patients from 15 different countries has calculated a global prevalence of 138/100,000²¹. The Collaborative Longitudinal Study on Keratoconus found an increased prevalence in male with a M:F ratio of 1.33²². Country- and ethnic-specific differences have been reported by numerous studies^{23–30}.

In consideration of the increasing demand of refractive surgery due to the rising prevalence of refractive errors worldwide and the more sophisticated screening systems of this disease performed before surgery, it is reasonable to expect higher values in the next future.

Aetiology

Many theories have been developed on the aetiology of keratoconus and the multifactorial is the most accepted one. Both genetics and environment seem to play a role in the developing of this disease.

A positive family history is reported in 5 to 20% of keratoconus cases²³. Prevalence in first degree relatives can be 67% higher than general population³¹. Both autosomal dominant and recessive transmission have been reported, but keratoconus can be sporadic as well^{31,32}.

Association with connective tissue diseases, such as Ehlers-Danlos, osteogenesis imperfecta, pseudoxanthoma elasticum, joint hypermobility, mitral valve prolapse, and many others have been proved^{31,33–35}. Complex genetic syndromes have also been associated with keratoconus such as Down, Noonan, Apert, Crouzon and Bardet-Biedl syndromes^{36,37}. Other ocular conditions can be associated with keratoconus: aniridia, floppy eyelid syndrome, Fuchs corneal dystrophy, Granular corneal dystrophy, iridoschisis, Lattice corneal dystrophy, Leber congenital amaurosis, posterior polymorphous dystrophy, progressive cone dystrophy, retinitis pigmentosa, retinopathy of prematurity, and vernal conjunctivitis^{38–42}. In particular, floppy eyelid syndrome (FES) and vernal keratoconjunctivitis (VK) seem to have high association with keratoconus: in FES prevalence can be as high as 17%⁴³ and in VK patients it varies from 11 to 23%^{44,45}.

Environmental and acquired factors also appear to be related with keratoconus development: obesity affects 52% of the patients that show 100 times more risk of this disease than general population^{46,47}. Ocular inflammation is associated with keratoconus since eye rubbing and atopy have been reported to be strongly associated^{48,49}. Obstructive sleep apnea is present in 24% of keratoconus patients⁵⁰ and hormonal influence may play a role since thyroid disfunction and pregnancy have been also observed as linked by some authors⁵¹⁻⁵⁵.

Genetics have been extensively investigated in keratoconus and numerous genes have been associated to this disease: SOD1, VSX1, SPARK, DOCK9, MIR184, CAST, LOX, and many others⁵⁶⁻⁶². Collagen genes COL4A1, A2, A3, A4 and COL8A1 and A2 genes were all investigated without direct results^{63,64}. Interestingly, a single gene has not been identified and all the others seem to be related to cellular activities such as keratocytes activation during wound healing, proteases production, natural corneal cross-linking, and inflammatory response (IL-1)⁶⁵.

Histology

Histological examination reveals three typical signs in keratoconus: Bowman membrane (BM) multiple interruptions, iron deposits at basal cell layers of the epithelium and stromal thinning⁶⁶. Interruptions of BM can be small (<20µm) to large (>100µm) and filled with epithelial downgrowth associated with acellular stromal matrix or migrating keratocytes. Corneal epithelium grows directly on underlying stroma in the gap areas and become thin over scars⁶⁷. A whorled pattern of epithelium highlighted by fluorescein staining can be seen at slit lamp examination along with prominent corneal nerves⁶⁸⁻⁷¹. On light and confocal microscopy, epithelium can shows signs of regenerative atypia in the basal cell layer

(variable degree of pleomorphism, cell elongation and cytoplasmic vacuolation, enlarged vesicular nuclei with dispersed chromatin and prominent nucleoli)⁷². Stroma is variably affected depending on the severity of the disease. Collagen lamellae and keratocytes are reduced in numbers^{73,74}. Superficial scars develop following BM interruption when keratocyte subsequently undergo fibroblastic transformation and produce variable amounts of fibrous tissue⁷⁵. Changes progressively spread transversely and vertically affecting all the stromal tissue in advanced stages, especially following corneal hydrops⁷⁶.

Diagnosis

Keratoconus can be diagnosed in its early stages only on suspicion, followed by further dedicated examinations, because macroscopic and biomicroscopic evaluations are usually normal. Affected patients refer to ophthalmologist for visual blurring, distortion and/or monocular diplopia⁶⁶. Refraction usually reveals myopic astigmatism, scissoring reflex on retinoscopy, abnormal keratometry, and stromal thinning at slit lamp biomicroscopy. The gold standard examination is corneal topography.

Topography shows a cone-shaped protrusion, generally eccentric and located in the inferior-temporal mid-periphery^{77,78}. Keratoconus is located in the central and paracentral corneal regions in 97% of the cases. Paracentral region comprises 72% of the cases while approximately 25% are in the central region⁷⁹. When located inferiorly, the corresponding area of steepening is associated with a superior area of flattening⁸⁰ (Figure 2). Two types of cones have been described: a round or nipple-shaped that is small in diameter and typically located in the center and a wider oval sagging cone usually located inferiorly⁸¹.

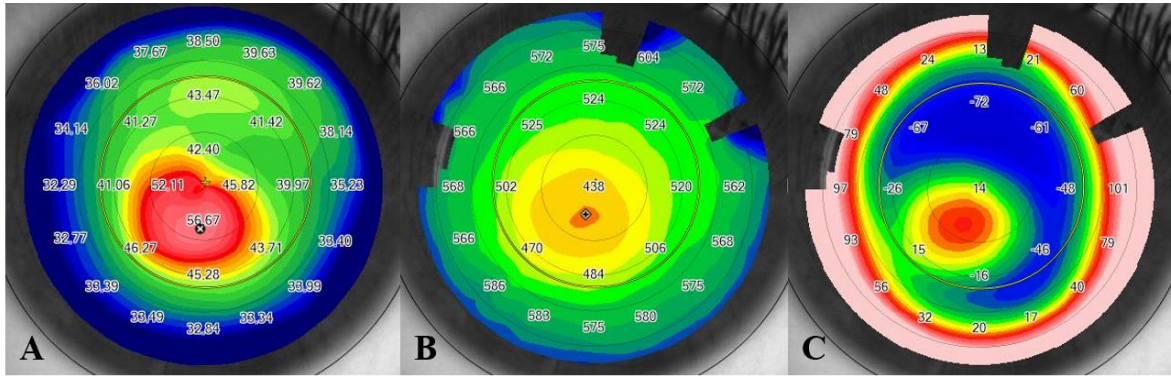


Figure 2. Keratoconus tomography. (A) Anterior surface tangential curvature. (B) Corneal pachymetry. (C) Posterior surface elevation.

In the past, a qualitative analysis could be made by evaluating changes of anterior curvature by means of a color-graded scale where a red area usually means abnormal high curvature⁸². Modern tomographers can provide thickness map and posterior elevation map useful to visualize stromal thinning and posterior ectasia respectively. When not evident, keratoconus can be suspected by evaluating quantitatively these curvature changes by means of univariate and multivariate detection systems^{83,84}. The Belin/Ambrosio Enhanced Ectasia Display III (BAD-III) is one of the most sophisticated one. It performs a regression analysis of nine indexes, comprising the pachymetric progression, posterior and anterior surface elevation with respect to reference-calculated surfaces and provides a single index that discriminate between normal, suspicious and ectatic cornea⁸⁵. Unfortunately, none of the indexes available can provide a specificity and a sensitivity of 100% in keratoconus diagnosis⁸⁴. Recently, corneal epithelial thickness⁸⁶ and corneal biomechanics⁸⁷ are being increasingly used as a more sensible tool to detect keratoconus, with much higher accuracy, especially in pre-refractive surgery ectasia screening.

Classification

Based on clinical signs, visual acuity and topographic parameters, multiple staging systems have been developed in the past. The most famous and widely adopted, despite being old, is the Amsler-Krumeich (AK) system. This grades keratoconus in four stages based on anterior corneal curvature, refraction, presence or absence of scarring and central corneal thickness (Table 1)⁸⁸. According to the Global Consensus Document on Keratoconus and Ectatic Diseases there is currently no adequate classification system to grade keratoconus⁷⁷. The AK system flaws are due to its old concept and grading parameters it uses. Central keratometry not always reflect the severity of the disease because frequently the cone is inferiorly displaced and keratometric readings are much higher at the apex⁸⁹. Moreover, no significance is given to the posterior corneal surface despite it has been demonstrated as the first surface to change in the keratoconus development.

Grades	Characteristics
Stage I	Eccentric steeping Myopia and astigmatism <5.00 D Mean central keratometry readings <48.0 D
Stage II	Myopia and astigmatism from 5.00 to 8.00 D Mean central keratometry readings <53.0 D Minimum corneal thickness >400 µm
Stage III	Myopia and astigmatism from 8.00 to 10.00 D Mean central keratometry readings >53.0 D Minimum corneal thickness from 300 to 400 µm
Stage IV	Refraction not measurable Mean central keratometry readings >55.0 D Central corneal scarring Minimum corneal thickness <300 µm

Stage is determined if one of the characteristics applies

Table 1. Amsler-Krumeich classification of keratoconus.

Recently, a novel grading system has been proposed by Belin et al. named the “ABCD” system⁹⁰. In this system, keratoconus four parameters are graded separately in four stages with a stage 0 meaning a normal cornea. Staging is performed by analysing an area of 3 to 4 mm of diameter centered on the cone calculated by the BAD algorithm. The “A” stands for Anterior radius of curvature, “B” for Posterior radius of curvature (Back surface), “C” for Corneal pachymetry at thinnest point, “D” for Distance best corrected visual acuity. Scarring is also considered as an extra sign. The classification ranges are reported in Table 2⁹⁰.

ABCD criteria	A ARC (3mm zone)	B PRC (3mm zone)	C Thinnest pachy (μm)	D BDVA	Scarring
Stage 0	> 7.25mm(< 46.5 D)	> 5.90mm	> 490 μm	≥ 20/20(≥ - 1.0)	
Stage I	> 7.05mm(< 48.0 D)	> 5.70mm	> 450 μm	< 20/20(< -, +, ++ 1.0)	
Stage II	> 6.35mm(< 53.0 D)	> 5.15mm	> 400 μm	< 20/40(< -, +, ++ 0.5)	
Stage III	> 6.15mm(< 55.0 D)	> 4.95mm	> 300 μm	< 20/100 (< 0.2)	-, +, ++
Stage IV	< 6.15mm(> 55.0 D)	< 4.95mm	≤ 300 μm	< 20/400 (< 0.05)	-, +, ++

Table 2. ABCD classification of keratoconus.

Keratoconus Management

Medical and parasurgical

Visual rehabilitation relies on spectacle lenses when keratoconus is in its early stages but, when ectasia evolves, contact lenses may be the only option to achieve acceptable visual acuity. When gas-permeable lenses fail on multiple attempts, surgical option should be considered. It is worth noting that, after surgery, over a third of patients have to continue wearing contact lenses due to astigmatism or anisometropia⁹¹. The use of contact lenses cannot prevent the evolution of keratoconus and it has been linked to apical scarring if they fit incorrectly^{92,93}.

Up until the beginning of the 21st century, keratoconus treatment was only surgical. Patients were supposed to wait until vision became unacceptable to undergo keratoplasty. In 2003 Wollensak et al. introduced an innovative conservative procedure to halt the progression of keratoconus by means of corneal collagen cross-linking (CXL)⁹⁴. In this procedure, corneal epithelium is removed and stromal tissue is soaked with a riboflavin solution (Dresden protocol). Subsequent application of ultraviolet light generates free radicals that “crosslink” collagen fibers resulting in corneal stiffening and reduction of ectasia progression⁹⁴. At present time, no other medical treatment is available for keratoconus.

Corneal collagen cross-linking should be considered in progressive keratoconus but, currently, there are no definitive criteria to predict risk of progression. Moreover, CXL treatment proved to reduce the risk of further evolution but cannot reverse corneal ectasia^{95,96}. Indications for CXL should include an increase of 1.00 D or more in the steepest keratometry measurement, an increase of 1.00 D or more in the manifest cylinder, and an

increase of 0.50 D or more in the manifest refraction spherical equivalent (MRSE)⁹⁷. Younger patients show faster progression and they more likely require corneal transplant in their life so, according to many authors, CXL should be performed without waiting clear signs of evolution in this group^{98,99}. A clear cut-off age is+ yet to be defined but some recent investigations tried to predict the need for CXL. Kato et al. reported that patients aged < 20 years with minimum radius of curvature (Rmin) inferior to 5.73 mm had a significantly higher probability of needing CXL treatment in the future. On the other hand, patients aged > 27 years with Rmin > 5.73 mm had no requirement for CXL later in life. In their study only 8.5% of eyes with Rmin > 5.73 mm (equivalent to 58.9 D) required CXL¹⁰⁰. A meta-analysis showed that patients aged less than 17 years, and those with a Kmax > 55 D, have a significantly high risk of keratoconus progression¹⁰¹.

Surgical

Although keratoconus can be staged according to many grading systems, none of them can provide indication for surgery. Sometimes, severe keratoconus can be perfectly managed with rigid contact lenses, providing excellent visual acuity, other times moderate cases might undergo surgery because of unacceptable visual quality or lens intolerance. Historically, keratoconus was treated with penetrating keratoplasty (PK) and currently this technique is still widely adopted. Deep anterior lamellar keratoplasty (DALK) is becoming the gold standard procedure because of the advantages of preserving recipient endothelium on graft survival. Currently, this procedure is performed in about 20% of the keratoconus undergoing keratoplasty worldwide¹⁰².

In order to avoid keratoplasty in moderate cases, in 2000 it was introduced a novel conservative procedure - named Intra-Corneal Ring Segment implantation (ICRS) - previously adopted to treat myopia, before refractive surgery became the gold standard¹⁰³. In this procedure, plastic rings of polymethylmethacrylate are implanted into peripheral arcuate stromal tunnels to induce flattening and regularization of central corneal curvature. The stromal tunnel is fashioned manually using a handheld corkscrew blade or automatically using a femtosecond laser and the ring segments are then inserted at around 70% of stromal depth¹⁰⁴. If tunnel is shallower than 60% there is an increased risk of ocular surface complications while deeper than 80% it may have no topographic effect at all¹⁰⁵.

