• Review Article •

Corneal collagen cross-linking in patients with keratoconus from the Dresden protocol to customized solutions: theoretical basis

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Abstract

● Keratoconus is an ectatic condition characterized by gradual corneal thinning, corneal protrusion, progressive irregular astigmatism, corneal fibrosis, and visual impairment. The therapeutic options regarding improvement of visual function include glasses or soft contact lenses correction for initial stages, gas-permeable rigid contact lenses, scleral lenses, implantation of intrastromal corneal ring or corneal transplants for most advanced stages. In keratoconus cases showing disease progression corneal collagen crosslinking (CXL) has been proven to be an effective, minimally invasive and safe procedure. CXL consists of a photochemical reaction of corneal collagen by riboflavin stimulation with ultraviolet A radiation, resulting in stromal crosslinks formation. The aim of this review is to carry out an examination of CXL methods based on theoretical basis and mathematical models, from the original Dresden protocol to the most recent developments in the technique, reporting the changes proposed in the last 15y and examining the advantages and disadvantages of the various treatment protocols. Finally, the limits of non-standardized methods and the perspectives offered by a customization of the treatment are highlighted.

● **KEYWORDS:** corneal collagen cross linking; keratoconus; custom fast protocol; Dresden protocol; lambert-beer law; Bunsen-roscoe law
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INTRODUCTION

Keratoconus is a degenerative disease characterized by progressive corneal thinning and steepening, irregular astigmatism, edema formation, scarring, and exhaustion of the corneal apex. All these events have a serious impact on visual acuity, especially at a young age[1].

Keratoconus is mainly bilateral, but often with asymmetrical appearance and development. The onset typically occurs in early adolescence with usual progression mostly in the following 15y; the onset is very variable: in some cases, it can occur earlier, in pediatric age, or later, in adult subjects[2].

Frequent changes in refractive error correction typically occur in these patients; over time the eyeglass correction is not suitable and it's necessary a switch to wearing gas-permeable or rigid contact lenses. The pathogenesis is still not totally clear: abnormalities in the organization and adhesion of collagen fibers, the main support of the corneal stroma, were found; continuous eye rubbing has also been indicated as a concomitant cause, generally due to chronic allergic conjunctival inflammation[2], determining changes in corneal shape and intraocular pressure (IOP) with significant reduction in keratocyte density[3].

The etiology is unknown. However, keratoconus is often associated with atopy, asthma, eczema, Down syndrome, Leber congenital amaurosis, Ehlers-Danlos syndrome, or other connective tissue disorders. The prevalence in the whole population, according to most recent epidemiological studies, is 1.38 per 1000 [95% confidence interval (CI): 1.14-1.62 per

1000)[4]. Although genetic predisposition to keratoconus has been observed, no specific gene has been identified yet. Histopathological studies have shown ruptures or complete absence of the Bowman membrane, collagen disorganization, scarring, and thinning. The cause of these changes is unknown, although some attribute it to changes in enzymes that lead to the degradation of corneal collagen. Although keratoconus does not meet the criteria of inflammatory disease, recent studies show a pathogenetic role of proteolytic enzymes, cytokines, and free radicals, in particular matrix metalloproteinase-9 (MMP-9), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), even in subclinical forms, highlighting pathologic characteristics of inflammatory type[5].

Clinical diagnosis is based on the frequent refractive changes, poor best corrected visual acuity (BCVA), deformation of the cornea and characteristic opacities at the slit lamp examination (Vogt striae, apical opacity), scissor reflex at retinoscopy, deformation of the sights in keratometry, ectasia at the corneal topography and tomography, and corneal thinning at pachymetry[4]. The initial approach to keratoconus involves glasses or soft contact lenses correction to improve visual acuity. With the progression of the disease, it is necessary to use gas-permeable rigid contact lenses for a satisfying correction; over time, the accentuation of the corneal curvature or the appearance of inflammatory complications can be an impediment for contact lenses use, especially in allergic subjects. In addition, central corneal scarring can limit visual acuity, despite the use of any optical device. Traditionally, when patients cannot obtain adequate vision either with glasses or contact lenses, surgical options are considered: penetrating keratoplasty (PK) and, more recently, deep anterior lamellar keratoplasty (DALK)[6-7].

In the last 25y, with the development of corneal cross-linking (CXL), an etiopathogenetic and non-invasive approach to keratoconus has been implemented in clinical practice. In the CXL riboflavin-based solutions are used in combination with ultraviolet A (UV-A) irradiation. Riboflavin plays the role of photosensitizer in the process of corneal photopolymerization and increases the formation of intrafibrillar and interfibrillar covalent bonds when combined with UV-A irradiation. This photochemical reaction increases corneal stiffness, collagen fibers thickness, and resistance to enzymatic degradation, especially in the anterior stroma[8-9]. Since the late 1990s, numerous papers published in international peer-reviewed journals have reported promising clinical results on the use of CXL in the treatment of progressive keratoconus[10-12]. Thanks