Different size, length and thickness are available and the best ring is chosen according to nomograms¹⁰⁴. Since keratoconus is usually located in the inferior portion of the cornea, asymmetric ring placement is often required and the thicker segment in these cases should be placed inferiorly¹⁰⁶. ICRS use is limited to eyes with maximum K values <58 D and at the same time they are not ideal for early keratoconus because of high risk of complications in the first case and poor visual outcomes in both cases. Complications include segment migration, extrusion, and stromal melting¹⁰⁷.

The effect of this treatment has been extensively investigated in order to elaborate more accurate nomograms, but the working principle has not been completely clarified yet. Two main theories have been provided from mathematical analysis of the geometrical effects. The first is the arc-shortening hypothesis. A spacing element introduced between collagen fibers increases the tension of the fibers by changing and lengthening their route, hence producing flattening of the affected meridian. Theoretical models based on finite element analysis have shown that this effect is directly proportional to the thickness of the element and inversely proportional to its distance from the center. It is worth remembering that

normal corneas present orthogonal arrangement of the collagen fibers, a physiological organization that is lost in keratoconus¹⁰⁸. The other theory is based on Barraquer's "Thickness Law" for refractive surgery procedures, which states that when tissue is added to the periphery or is removed from the center of the cornea, a flattening of corneal tissue is produced¹⁰⁹. Probably both theories are involved in the final effect and sophisticated mathematical models are needed to better predict the results.

The results of PK in keratoconus are satisfactory in term of visual rehabilitation: final uncorrected visual acuity (UCVA) ranges from 20/50 to 20/100, with 40% of patients reading 20/50¹¹⁰. Spectacle correction gives mean corrected distant visual acuity (CDVA) of 20/30 and 20/40¹¹¹. After 15 years, a CDVA of 20/40 is present in 66% of eyes but 18.9% had VA lower than 20/200. Contact lens is needed in 5-60% cases postoperatively¹¹². No study has shown a superiority of one style or pattern of graft suturing in terms of BCVA¹¹³.

DALK, with proper dissection plane, provides visual results equivalent to PK. The evidences available show that uncorrected and corrected visual outcomes, as well as percentage of patients requiring contact lenses, are the same¹¹⁴. Residual stromal bed thickness appears to be a factor influencing visual recovery¹¹⁵. Compared to PK, visual rehabilitation appears to be quicker thanks to the possibility of earlier suture removal¹¹⁶. Suture removal is typically performed after 12 months in PK, delaying visual recovery. One of the most important advantages of DALK over PK in keratoconus is the possibility of performing large diameter graft (9 mm) with better visual results, low rejection risk, and as much as possible removal of the area of ectasia^{117,118}. Graft survival expectancy has to be considered, especially in keratoconus surgery, owing to the young age of patients. Predicted percentage of surviving grafts differs significantly with 63.2% of DALK grafts vs 23,9% of PK at 20 years⁴.

Stromal Keratophakia

History

The optical function of the cornea relies on the high regularity of its central area and small changes of this portion produce great modification of its dioptric power^{119,120}. Thanks to the accessibility of this component of the eye and based on some observations following first cataract surgeries and traumas, surgical attempts to induce intended modifications of the corneal curvature to correct ametropia were tried since 1800s¹²¹. Surprisingly, one of the first experiments to correct corneal curvature was performed by J. I. Barraquer in 1949 on a keratoconus affected patient that obtained significant reduction of his ametropia through flattening of the central corneal curvature¹²². This laid the foundation of modern refractive surgery and led to the introduction of a novel concept: manipulation of corneal shape to obtain refractive results.

Due to limitations of available surgical instruments, the first procedures were invasive and visual results were poor. The term keratomileusis derives from Greek words “kerato” for cornea and “mileusis” for chiseling. The first technique of myopic keratomileusis, introduced by Dr. Barraquer, was performed freehand with a Paufigue knife. Later, he designed a microkeratome to enable automated lamellar dissection with improved outcomes¹²³. In the original procedure, the removed anterior lamella had to be frozen in order to be chiseled by a dedicate instrument, named cryolathe, then thawed and sutured back on recipient stromal bed (autoplasmic keratomileusis)¹²⁴. To treat myopia, tissue must be removed from the center of the lamellar cap, while to treat hyperopia, tissue has to be

removed from the periphery. With this procedure, central corneal curvature can be increased or decreased to treat spherical refractive errors.

Refractive surgery experienced a great leap forward when excimer laser technology was introduced in ophthalmology in the late 1980s thanks to Trokel and McDonald. The excimer laser utilizes an argon fluoride gas mixture that emits a 193 nm ultraviolet beam when properly excited. Photorefractive keratectomy (PRK) was first performed in 1985 and rapidly became the reference technique for the treatment of ametropia¹²⁵⁻¹²⁷. In 1986 it was introduced an alternative combined technique based on both lamellar dissection and laser ablation. The laser in situ keratomileusis (LASIK) uses both the concept of Barraquer microkeratome to create a lamellar cut and excimer laser ablation to reshape exposed stromal bed¹²⁸⁻¹²⁹. Both technique gives excellent outcomes and are now widely adopted by all refractive surgeons¹³⁰.

The concept of cornea remodelling has been extensively investigated in the past decades to improve visual results and further advanced with the introduction of femtosecond laser technology in early 2000s. By means of photodisruption mechanism, femtosecond laser (FSL) is able to create complex intrastromal dissection plane geometries previously unreachable with mechanical blades¹³¹. In 2011, following a rapid evolution of this technology, an innovative refractive surgery technique was introduced: the Small Incision Lenticule Extraction (SMILE)¹³².

The cornerstone of all refractive procedures available today is tissue subtraction. Despite excellent results in normal eyes, this approach is not suitable for thin corneas with high refractive errors or tissues with impaired biomechanics, conditions that are a well-known contraindication to refractive surgery¹³³.

Amid the first experiments in this exciting surgery field, Barraquer proposed an alternative approach to reshape corneal curvature by means of tissue addition rather than subtraction. The original “thickness law” coined by the same author stated that if tissue is removed from the center or an equal amount of material is added to the periphery, the central corneal curvature becomes flattened and vice versa¹²³. This law is the base of modern refractive surgery.

On the wake of this concept, another technique termed keratophakia (from Greek words “kerato” for cornea and “phakia for “lens”) was introduced in late 1970s^{134,135}. The first concept implied the implantation of a corneal disc of about 6 mm diameter, carved from a donor cornea, within the patient’s corneal stroma. The procedure was complex at first and after lenticule preparation with a cryolathe, it involved lamellar cap dissection of the recipient cornea, donor lenticule implantation on the keratectomy bed and then repositioning and suturing of the recipient cap^{136,137}. Parallel to this work, a similar approach was proposed by Kaufman based on donor lenticule suture to the anterior corneal surface after epithelium removal in a procedure called “epikeratophakia”^{138,139}.

Results of these early experiments were not exciting due to many technical difficulties. Dissection depth of early microkeratomes was quite variable and inadvertent perforation was even possible. Wound dehiscence of the lamellar cap was also possible with risk of infections, epithelial ingrowth, and irregular astigmatism development. Visual recovery was generally poor due to structural lenticule cryodamage. In a group of 32 patients, Troutman reported CDVA > 20/40 in 84% of patients and a target refraction within 1.00 D in only 38.5%¹⁴⁰. Similarly, Swinger and Barraquer achieved CDVA > 20/40 in only 46.4% of their 46 patients¹³⁴. Refractive results were far away from the desired target with a mean postoperative astigmatism of 2.45 ± 0.37 D¹⁴¹. Given the increasing accuracy and

reproducibility of the laser subtractive techniques in that years, keratophakia and epikeratophakia were soon abandoned.

Femtosecond Laser in Ophthalmology

Femtosecond Laser (FSL) is capable of creating planes of dissection rather than ablating the recipient tissue like excimer laser thanks to the photodisruption principle. FSL is a solid-state infrared laser that produces a light pulse with a duration of 220–580 femtoseconds. Thanks to the photoionization phenomenon, plasma state is induced in a precise focal spot, generating expanding cavitation bubbles of vaporized tissue. By spatially organizing numerous micron-sized spots along the desired resection planes, this laser can produce intrastromal dissections while preserving vitality of nearby tissues¹³¹ (Figure 3).

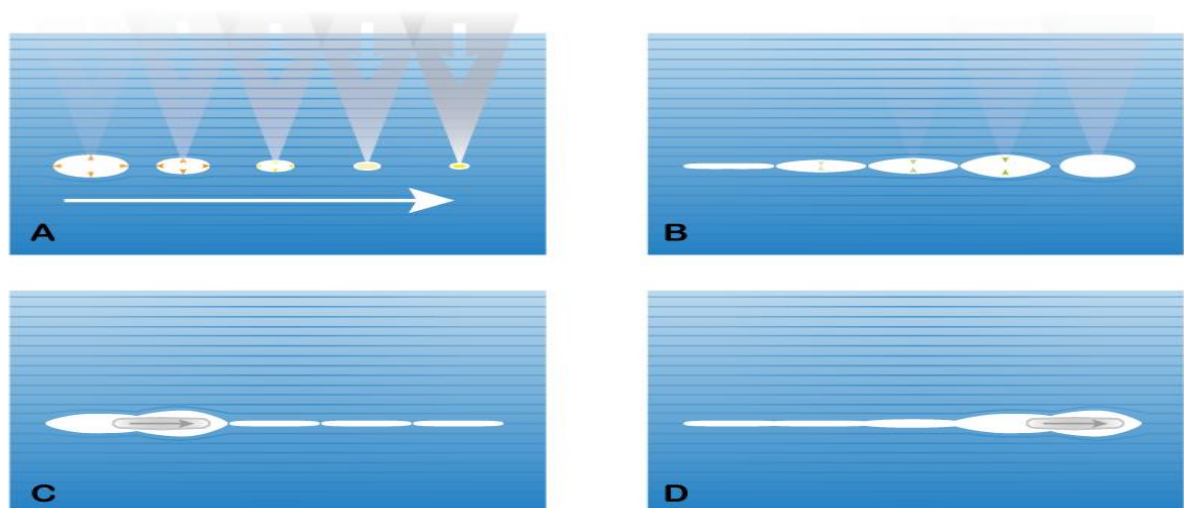


Figure 3. Femtosecond laser mechanism of action: photodisruption. (A) FSL beam creates a bubble of vaporized matter, called “plasma” that expands (orange arrows) due to fast increase of temperature and pressure within the bubble. (B) Collapse of the bubble (green arrows) to create focal dissection of the stroma (cavitation). (C – D) Thousands of adjacent cavitation bubbles determine dissection planes.

The first clinical application of FSL technology was in arcuate keratotomy and corneal flap creation for LASIK, which is still in use^{131,142,143}. FSL is also still used to create complex host-donor junction configurations for penetrating and deep anterior lamellar keratoplasty, but visual outcomes showed to be comparable to traditional mechanical trephination^{144–147}. The most important role of this technology in ophthalmology perhaps was reached with the introduction of SMILE procedure in 2011¹³². As already mentioned, with this all-femtosecond technique it is possible to sculpt intra-corneal lenticules of stromal tissue to induce curvature changes by subtraction rather than ablation.

The lenticule extraction concept was attempted earlier by Krueger¹⁴⁸ in 1998 and Heisterkamp and Ratkay-Traub in 2003, in non-corneal blind patients and animal model respectively^{149,150}. In 2007, further improvements of this technology by Sekundo et al. led to development of the first commercially available procedure: the Femtosecond Lenticule Extraction (FLEx)¹⁵¹. This procedure is comparable to LASIK but both corneal flap and lenticule are laser sculpted in the first surgical step. The flap is manually lifted by the surgeon, the lenticule removed and then, the flap repositioned¹⁵². The underlying concepts of SMILE were already present in this idea. By beginning with a transition technique, where surgeons tried to perform the procedure without lifting the flap through a limited opening (pseudoSMILE), the evolution of the procedure eventually arrived to the current SMILE technique, where incision is reduced to only 3-4 mm¹⁵³. The main advantage of avoiding flap creation and lifting is that anterior corneal lamellae remain unaffected by the procedure and corneal biomechanics is better preserved compared to LASIK¹⁵⁴.

Lenticule Implantation

It was soon clear that femtosecond lenticule extraction procedures could offer other interesting aspects beyond refractive results. In 2011, Mohamed-Noriega et al. proved that fresh extracted tissues after FLEx are still vital and their vitality and optical efficiency were maintained after cryopreservation¹⁵⁵. Angunawela et al. showed that lenticules could be safely reimplanted to reverse the refractive procedure in a rabbit model¹⁵⁶ and Riau et al. showed that this procedure was feasible also in non-human primate model¹⁵⁷. Following this early insights, many authors began to show interest in this field and numerous experiments took place. Liu H et al¹⁵⁸. presented a similar experiment of autologous lenticule implantation in rabbit cornea the same year of Angunawela and later, in 2015, Zhang et al. and Liu R et al. showed the results of allogenic lenticule implantation in rabbit and monkey animal models^{159,160}.

Thanks to the possibility of increasing corneal curvature with convex positive meniscus shaped lenticule implantation, the first clinical application of this concept was in hyperopia treatment. Williams et al. compared the results of three different procedures for hyperopia correction in a non-human primate model¹⁶¹. The lenticules were derived from -2.0 D and -4.0 D SMILE procedures, and were implanted beneath a $160\text{ }\mu\text{m}$ corneal cap. At 3 months, the lenticule implanted group had the greatest regression and a greater tendency to regression was seen in the $+2.0$ D group than in the $+4.0$ D group. In vivo confocal imaging revealed hyper-reflectivity of the lenticule interfaces that gradually subsided and returned to the pre-operative level after 3 months. Immunohistochemistry further showed that the inflammatory, fibrotic, wound healing, apoptotic, and tissue stress responses were minimal at post-implantation 3 months.

The first human case of FSL-derived allogenic lenticule implantation was reported by Pradhan et al. in 2013¹⁶². In this first case of aphakic patient, authors implanted a convex lenticule produced by myopic SMILE of -10.50 D ($127\text{ }\mu\text{m}$ central thickness) with 5.75 mm diameter in a lamellar pocket at $110\text{ }\mu\text{m}$ depth, obtaining a partial correction of the refractive error one year postoperatively. Explanation of the under-correction proposed by the authors was posterior surface bulging with loss of anterior effect and epithelial remodeling of the corneal apex.

In 2014 Ganesh et al. performed the first allogenic cryopreserved lenticule implantation series on a group of 9 hyperopic patients matched with a group of 9 myopic donors undergoing SMILE. Mean spherical equivalent of hyperopic eyes treated was $+4.50 \pm 1.1$ diopter (D). Mean keratometry changed from 43.9 D preoperatively to 47.4 D postoperatively and central corneal thickness increased from $532\text{ }\mu\text{m}$ to $603\text{ }\mu\text{m}$. Mean residual spherical equivalent for hyperopic eyes was $+0.6$ D ($+4.1$ D for the aphakic eye). The authors reported uneventful surgery in all cases without evidence of rejection or loss of best-corrected visual acuity during the entire follow-up period¹⁶³.

Fresh autologous transplantation of lenticules to treat hyperopia was investigated also by Sun et al. in 2015¹⁶⁴. They enrolled 5 patients who had one myopic eye and one hyperopic eye. Recipient eye underwent excimer laser ablation prior to receive the autologous lenticule from the fellow myopic eye to treat the expected residual refractive error. Lenticules were produced by myopic SMILE and implanted under a $110\text{ }\mu\text{m}$ flap in the recipient eye. All lenticules remained secured in place and transparency was preserved throughout the one-year follow-up. Refraction stabilized after one month, but residual spherical equivalent was high (range from $+1.13$ to -2.63 D)¹⁶⁴.

In the same year, at the Eye Clinic of Marburg University, Sekundo performed the first case in Germany of high hyperopia treatment by means of lenticule implantation derived from a myopic donor in a similar fashion to Ganesh, but with fresh tissue¹⁶⁵. As reported by other authors, there was also in this case approximately a 60% undercorrection¹⁶⁵.

Like hyperopia, presbyopia is a positive refractive error. The physiological decline of accommodation occurs in individual older than 40 years and it is usually corrected with spectacles. The concept of refractive lenticule implantation was investigated also in presbyopia by Jacob et al. in 2017 and Liu et al. in 2018. The first group proposed a modified lenticule derived from -3 D SMILE with a central 1 mm trephination implanted into a stromal pocket at 120 µm depth of the non-dominant eye. They reported improvements in both near and intermediate uncorrected visual acuity with no signs of rejection nor decentration over a 6-months period¹⁶⁶. The second group investigated the feasibility of allogenic and xenogeneic decellularized lenticule implantation in a non-human primate model. They implanted a 3-mm trephined lenticule derived from a -3.00 D SMILE into a corneal pocket at 120 µm depth of a *Macaca fascicularis* cornea (*cynomolgus macaque*)¹⁶⁷. The xenogeneic lenticules were taken from porcine eyes. Both simulated keratometric values and asphericity increased by 1.8–2.3 diopters and by –0.26 to –0.36 respectively. Allogenic and decellularized lenticules proved to be biocompatible whereas one eye in the xenogeneic group developed corneal stromal rejection¹⁶⁷.

Therapeutic use of SMILE-derived lenticules is another possible application of this innovative concept of keratoplasty. Ganesh et al. proposed in 2015 the implantation of modified lenticules combined with corneal collagen cross-linking to treat grade 1 to 3 keratoconus (with or without documented evidence of progression) in a modification of the original technique he proposed in 2014 named femtosecond intrastromal lenticular

implantation (FILI)^{163,168}. Cryopreserved lenticules (whose diameter ranged from 6.0 to 7.0 mm) were thawed and washed, soaked with riboflavin, and then underwent a central 3-mm trephination to obtain a donut-like shape. Intra-stromal pocket was created into the recipient cornea at 100- μ m depth with 7.0- to 8.0- mm diameter (1 mm larger than the donor lenticule) and a 4-mm superior incision. Riboflavin was injected into the stromal pocket and, after lenticule implantation, UV-A irradiation was performed using the Avedro CXL system (Avedro KXL[®] system, Waltham, MA, USA), at 30 mW/cm² for 3.3 minutes, delivering a total dose of 6.3 J/cm² to the corneal surface. Improvement was reported for uncorrected distance visual acuity (1.06 ± 0.48 to 0.38 ± 0.27 logMAR) and corrected distance visual acuity (0.51 ± 0.20 to 0.20 ± 0.24 logMAR), and manifest spherical equivalent (23.47 ± 1.15 D to 21.77 ± 1.7 D). Mean keratometry in 3-mm and 5-mm zones reduced by 3.42 ± 2.09 D and 1.70 ± 1.31 D, respectively. Mean pachymetry in the central and midperipheral zones increased by 18.3 ± 7.3 μ m and 33.0 ± 8.8 μ m, respectively. All eyes had reduction of higher-order aberrations, specifically coma, with no loss of lines of corrected distance visual acuity. No adverse events were reported¹⁶⁸.

Therapeutic application of refractive lenticule implantation has been also attempted in corneal perforation sealing¹⁶⁹ or stromal thickness restoration in complicated LASIK before retreatment¹⁷⁰.

Stromal Lenticule Addition Keratoplasty (SLAK)

Contemporary to the first lenticule implantation experiments performed by many authors for refractive error correction, Mastropasqua et al. hypothesized the possibility of treating corneal ectasia by exploiting the tissue addition concept. Tissue addition could be a valuable alternative to tissue subtraction for changing corneal curvature, as stated by Barraquer in the 1940s. Femtosecond laser is able to overcome the limits of the traditional blade systems and appeared to be the answer to precise and accurate cut of stromal tissue with desired geometry. All authors moved towards refractive correction of positive ametropia but few considered ectatic diseases. Lenticule extraction procedures available at that time (i.e. FLEx and SMILE) were able to treat negative ametropia (myopia) by producing positive meniscus lenticules with a convex thickness profile. The intra-stromal implantation of that type of lenticule appeared to be ideal for hyperopia correction because, according to the Barraquer thickness law, by adding tissue to the central corneal area, an increase of central corneal curvature is obtained.

Taking into account the results of early experiments with positive meniscus shaped lenticule, Mastropasqua et al. hypothesized that a lenticule with a negative profile can induce opposite changes to corneal curvature, that is, corneal flattening. This concept is part of the knowledge learned from intra-corneal ring segments implantation, a well-known technique whose working principle appears to be the result of a combination of corneal volume displacement and collagen fiber tensile strength redistribution¹⁷¹. According to this hypothesis and the ongoing development of a novel negative meniscus shaped lenticule model for SMILE in hyperopia treatment¹⁷⁰, they proposed the use of such lenticule geometry to obtain the desired effect in a novel procedure of keratoplasty. Since all these lenticule implantation techniques share the same concept – tissue addition – they proposed that this kind of

procedure may be named “Stromal Lenticule Addition Keratoplasty” (SLAK)¹⁷³. More specifically, negative meniscus lenticule presents a concave profile with a maximum thickness in the peripheral region of the optical zone that gradually reduces towards the thinnest point, located in the center (Figure 4).

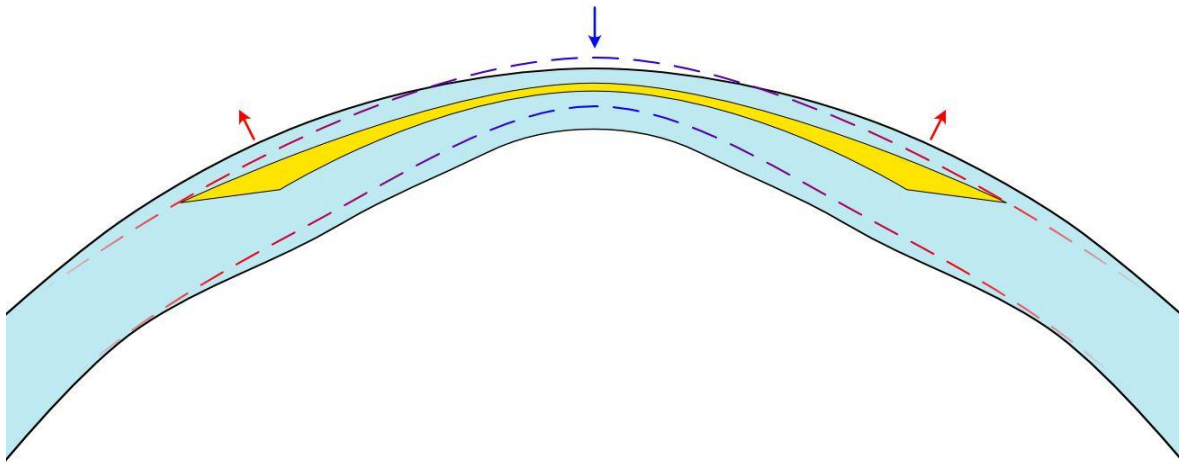


Figure 4. Corneal modifications induced by SLAK surgery. The implantation of a negative meniscus lenticule induces central flattening (blue arrow) as a result of peripheral steepening (red arrows).

The first ex-vivo preliminary SLAK study on normal corneas was designed in 2015 to understand the effect of negative meniscus lenticule addition¹⁷². Following the promising results experienced ex-vivo, a human in-vivo non-comparative interventional case series was performed in a group of subjects affected by advanced central keratoconus who were already candidates for keratoplasty in 2018¹⁷³. Treatment was reserved to central keratoconus because only symmetrical lenticule geometry was available.

Surgical technique

The first phase of the surgical procedure consists of lenticule creation in the donor cornea by means of a femtosecond laser (FSL). Corneo-scleral buttons are mounted on an artificial anterior chamber and pressure is standardized with a BSS bottle at 180 cm of height to inflate the anterior chamber.

All surgical procedures are performed under topical anesthesia. Corneal epithelium is removed using a blunt spatula and a modified femtosecond laser flap-cut procedure (flocket)¹⁷⁴ is performed to create an intrastromal pocket with a VisuMax femtosecond laser platform (Carl Zeiss Meditec, Jena, Germany) centered on the corneal central zone. A hinge length of 21.7 mm is set in order to produce a circular planar plane of dissection with a single 4-mm superior opening, a diameter of 8.2 mm, and depth from surface of 120 μ m. After the laser phase, the patient is moved to the surgical microscope and the flocket opened by means of a blunt dissector^{172–173}.

Donor lenticule is produced by means of femtosecond lenticule extraction procedure (FLEx) with VisuMax femtosecond laser platform (Carl Zeiss Meditec, Jena, Germany). Lenticule parameters are set as following: flap thickness 110 μ m, flap diameter 8 mm, hyperopic correction 8.00 D, optical zone 6 mm. The maximum lenticule thickness obtained is 148 μ m with a central lenticule minimal thickness of 30 μ m. The overall transition zone in the periphery of the lenticules is 0.70 mm in diameter^{172–173} (Figure 5).

After completion of the FSL dissection phase, the flap is lifted with a blunt spatula under the surgical microscope and then the lenticule is separated and transferred to the patient's eye maintaining its original orientation. The incision of the recipient pocket is reopened with a Seibel spatula and the plane dissected with a blunt dissection spatula (Mastropasqua SMILE

kit, Janach, Como, Italy). The lenticule is placed on the patient's cornea close to the incision opening and, while grasping its distal edge, it is dragged into the pocket through the incision and spread out. Final distention is achieved with gentle caressing from the surface using a spatula. The lenticule is carefully centered onto cone apex and correct distension and centration are assessed by the operator using an intraoperative microscope, AS-OCT, and tomography^{172–173} (Figure 6).

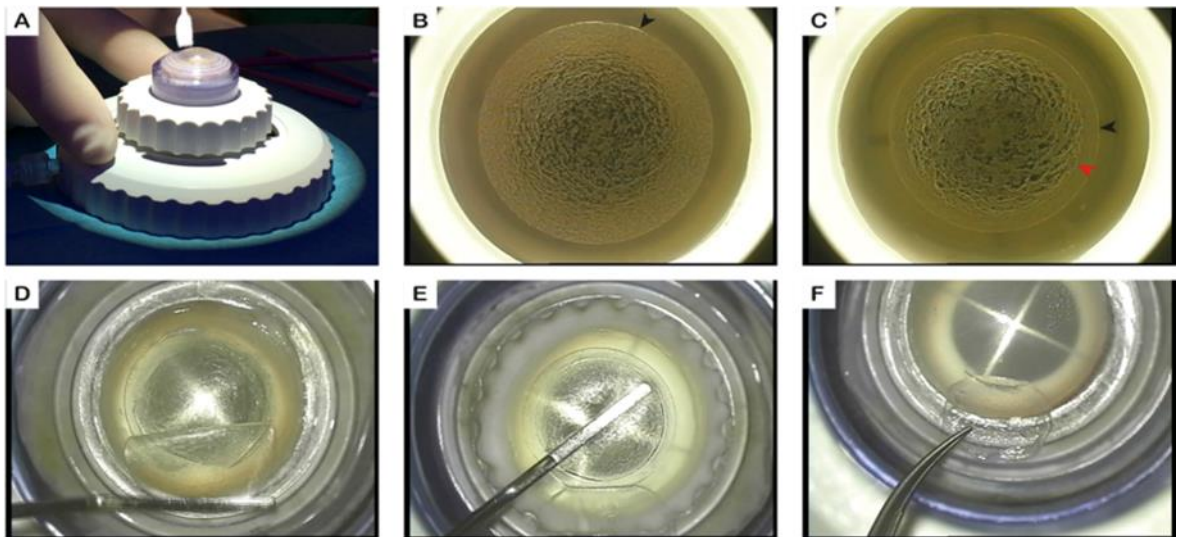


Figure 5. SLAK donor preparation – FLex procedure. (A) Donor tissue mounted on the artificial anterior chamber. (B – C) FSL shapes the lenticule (red arrow) and the side cut (black arrow) used for FLEEx extraction. (D) Lifting of the stromal cap. (E) Dissection of the lenticule with a blunt spatula. (F) Extraction of the lenticule.

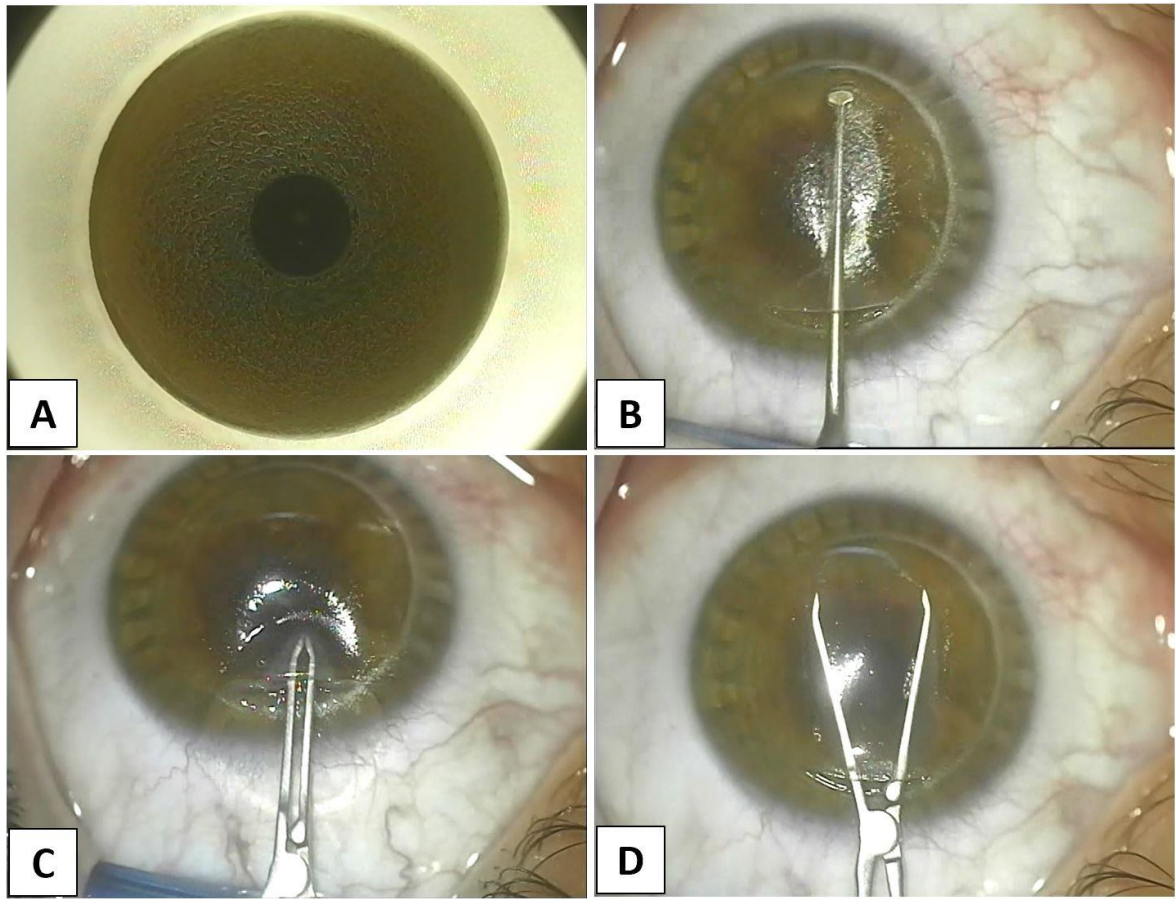


Figure 6. SLAK surgery. (A) FSL sculpts the pocket in which the lenticule is implanted. (B) Recipient pocket opened with blunt spatula. (C) Lenticule insertion in recipient cornea. (D) Lenticule distention.

Results

The results of the first human in-vivo non-comparative interventional case series of SLAK proved that corneal flattening is possible by selective tissue addition. Despite the lack of customization, addition of a standardized negative meniscus lenticule, centered on the cone apex, produced a significant improvement of corneal topography along with best corrected visual acuity in their first case series in 2015¹⁷³. Uncorrected distance visual acuity (UDVA) at six-months improved in 8 of 10 eyes ($P < .01$; Fisher's exact test), whereas all but one eye

had improvement of corrected distance visual acuity (CDVA). UDVA improved from 1.58 ± 0.36 to 1.22 ± 0.37 logMAR ($P = .024$; Wilcoxon signed-rank test), whereas CDVA improved from 1.07 ± 0.17 to 0.70 ± 0.23 logMAR ($P = .007$). One eye gained three lines, 3 eyes gained two lines, 5 eyes gained one line and 1 eye had no change in lines of CDVA. Spherical equivalent significantly reduced from -7.46 ± 2.49 D to -3.61 ± 1.99 D at 6 months ($P = .021$).

Corneal Topography

On corneal topography, a generalized flattening of the cone can be observed after SLAK. They reported a mean anterior keratometry value reduction from 58.69 ± 3.59 to 53.59 ± 3.50 diopters at 6 months after surgery in the first in-vivo study ($P < .05$; Wilcoxon signed-rank test), along with negligible variation of the posterior corneal mean keratometry. Anterior corneal asphericity (Q value) was reduced, indicating a reduction of corneal irregular high prolated morphology ($P < .05$; Wilcoxon signed-rank test)¹⁷³. Corneal topography after SLAK shows an area of central curvature reduction surrounded by a red ring of increased curvature corresponding to the transition zone from the addition area to the peripheral recipient cornea. Anterior topographic flattening appears similar on color-graded map to corneal flattening pattern experienced after conventional myopic refractive laser treatment¹⁷³ (Figure 7).

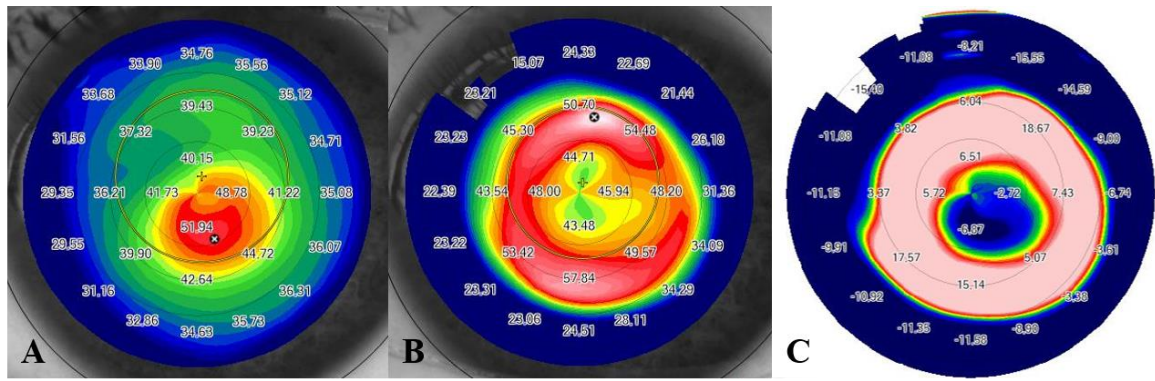


Figure 7. SLAK surgery results. (A) Pre-surgery anterior surface tangential tomography. (B) Post-surgery anterior surface tangential tomography. (C) Anterior surface tangential differential map of the previous two maps.

In vivo Confocal Microscopy

Intrastromal FSL-based refractive surgery procedures proved to induce a lower level of inflammation and apoptosis, combined with a better preservation of anterior stromal lamellae structure and nerve plexus compared to traditional ablative techniques^{131,153–154,175–177}. Interfaces produced by FSL cut appear optically clear and highly regular¹⁷⁸. Thanks to the low energy delivered into the recipient stroma, a low incidence of corneal haze was reported after ReLEx treatments. Preservation of anterior lamellar structure proved to be relevant to corneal biomechanics while subepithelial nerve sparing induces faster recovery of corneal sensitivity and epithelial integrity^{154,175,179,180}.

On IVCN, corneal epithelium appeared regularly stratified after SLAK throughout the follow-up¹⁸¹. Mean sub-basal nerve density was restored to preoperative values at 3 months after surgery and no significant variations of dendritic immune cell density was reported. Mild anterior stromal oedema was present in all cases but rapidly subsided during the first month. At 6 months postoperatively, no signs of stromal reaction or keratocyte activation

were visible. Interface reflectivity significantly reduced at 3 months with persistence of some cellular debris consisting of roundish appearing, reflective elements with small diameter. Interfaces remained visible up to the end of the follow-up of 12 months on IVCN but with decreasing reflectivity. Interface appearance was similar to what was previously documented after refractive lenticule extraction procedure.

No significant endothelial or keratocyte cell density changes nor signs of rejection were reported following SLAK. Lenticule microfolds were variably visible in some cases on IVCN but never documented on slit lamp examination¹⁸¹.

Epithelial and stromal remodeling

Remodeling of corneal structure is a progressive phenomenon that requires time after surgery^{182–186}. Epithelial compensation is a documented process that can affect final corneal curvature and induce refractive changes after corneal surgery and laser treatments^{187–188}. Epithelial thickness map one month after SLAK showed a central thickness increase and a mid-peripheral thickness reduction corresponding to the zone of increased curvature¹⁸⁹. Epithelial thickness outside the addition area experienced a progressive increase up to 6 months after surgery. No significant changes were reported on optical coherence tomographic analysis regarding lenticule thickness profile and anterior or posterior recipient stromal thicknesses throughout the follow-up¹⁸⁹.

Excimer laser lenticule shape customization

This paragraph describes the first set of experiments aimed to reproduce a model of keratoconus using eye-bank corneal tissues, and to customize lenticules to be implanted by using excimer laser technologies. The original work described in the following paragraphs is integral part of this PhD thesis and the results were recently published¹⁹⁰. Stromal lenticule addition keratoplasty (SLAK) has proven to be a promising minimally invasive treatment option for keratoconus, able to induce central corneal flattening through stromal tissue addition^{172,173}. In this technique, central corneal curvature reduction is achieved using peripheral tissue addition produced by intrastromal implantation of negative meniscus-shaped stromal lenticule created by femtosecond laser (FSL) cut^{172,173}. SLAK is still limited to central keratoconus because current FSL technology can produce only stromal lenticules with a symmetric thickness profile¹⁷³. As a result, only 25% of the eyes affected by keratoconus are suitable for this procedure⁷⁹. Moreover, the lack of lenticule shape customization algorithms based on cone specifications makes it difficult to optimize curvature outcomes even in central conus. Alió and colleagues, by investigating the feasibility of corneal stroma regenerative therapy with intrastromal implantation of large decellularized planar lenticules (with or without mesenchymal stem cells recellularization) in advanced keratoconus, observed some corneal flattening regardless of the cone¹⁹¹. To clarify this concept, Nubile et al. investigated the effect of different types of customized lenticule implantation on an ex vivo eccentric keratoconus model¹⁹⁰. No other validated methods were described in literature, and only pilot studies describing the use of an excimer laser to model corneal stroma in a keratoconus-like fashion had been reported¹⁹². Therefore, it was proposed to simulate the decentered curvature of eccentric keratoconus, comparing two different technical approaches of donor cornea reshape aiming at reproducing a realistic

geometry in an ex vivo model, created by using excimer laser ablation of both anterior and posterior surfaces of the cornea. In addition, the use of excimer laser ablation to produce custom lenticules suitable for eccentric keratoconus correction was investigated. The aims of this investigation were to create a model that mimics only the corneal keratoconus shape and to explore the optimal lenticule shape that could make SLAK available for off-centered ectasia¹⁹⁰.

Surgical technique

In this investigation, lenticules obtained from the hyperopic-refractive lenticule extraction (ReLEx) procedure were customized using excimer laser ablation to produce different asymmetric shaped patterns. They were implanted into FSL-intrastromal pockets produced in re-shaped eye bank donor corneas. A model for eccentric keratoconus was created in recipient corneas through excimer laser ablation, to evaluate the flattening effect of lenticule intrastromal implantations.

Two different methods (defined below) were used to reproduce the eccentric keratoconus model: both were compared with a pool of topographies obtained from real keratoconus eyes to establish which was the most reliable.

A total of 28 human eye bank donor corneas with scleral rims, non-suitable for human transplantation, were harvested from the eye bank and twelve of them were assigned to the recipient group. Eight tissues were sculpted to create two eccentric keratoconus models (4 for each type) with different specifications. After accurate topographic assessment, the remaining four corneas were shaped with the most efficient technique capable of consistently

reproducing the desired parameters of the keratoconus model. The remaining 16 donor corneas were assigned to lenticule production.

Donor corneas assigned to the recipient keratoconus model group underwent excimer laser ablation re-shaping with MEL-80 excimer laser to simulate the curvature steepening and stromal thinning typical of keratoconus before femtosecond laser pocket creation. The intended curvature parameters were the following:

- Kmax ranging from 60 and 70 diopters (D).
- Decentration of the thinnest point of about 1.5 mm (ranging between 0.7 and 1.8 mm).
- Thinnest pachymetry ranges between 380 and 490 μm .

The anterior surface reshaping was performed using an inferiorly decentered hyperopic photorefractive keratectomy (PRK) ablation in model 1 (+5 D with an optical zone of 6 mm, performed on an off-centred mark, to reproduce an off-centered steepening of the anterior corneal curvature) or phototherapeutic keratectomy (PTK) ablation with cone apex masking in model 2 (PTK ablation of 100 μm and 8 mm diameter centered on the mark with the exclusion of the central 4 mm area that was masked using a white paper disc placed onto the anterior corneal surface according to the mark position). Stromal thinning was reproduced using an inferiorly decentered myopic PRK ablation of the posterior corneal surface after corneal eversion in both models (1.5 mm inferiorly off-centered myopic PRK treatment of -18 D in total with an optical zone of 5 mm) (Figure 8).

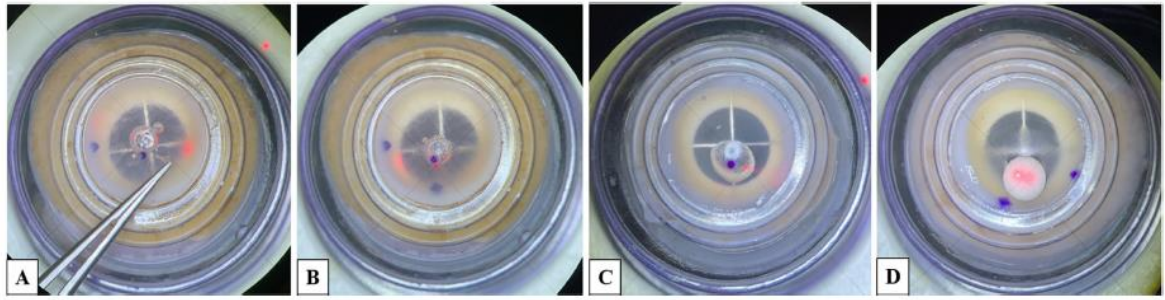


Figure 8. Keratoconus models creation. (A) Everted cornea mounted onto the anterior chamber. (B) Off-centered myopic ablation (-18 D) performed on the posterior surface to simulate posterior ectasia and stromal thinning. (C) Model 1: anterior surface hyperopic ablation (+5 D, 5 mm optical zone) performed on the mark. (D) Model 2: anterior surface PTK ablation performed with a 4 mm mask (8 mm diameter and 100 μ m depth).

The selected keratoconus model for recipient tissues underwent a modified FSL flap-cut procedure with a 500 kHz FSL platform to produce the stromal pocket at a depth of 120 μ m from the anterior surface with a single small incision of 4 mm.

All lenticules were created using ReLEEx-FLEEx hyperopic procedure; the first subset of lenticules with a planar shape (type I – planar lenticules) as described by Alió et al¹⁹¹. The type II (negative lenticules) were extracted after FSL treatment reproducing the same specifications used in the previous clinical study realized by Mastropasqua et al¹⁷³(Figure 9).

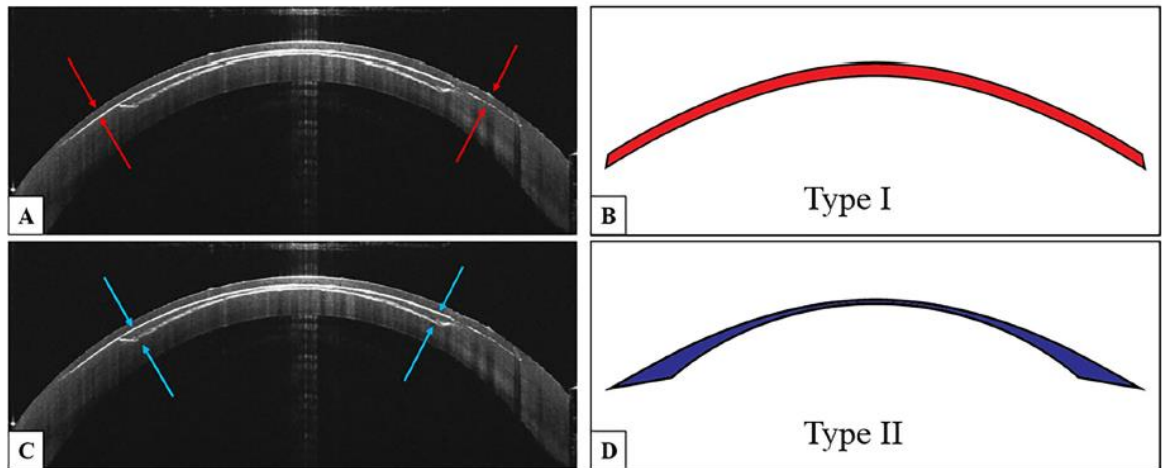


Figure 9. Non-customized lenticules. (A, B) Type I lenticule, with a planar shape (B) is obtained from the 120 μm stromal flap (A; *red arrows*) of the ReLEEx-FLEEx procedure. (C, D) Type II lenticule, with a negative meniscus shape (D), is sculpted with a +8 D hyperopic FSL ReLEEx-FLEEx treatment (C; *blue arrows*).

Type III (or customized planar) and type IV (or customized negative) lenticules underwent further modification with excimer laser ablation before their removal to obtain an asymmetric shape (Figure 10).

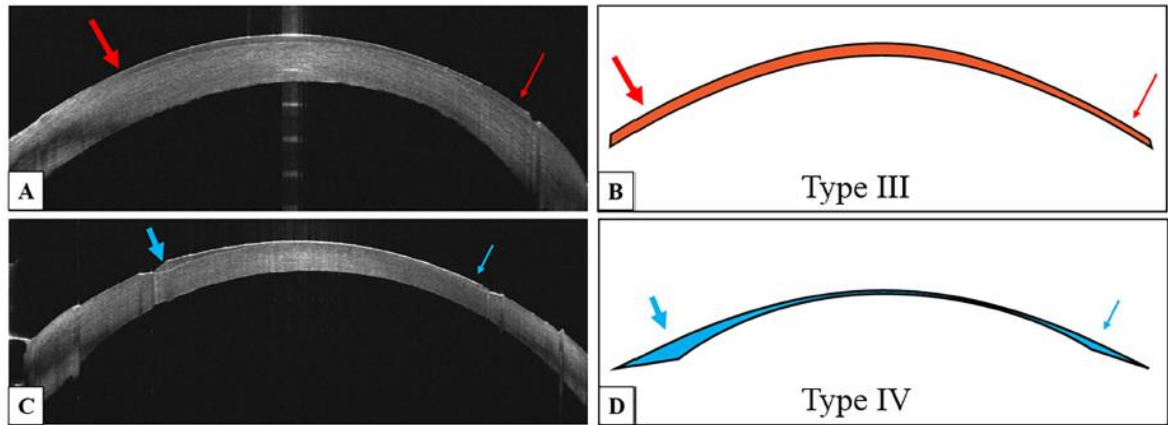


Figure 10. Planar and negative customized lenticules. (A, B) Type III lenticule (asymmetric planar shape – B) obtained through masked excimer laser ablation of the ReLEEx-FLEEx stromal flap (A). The thick red arrow indicates the masked zone and the thin red arrow indicates the ablated zone. (C, D) Type IV lenticule (asymmetric negative meniscus shape – D) obtained through masked excimer laser ablation of the stromal lenticule with the ReLEEx-FLEEx treatment after flap removal (C). The thick blue arrow indicates the masked zone and the thin blue arrow indicates the ablated zone.

In detail, after the aforementioned FLEEx procedure, type III lenticules were created by placing a 10 mm-wide mask onto the inferior half of the flap and performing a +3.50 D, axis 180 degrees, astigmatic PRK treatment with an optical zone diameter of 7.00 mm, to produce a bow-tie ablation of the superior half only. Type IV lenticules were produced with the same modality adopted for type III lenticules. After removal of the stromal flap, the anterior surface of the negative meniscus lenticule underwent +2.50 D, axis 180 degrees astigmatic masked PRK treatment with an optical zone diameter of 6.00 mm (Figure 11).

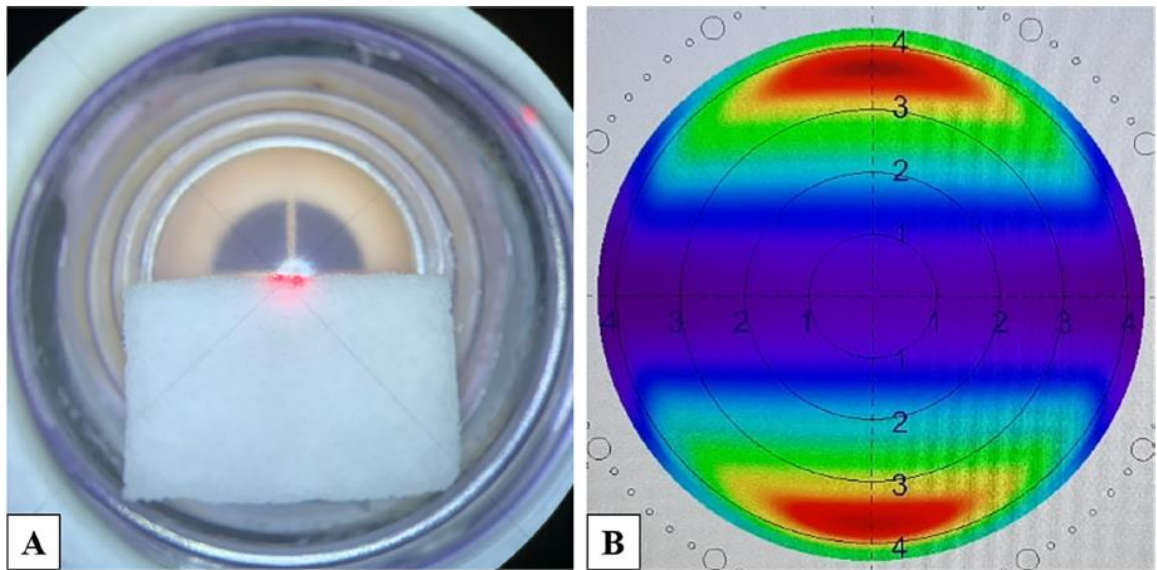


Figure 11. Planar and negative lenticules customization process. After placing a 10 mm-wide mask onto the inferior half of the lenticule (A), an astigmatic PRK treatment (B) is performed to produce a bow-tie ablation of the superior half only.

The thickest side of the lenticule (located inferiorly) was marked with gentian violet to recognize its position after implantation. Lenticules were finally implanted into keratoconus model-shaped corneas and centred carefully with a mark-on-mark technique on the conus apex.

Results

An ex vivo keratoconus model was successfully reproduced in all corneas. An additional number of 4 corneas were assigned to Model 2 group, which reproduced better the corneal curvature of a real keratoconus than Model 1 group.

Topographic analysis showed a significant inferiorly decentered corneal steepening in both keratoconus models. Model 1 produced a smoother curvature regression from the cone apex towards the periphery compared to model 2 but was affected by a semilunar steepening in the superior half of the cornea corresponding to the shoulder of the ablation area. Model 2 produced a sharper cone area with a more regular peripheral curvature compared to Model 1 but the cone apex was affected by an isle of minimal flattening due to the total masking of that area during laser ablation. Topographic assessment was repeated after FSL stromal pocket creation and no significant changes in the aforementioned parameters were observed (Figure 12).

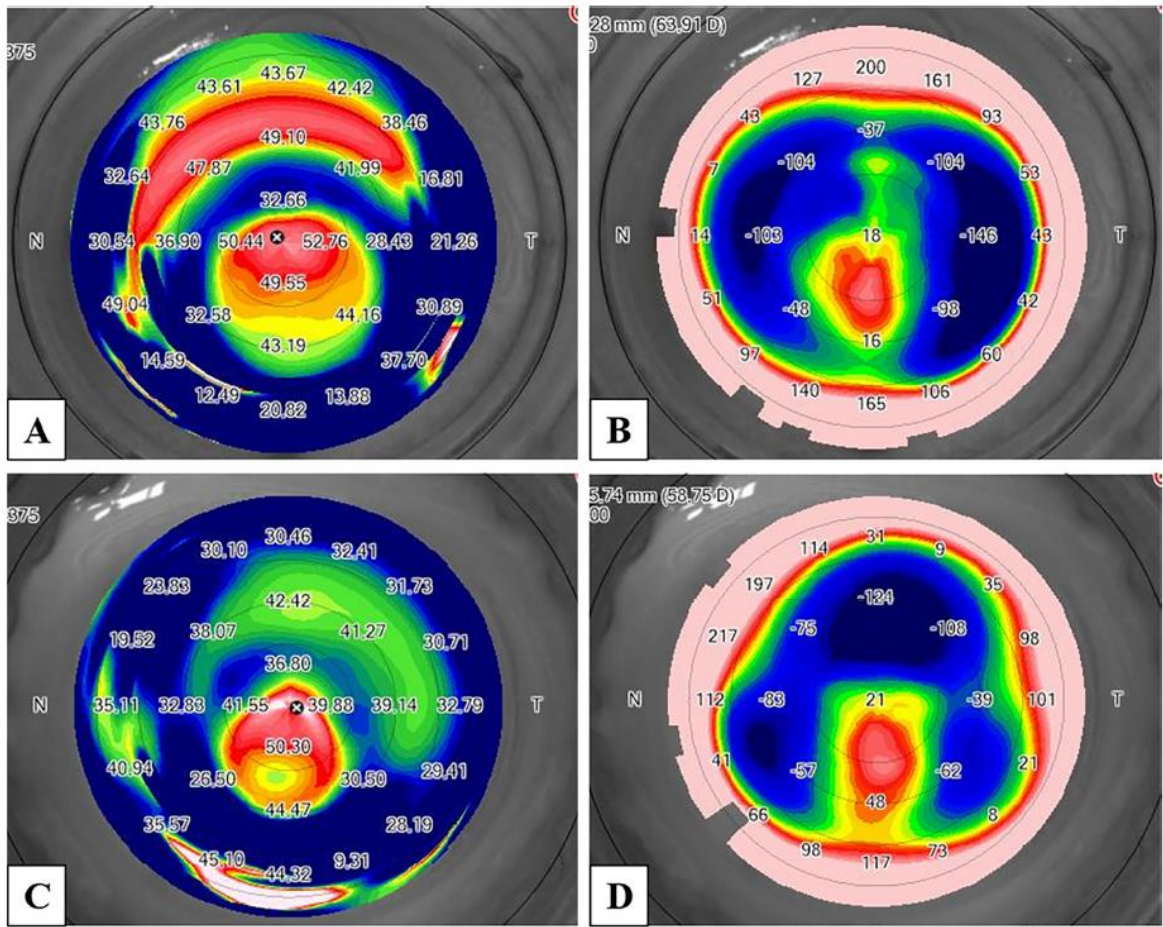


Figure 12. Pre-implantation topographies of keratoconus models. (A, B) Model 1: in the anterior tangential map (A), a paracentral inferior oval steep area (corresponding to the cone apex) and a peripheral superior arcuate steep zone (corresponding to the superior edge of the anterior hyperopic excimer laser ablation) is evident. The posterior elevation map (B) shows the inferior paracentral ectasia (corresponding to the cone apex). (C, D) Model 2: in the anterior tangential map (C), a paracentral inferior oval steep area (corresponding to the cone apex) is evident. The posterior elevation map (D) shows the inferior paracentral ectasia (corresponding to the cone apex).

Each recipient cornea was implanted with all four lenticule types in random order. Lenticule extraction and implantation with proper centering and distension of the tissues inside the intrastromal pocket were obtained in all cases.

The pachymetric assessment showed an overall increase of corneal thickness in the lenticule addition area in all cases, with different patterns dependent on each lenticule shape. Planar lenticules induced a homogeneous corneal thickening, whereas customized planar lenticules produced higher thickening in the inferior half of the cornea consistent with the lenticule thickness profile and its rotational orientation. Negative lenticules determined a maximal increase of pachymetry at their periphery, with a gradual reduction towards the center. The peripheral thickening was asymmetric after customized negative lenticule implantation, with the thicker area of the lenticule positioned inferiorly (Figure 13).

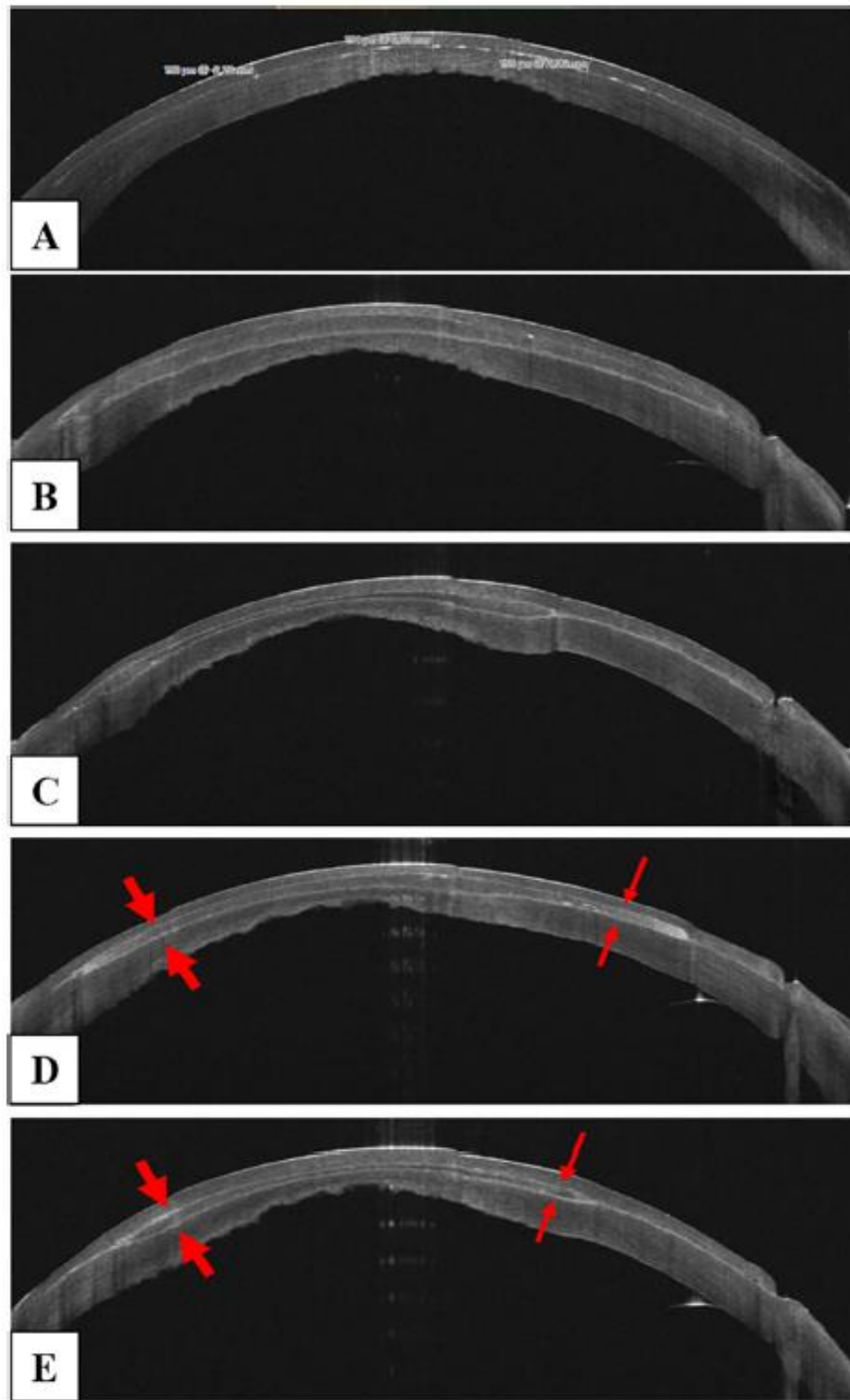


Figure 13. Anterior segment OCT scan of recipient cornea (keratoconus model 2) after stromal pocket creation (A) and lenticule implantation (B–E). (B) Type 1 lenticule with a planar profile. (C) Type II lenticule with a negative meniscus profile. (D) Type III lenticule with an asymmetric planar profile. (E) Type IV lenticule with an asymmetric negative meniscus profile. Thick and thin red arrows (D, E) highlight masked and ablated areas of customized lenticules respectively.

In all cases of lenticule implantation, corneal topography showed a flattening of the cone apex regardless of the lenticule type. Flattening was confirmed by a significant reduction of maximum keratometry in the central cornea which was more pronounced in groups 2 and 4 compared to groups 1 and 3 ($P < 0.01$). An increase of anterior curvature was detected in correspondence of negative lenticules periphery, where the maximal intrastromal pocket spacing was produced by the thickest part of the lenticule. Although type II lenticules caused a uniform annular steepening along the whole periphery, customized negative lenticules (type IV) induced a lower curvature increase in the upper peripheral half of the cornea where the thinner part of the lenticule was located. In addition, planar lenticules (types I and III) induced a peripheral steepening that was less pronounced but wider compared to negative lenticules. Average keratometry did not change significantly in group 2 compared to pre-implantation model ($P = 0.12$). A reduction of average keratometry, instead, was observed with planar and customized lenticule, with a mean difference between pre- and post-implantation of 4.05 D ($P < 0.01$) with type I, of 4.4 D ($P < 0.01$) with type III, and of 3.2 D ($P = 0.02$) with type IV (Figure 14).

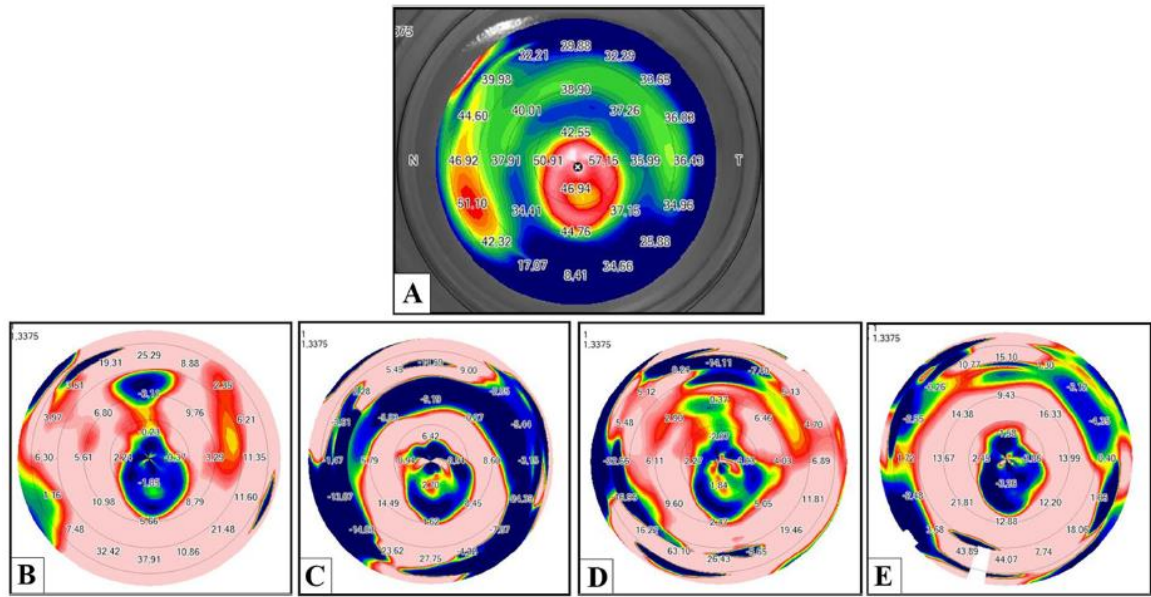


Figure 14. Topographies before and after lenticule implantations. (A) Keratoconus model tangential anterior map. (B–E) All differential maps after lenticule implantations show reduction of cone apex curvature and the peripheral steepening, that is annular and pericentral after type II (C) and type IV (E) lenticules implantation, whereas type I (B) and type III (D) lenticules induce a wider steepening. However, both type III (D) and type IV (E) lenticules implantation result in a less pronounced steepening in the superior hemisphere compared to type I (B) and type II (C), respectively.

Limitations

While this investigation showed that a good flattening of an off-centered cone could be achieved, an ideal lenticule shape suitable for every keratoconic eye cannot be created, considering the wide range of asymmetry of real keratoconus morphologies. This is also due to technical difficulties, as the “manual” nature of both keratoconus models creation and lenticules sculpting, using symmetrical off-centered ablation patterns and “homemade” masks, cannot precisely reproduce the whole complex corneal pathology and create a

specific lenticule geometry for optimal corneal regularization. Nevertheless, the approach to customize lenticules by increasing their asymmetry and tailoring the re-shaping, refractive, and structural effects in eccentric cones may be potentially feasible. The development of customization algorithms, hopefully supported by technology advancements to optimize their reproducibility and ease of creation, which is still lacking as of today, may determine a further refinement of such approach.

Chapter 2: The experimental study

Introduction

To date, SLAK is still limited to central forms of keratoconus because current femtosecond laser technology is not able to produce stromal lenticules with an asymmetric thickness profile¹⁷³. In addition, the lack of in vivo, and ex vivo, keratoconus models, as well as the lack of specific algorithms based on cone characterization to produce customized lenticules, makes it difficult to optimize the desired corneal curvature changes according to the characteristics of the corneal ectasia. Several studies, using different approaches, have investigated the possibility of creating animal models of corneal ectasia with sufficient similarities to keratoconus with respect to clinical characteristics, such as decreased central corneal thickness and increased corneal curvature. The most used methods to induce keratoconus in animals are corneal treatment with collagenase and exposure to ultraviolet radiation¹⁹³. However, consensus on reliable and accurate animal models for keratoconus is still lacking, thus limiting research approaches mainly to clinical studies and ex vivo investigations.

As a different approach, the possibility of using human donor corneas from eye banks to reshape stromal morphology similarly to keratoconus characteristics has been recently attempted. However, as of today, no validated methods are described in literature, and only one pilot study described an ex-vivo model based on excimer laser ablation used to modify the thickness of the cornea by decentralized posterior excimer laser ablation¹⁹².

Nubile et al. have recently proposed simulating the decentered curvature of eccentric KC, comparing two different technical approaches aimed at remodeling human donor corneas to reproduce a stromal geometry like KC curvature and thickness profiles, in an ex vivo model. The anterior corneal steepening was obtained through a hyperopic photorefractive keratectomy (PRK) in Model 1, or through masked phototherapeutic keratectomy (PTK) on the anterior corneal surface in Model 2, while the stromal thinning, in both models, was obtained through myopic ablation of the posterior corneal surfaces¹⁹⁰.

Tomographic analysis showed a significant inferiorly decentered corneal steepening in both models. Each model presented peculiar morphological characteristics, both adequate to reproduce the gross shape of a keratoconic cornea, Model 2 being more satisfactory, with a sharper cone area and a more regular peripheral curvature compared to Model 1. On the other hand, the anterior curvature steepening profile was characterized by an isle of minimal flattening due to the partial masking used during the anterior corneal surface laser ablation¹⁹⁰. In addition, both approaches were suitable for creating central or minimally eccentric keratoconus models, but the reproducibility in producing more eccentric cone location is still insufficient, due to the lack of customized automated asymmetric ablation patterns and the use of “homemade” masking systems that are in no way able to guarantee a reliable eccentric corneal conical profile.

The present study aims to create a new keratoconus model using the MEL80 excimer laser (Carl Zeiss Meditec, Jena, Germany) to increase the decentration of the cone. The basic shape of the model was derived from the principles lined with the study previously published¹⁹¹, and from further analysis of the topography and pachymetry from real keratoconus patients. The model alters the corneal shape of the human donor corneas in the

central zone to closely mimic that of a keratoconic cornea; however, it is not aimed to reproduce the biomechanical properties of typical corneal ectasia in donor human tissues.

The innovation in this study is the use of preset excimer laser shot-files pattern for both the anterior surface and the posterior surface ablations, based on real tomographies of decentered keratoconus, provided by an eminent company in the refractive surgery field (not to be disclosed for multi-parties NDA). Such company developed pattern specific asymmetrical shot-files and delivered them in order to create reproducible keratoconus models from eye bank sclero-corneal discs.

Materials and methods

This was an ex vivo investigation of corneal curvature re-modeling, with the aim to create a reproducible model for eccentric keratoconus in eye bank donor corneas, through the use of asymmetrical excimer laser ablation.

In the first experimental part of the study, customized asymmetrical excimer laser shot files for both anterior and posterior corneal surface, based on real keratoconus tomographies, were created, based on the above-mentioned system provided by the company. Those shot files were elaborated by software engineers, according to the calculations aimed to hyperprolate the anterior corneal surface in the eccentric zone, with corresponding stromal thinning.

A total of 10 human corneas with scleral rims, non-suitable for human transplantation, were harvested from the eye-bank institute (Fondazione Banca Degli Occhi Del Veneto Onlus – FBOV, Venezia, Italy). All tissues had no evidence of irregularities or other opacities and

were stored for less than 48 hours, with a mean storage time of 30 hours. However, an additional 8 hours of storage in a dextran-enriched medium (Thin-C, Alchimia, Italy) was performed to minimize the storage-induced stromal edema. Tissues with a CCT higher than 600 μm were excluded from the present study. An accurate topographic and pachymetric assessment (MS-39, CSO, Italy) was performed before and after the intervention. Prior to beginning the surgical maneuvers, asymmetrical excimer laser shot files were retrieved and loaded into the MEL 80 system.

Surgical technique

Donor corneo-scleral discs were mounted on artificial anterior chambers (Network Medical Products Ltd., Coronet House, UK) and the epithelium was removed with a blunt spatula. To obtain adequate pressure and stability of the corneal tissue, the BSS bottle was standardized at 180 cm of height, and system pressure was assessed with an applanation tonometer to guarantee that the internal pressure of the corneo-scleral rings was within the expected range (25–30 mmHg).

Subsequently, corneas underwent excimer laser ablation re-shaping (MEL-80, Carl Zeiss Meditec, Germany), on both anterior and posterior corneal surface to reproduce and simulate the anterior corneal curvature steepening and stromal thinning, typical of keratoconus. Transformation was achieved through consecutive application of two separate ablation patterns on the anterior and posterior side of a donor cornea using the MEL 80 excimer laser. Posterior ablation was achieved, immediately after the anterior one, by flipping the corneoscleral tissue upside down on the artificial anterior chamber, then the donor discs were reverted to the original position. The anterior ablation pattern was used to create the intended

anterior topography with a decentered hyperprolate zone on the donor cornea, while the posterior ablation pattern was used to reduce the corneal thickness at the same position of the corneal protrusion. This was possible thanks to the peculiar preset customized shot file that was provided for the study, in which the anterior asymmetrical ablation, saving a small portion at around 1,5 mm distance from the center in the inferotemporal area, was combined with a small posterior ablation that was created in the exact same corneal spot after flipping the donor corneo-scleral rim (that is, excimer laser was applied 1,5 mm inferonasally from the center) (Figure 15).

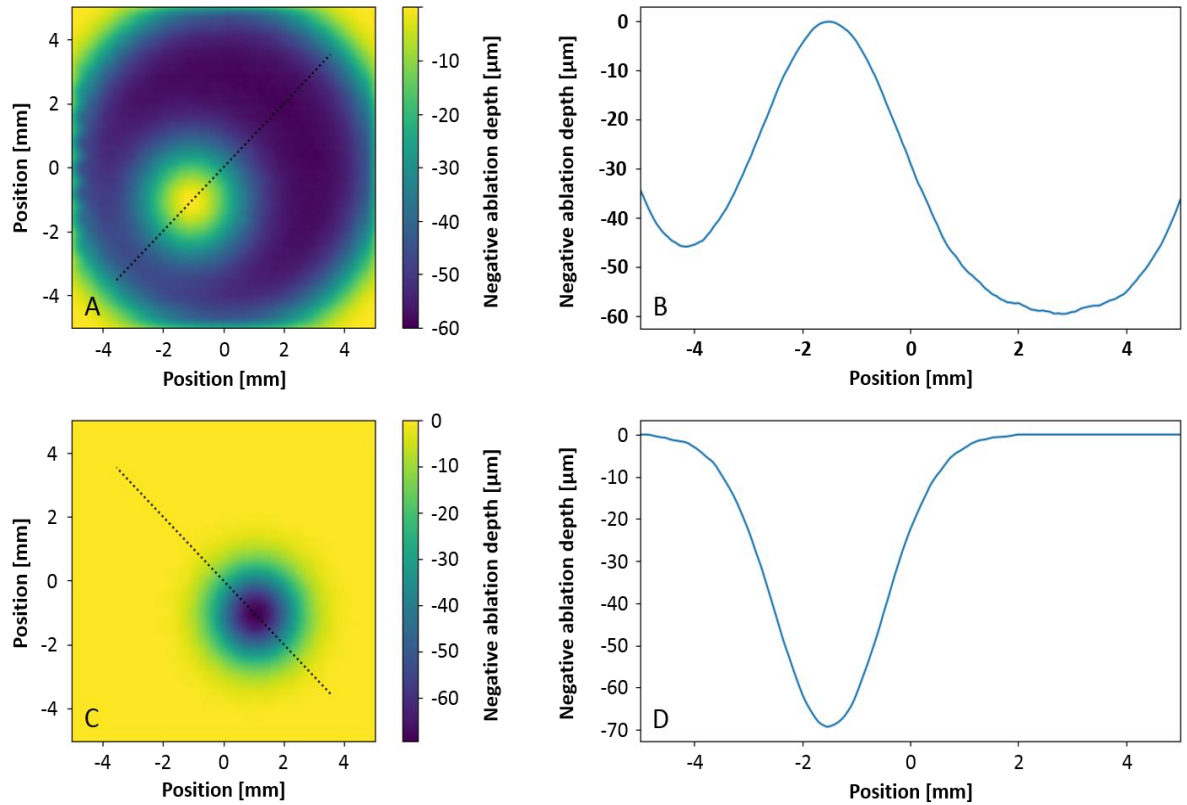


Figure 15. Ablation patterns applied to create the keratoconus model with eccentricity of 1.5 mm. (A) Anterior ablation pattern with off-centered protrusion (yellow), dotted line indicates cross-section. (B) Cross-section of the two-dimensional anterior ablation pattern at the 45° angle. (C) Posterior ablation pattern for corresponding material removal from the posterior surface, dotted line indicates cross-section. (D) Cross-section of the two-dimensional posterior ablation pattern at the 135° angle.

To facilitate and standardize the intended stromal corneal ablations, tissues were marked on both the anterior and posterior corneal surfaces, before excimer laser use. In addition, the marking of additional reference points (purple dots) was made on the corneal edges to define the inferior, superior, and nasal position of the cornea (Figure 16). Marking procedure was

added stepwise during the procedure. After the anterior ablation, marking procedure was executed. Firstly, the inferior position was differentiated with two dots to enable a clear orientation of the cornea. Then, the superior corneal position was located towards the surgeon's position in front of the excimer laser and, finally, the nasal mark was placed. Afterwards, the position of the excimer laser aiming laser (red dot) was marked, coincident to the center of the previous ablation (Figure 16). Subsequently, the cornea was flipped around along the superior-inferior axis (yellow line) and turned inside out so that the posterior surface was facing upwards while maintaining the superior-inferior orientation.

Before the posterior ablation, the aiming laser beam (red dot) was centered on the central corneal mark, previously placed, to ensure that the posterior ablation was applied in a symmetrical fashion to the anterior ablation (that is, the posterior ablation was coincident to the same spot in the anterior ablation that was not ablated), then the ablation was started. After completion of the posterior ablation, the cornea was flipped back to its original position along the inferior superior axis (yellow line) maintaining the superior-inferior orientation (Figure 16).

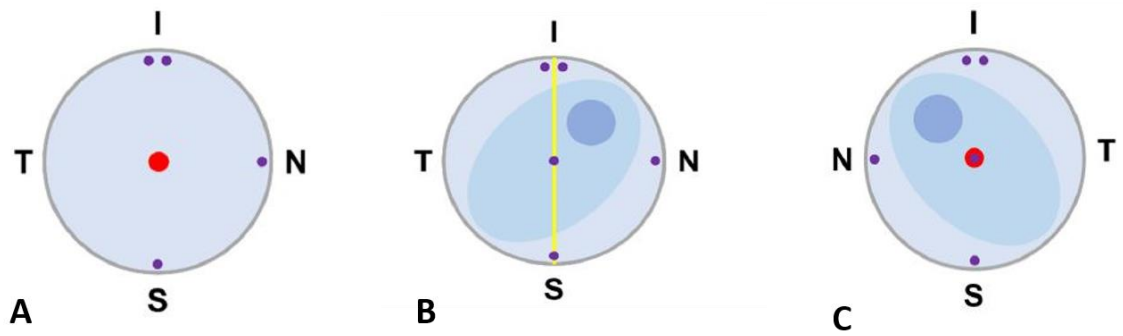


Figure 16. Marking and orientation of the cornea. (A) Before anterior ablation, marks on the corneal edges the inferior, superior, and nasal positions are placed. (B) Post anterior ablation, marking the position of the MEL80 aiming laser (red dot) is placed. (C) Before posterior ablation, the marking of the aiming laser beam of the MEL80 (red dot) on the central cornea is achieved.

Tomographic analysis and wavefront aberrometry measurements of the anterior corneal surface using AS-OCT (MS-39, CSO, Florence, Italy) were performed in all corneas before and after the intervention, using a specific realized holder for the artificial anterior chamber to keep corneas correctly aligned in front of the instrument while scanning.

Curvature changes were assessed using the average keratometry (AvgK) values in the central 3-mm area, maximum keratometry (Kmax) on the anterior and posterior corneal surfaces, and the cylinder changes. In addition, peripheral curvature in the 6 mm zones for the inferior and superior hemi-meridians and the Symmetry Index of the anterior curvature (SI_f – Symmetry Index_{FRONT}) were recorded. The latter parameter is defined as the difference of curvature (expressed in diopters) of two circular zones with a radius of 1.5 mm centered on the vertical axis in the inferior and superior hemispheres.

Corneal asphericity variations were assessed using the Q-value in the 6 mm peripheral-inferior zone (where the cone apex is located), the superior hemi-meridians, and the average Q-value of the four hemi-meridians.

The total root mean square per unit of area (RMS/A) at a maximum of 6 mm diameter, defined as the deviation in regularity/aberrations of the corneal surface from the best fit asphero-toric surface, was used as a shape index to express quantitatively corneal aberrations. Furthermore, RMS/A for spherical aberration, coma, and astigmatism at a maximum of 6 mm diameter were investigated before and after the intervention. Spherical aberration evaluates the blurred or distorted image, and coma evaluates changes in contrast sensitivity and halos. Finally, astigmatism was evaluated to estimate corneal irregularities.

Statistical analysis

To determine the appropriate statistical test, all the distributions were firstly checked. Then, the Shapiro-Wilk test was carried out to assess whether the residuals followed a normal distribution. If the p-value was greater than 0.05, the Student's t test for repeated measures was used. If normality assumption was violated, the non-parametric Wilcoxon test was used as it is more suitable for non-normal distributions. If the p-value was 0.05 or higher, indicating that the data did not significantly deviate from a normal distribution, the T-Student test was used to compare means. This method ensured accurate statistical analysis based on the distribution characteristics of the data. All the statistical tests were carried out by using the open-source R-based JAMOV software (<https://www.jamovi.org/>).

Results

The use of custom, asymmetric, excimer laser shot files to create a keratoconus model, successfully reproduced a real world conical off-centered protrusion with good predictability, reproducibility and relative ease. A 1,5 mm off centered ectasia was detectable in all models, consisting of a coincident anterior curvature steepening, thickness reduction and posterior surface protrusion (Figure 17). Anterior segment OCT showed a small notch in the paracentral inferior area of the posterior surface due to laser ablation (Figure 18). The automated software for keratoconus detection, provided with the MS-39 Tomography and validated for clinical use, recognized as normal corneal patterns all the tissues before the shotfile ablations, and as “keratoconus” the corneas after excimer laser tissue modifications.

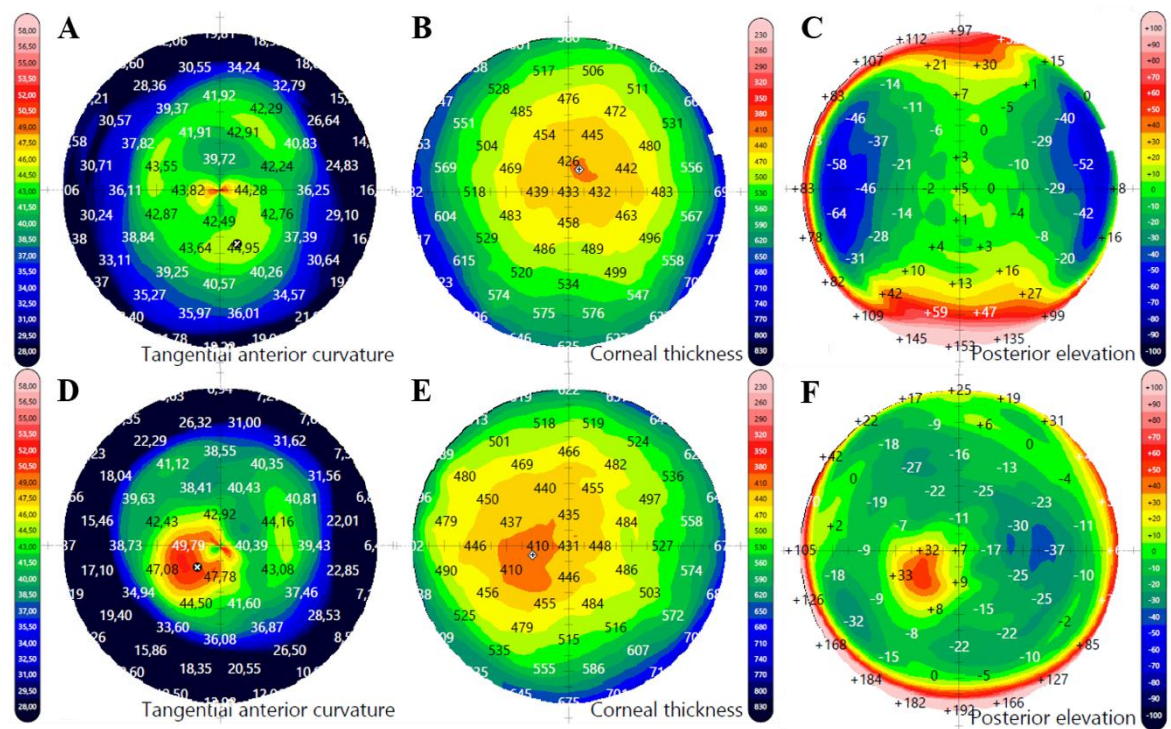


Figure 17. Keratoconus model with custom asymmetric excimer laser shot file ablation. (A) Preoperative tangential anterior curvature, showing normal prolate cornea. (B) Preoperative normal corneal thickness. (C) Preoperative normal posterior elevation. (D) Postoperative tangential anterior curvature, showing off-centered corneal steepening, similar to real world keratoconus. (E) Postoperative corneal thickness, showing coincident corneal thinning, similar to real world keratoconus. (F) Postoperative posterior elevation, showing coincident posterior ectasia, similar to real world keratoconus.

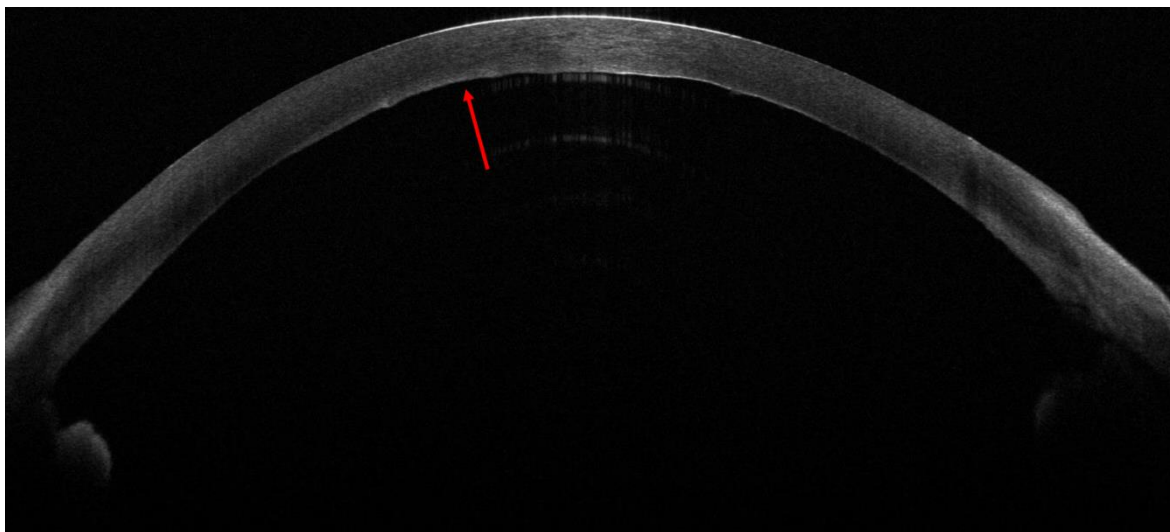


Figure 18. Keratoconus model AS-OCT. The red arrow shows the endothelial “notch”, where posterior ablation was executed.

Data for each keratoconus model was analyzed. Mean corneal apex thickness postoperatively was 435.2 μm (Table 3). We observed a statistically significant decrease (p-value = 0.006) between the preoperative and postoperative values, with a mean difference of 31.5 μm and an effect size $r = 0.927$.

Postoperatively, mean anterior and posterior maximum keratometry (Kmax) values were 58.023 D and -9.555 D, respectively (Table 3). We observed a statistically significant increase in both anterior and posterior Kmax (p-value = 0.002) between the preoperative and postoperative values, with mean differences of 6.545 D and 2.04 D, respectively, and an effect size $r = 1$. Additionally, mean average keratometry (AvgK) value postoperatively was 45.825 D (Table 1). We obtained a statistically significant increase (p-value < 0.001) between the preoperative and postoperative times, with a mean difference of 1.764 D and an effect size $d = 1.74$.

Mean cylinder (Cyl) value postoperatively was -2.281 D (Table 3). Although there was a decrease in the Cyl between the preoperative and postoperative values, the change was not statistically significant (p-value = 0.644), with a mean difference of 0.198 D and an effect size $d=0.151$.

Mean symmetry index front (SI_f) value postoperatively was 3.759 (Table 3). A statistically significant increase was observed (p-value < 0.001) between the preoperative and postoperative periods, with a mean difference of 4.016 and an effect size $d = 1.672$.

Mean values for inferior and superior peripheral curvature at 6 mm were 7.233 mm and 8.166 mm, respectively, with a mean corneal asphericity Q-value of -1.337 (Table 3). A statistically significant decrease in the inferior peripheral curvature was observed (p-value = 0.002) between the preoperative and postoperative time, with a mean difference of 0.548 mm and an effect size $d=1.359$. Additionally, there was a statistically significant increase in the superior peripheral curvature (p-value = 0.027) and the Q-value (p-value = 0.06) between the preoperative and postoperative periods, with mean differences of 0.253 mm and 0.585, respectively, and effect sizes of $d = 0.834$ and $d = 0.679$.

Mean values of maximum anterior and posterior elevation (ΔZF_{Max} and ΔZB_{Max} respectively) were 31.92 μm and 49.84 μm , respectively (Table 3). A highly statistically significant increase was observed for these outcomes ($p < 0.001$) between the preoperative and postoperative times, with mean differences of 18.42 μm and 31.88 μm , respectively, and effect sizes of $d = 1.898$ and $d = 1.59$.

Mean values of total root mean square per area (RMS/A) and RMS/A for spherical aberration, coma, and astigmatism were 2.596 μm , 0.278 μm , 1.867 μm , and 2.425 μm , respectively (Table 3). Mean differences were 1.3 μm , 0.03 μm , 1.336 μm , and 0.442 μm ,

with effect sizes of 0.745, 0.117, 1.827, and 0.238, respectively. A statistically significant increase was observed for RMS/A and coma for these outcomes (p-value = 0.037 and < 0.001, respectively) between the preoperative and postoperative times. In contrast, the increases in spherical aberration and astigmatism were not statistically significant (p-value = 0.719 and 0.394, respectively).

	Mean	Median	SD	IQR
Pre-op corneal apex thickness (μm)	477.200	464.500	47.609	56.7500
Post-op Corneal apex thickness (μm)	435.200	431.500	43.599	18.0000
Pre-op Ant Kmax (D)	50.252	49.515	2.239	2.1700
Post-op Ant Kmax (D)	58.023	56.265	6.271	3.6900
Pre-op Post Kmax (D)	-7.126	-6.780	0.909	0.6000
Post-op Kmax (D)	-9.555	-9.135	1.709	2.1750
Pre-op AvgK (D)	44.061	44.125	1.011	0.7625
Post-op AvgK (D)	45.825	45.245	1.312	1.9375
Pre-op Cyl (D)	-2.083	-1.965	1.048	1.6675
Post-op Cyl (D)	-2.281	-2.030	1.382	1.4700
Pre-op SI _f (D)	-0.257	0.505	2.609	0.8875
Post-op SI _f (D)	3.759	4.025	2.435	2.7100
Pre-op 6mm-inferior peripheral curvature (mm)	7.781	7.800	0.230	0.2875
Post-op 6mm-inferior peripheral curvature (mm)	7.233	7.275	0.439	0.8225
Pre-op 6mm-superior peripheral curvature (mm)	7.913	7.910	0.217	0.2550
Post-op 6mm-superior peripheral curvature (mm)	8.166	8.195	0.291	0.3500

	Mean	Median	SD	IQR
Pre-op 6mm-peripheral Q-value Avg	-0.752	-0.780	0.611	0.9625
Post-op 6mm-peripheral Q-value Avg	-1.337	-0.890	0.956	1.0575
Pre-op ΔZF_{Max} / Max. Ant. Elevation (μm)	13.500	11.600	7.166	4.6500
Post-op ΔZF_{Max} / Max. Ant. Elevation (μm)	31.920	30.400	7.465	11.0750
Pre-op ΔZB_{Max} / Max. Post. Elevation (μm)	17.960	15.050	9.215	5.2500
Post-op ΔZB_{Max} / Max. Post. Elevation (μm)	49.840	47.550	21.035	22.6000
Pre-op RMS/A (μm)	1.554	1.125	1.004	0.5025
Post-op RMS/A (μm) (2)	2.596	2.460	0.621	0.5400
Pre-op Spherical Aberr. [RMS] (μm)	0.248	0.170	0.183	0.2500
Post-op Spherical Aberr. [RMS] (μm)	0.278	0.245	0.159	0.0925
Pre-op Coma [RMS] (μm)	0.531	0.550	0.236	0.3025
Post-op Coma [RMS] (μm)	1.867	1.755	0.680	0.8800
Pre-op Astigmatism [RMS] (μm)	1.983	2.000	1.233	2.0375
Post-op Astigmatism [RMS] (μm)	2.425	2.835	1.374	2.0800

Table 3. Mean Outcome Values between Preoperative and Postoperative times in keratoconus model corneas.

Discussion

The principle of the SLAK technique is to reshape the anterior corneal profile by stromal tissue addition, increasing thickness, and improving the anterior corneal curvature, which is the main determinant of visual impairment in keratoconus. Although *in vivo* remarkable results were obtained in central keratoconus with symmetric lenticules¹⁷³, as of today, *in vivo* customized stromal tissue addition, to improve outcomes in eccentric ectasia, has not been described yet in an *in vivo* real eccentric keratoconus. The currently available FSL platforms do not allow the creation of stromal dissections with peculiar asymmetric profiles. Pedrotti et al. investigated, *ex vivo*, the feasibility of intrastromal lenticule insertion to restore corneal shape in a model of ectatic human cornea¹⁹². Assuming that ectasia in keratoconus starts from the posterior stroma, they created posterior corneal surface ectasia model with excimer ablation aimed at achieving flattening of the posterior surface by myopic lenticule implantation. In their model, excimer laser ablation on the endothelial side only produced paracentral stromal thinning and a minimal non-predictable increase in anterior corneal curvature was obtained by high-pressure inflation of the artificial anterior chamber with BSS up to 100 mm Hg. Despite posterior flattening, the implantation of myopic lenticules induced an increase in the anterior corneal curvature with a trend to a more prolate profile¹⁹². It has to be noted that one of the main limitations of *ex vivo* studies investigating intrastromal lenticule addition for treating corneal ectasia is the fact that *ex vivo* models of keratoconic corneas have not been established yet. Afterwards, Nubile et al. resorted to masked excimer laser ablation to reshape FSL-derived stromal lenticules¹⁹⁰. In this study, we aimed to provide a standardized and controlled keratoconus corneal model from donor sclero-corneal discs, taking into account both the pachymetric reduction and the localized increase in anterior surface curvature, typical of the disease, obtained by posterior and

anterior excimer laser ablations, without the need to modify AAC pressure beyond physiological values. Additionally, we compared the effects on corneal curvature of intrastromal implantation of different lenticule profiles in a created ex vivo model of eccentric keratoconus. The first issue to be addressed was the creation of a reliable eccentric keratoconus model using excimer laser corneal reshaping. We also compared tomographic results of two different ablation procedures with a pool of real eccentric keratoconus topographies from patients' database. In both models, a myopic PRK on the endothelial side was performed to achieve the cone apex thinning. To obtain the apical steepening, the anterior corneal surface underwent a hyperopic PRK in model 1, and a PTK with a paper disc mask on the cone apex in model 2. In both cases, the typical inferior paracentral oval pattern on the anterior tangential map was obtained. Model 2 showed higher keratometry values and a steeper curvature progression from the cone to the periphery and it more accurately reproduced the profile of a real keratoconus because it did not alter significantly the superior hemi-cornea. In addition, the higher cone apex thickness observed in model 2 was more consistent with curvature parameters referring to real keratoconus. For these reasons, model 2 was chosen to produce recipient corneas for lenticule implantations. In the chosen ex vivo model, four different lenticule profiles were therefore evaluated. The planar lenticule (type I) and the negative meniscus shaped lenticule (type II), produced by FSL only, were already tested in the previous clinical studies that proved their efficacy in the treatment of keratoconus^{173,191}. The customized type III and type IV lenticules were obtained from the formers through further excimer laser ablation. As expected, all lenticules improved corneal pachymetry proportionally to the amount of the tissue addition. Topographic assessment showed detectable reductions in central anterior corneal curvature, pointing out a noteworthy relative flattening of the cone apex achieved in all cases after implantation,

although it was higher with negative and customized negative lenticules, compared to planar and customized planar ones. Types I, III, and IV lenticules led to a reduction in keratometry, whereas negligible differences were observed after type II lenticule implantation. This can be explained by curvature changes induced by the thickest part that superiorly falls in the paracentral corneal region. It follows a keratometric redistribution with inferior flattening and superior steepening that results in an almost unchanged keratometry. Differently, customized negative lenticules produced less curvature increase in the paracentral cornea thanks to their lower local tissue volumes, due to customized ablation, whereas planar and customized planar lenticules, having a wider diameter and a more regular profile, influence paracentral corneal curvature much less. A similar trend was observed also for asphericity changes following tissue addition. The concept of adding a higher amount of tissue around the cone apex, exploiting the law of thickness, and the arc-shortening effect^{109,123}, proved again to be the most effective in reducing its curvature. The main issue was the impact that negative meniscus lenticules had on the central corneal curvature, which is crucial for visual performance. Planar lenticules partially prevent this problem at the expense of lower cone flattening. Lenticule customization allowed to overcome these drawbacks and, at the same time, to exert remarkable effects on corneal asphericity and regularity. Customized negative lenticules induced the highest improvement in corneal asphericity, resulting in a less hyperprolate shape, whereas customized planar lenticules proved to be the most effective in restoring a corneal profile, closer to the best-fit asphero-toric surface¹⁹⁰. Improving regularity and reducing the excessive curvature in the central cornea, along with increasing the stromal thickness, is probably the most challenging goal of the intrastromal addition procedures aiming to reshape keratoconic corneas, having a profound impact on non-correctable high-order aberrations, and therefore on the corrected visual acuity of patients.

In our second investigation we tried to overcome and improve the limitations of the first study with the aim to produce a more regular and ideal model of keratoconus, starting from eye-bank naïve corneas. Despite the capability of mimicking keratoconus corneal shape by calibrated and geometrically distributed anterior and posterior excimer laser ablations, this approach still presented the described main limitations on the curvature gradient in the mid periphery of the anterior corneal surface¹⁹⁰, we aimed to improve the model in the second experiment based on customized shot-files for the corneal ablations. We found out that the use of asymmetric customized excimer laser shot files to create a keratoconus model from eye bank donor corneas is an effective and reproducible method to obtain ectatic corneas that could be subsequently used for implantation purposes. Corneal curvature and posterior elevation predictably increased and corneal thickness decreased, obtaining an off-centered conical protrusion, more similar to real world keratoconus. The use of asymmetric customized shot files enables simple modeling of a normal sclero-corneal button with few surgical passages that may influence good conical reproduction of a keratoconus. No evident signs of superior paracentral steepening or abrupt zone of ablation were found, so that a keratoconic profile was successfully and repeatedly achieved with ease. Although this ex vivo model simulates eccentric keratoconus conditions, it offers an environment significantly different from the in vivo application. The in vivo phenomena, such as the post-implantation wound healing processes, inflammatory reaction, keratocyte activity and collagen remodeling, and nerve degeneration as well as regeneration processes, occurring in the early and late period after surgery cannot be evaluated in such an ex vivo study. Further investigation should be undertaken to achieve customization of corneal lenticules that would be implanted inside a keratoconic eye for ectasia correction. The ideal hypothetical surgery is to obtain a specific excimer laser shot file pattern, based on patient specific keratoconus

morphology, to model a stromal lenticule, so that it acts as a “negative” to that specific keratoconus, and to easily implant it in the patient. Consideration should also be taken with regards to centration, since the lenticule, to exert its full correction potential, needs to be precisely centered on the cone apex. In a central cone, centration is a simple procedure, because the only requirement is to align the lenticule with the corneal center to achieve a satisfactory correction. This is not true for eccentric cones, as it is extremely difficult to align the steepest and thinnest point with the flattest lenticule point. Manual (gentian violet) centration may help in this process, but it is still a suboptimal solution for this issue. Refinements in centration processes (possibly automated to make the procedure even simpler) may achieve such objective. Our group has also begun further experiments to create stromal lenticules relative to a predetermined cone morphology and to implant them in the same keratoconus models. The aim of this experiments is to evaluate not only corneal thickness restoration, but also anterior surface regularization, which we believe is paramount for optimal visual correction of keratoconus and may revolutionize keratoconus treatment, in order to avoid a much more invasive keratoplasty procedure and its complications.

Conclusions

Corneal remodeling should be certainly further investigated to develop customization algorithms, hopefully, supported by technology advancements to optimize their reproducibility. Because the process of lenticule production might be complex and require a specific setup, other approaches can be considered such as eye bank lenticule production, cryopreservation, and delivery of customized lenticule. Future studies to assess the functional impact of custom-made lenticule implantations in patients with eccentric keratoconus and the wound healing process after excimer laser ablation are, therefore, needed. To provide satisfying best corrected visual acuity and contact lens tolerance, minimally invasive additive procedures, tailored to the patient specific cone morphology, may reduce the need for the more invasive approaches, such as traditional keratoplasty.

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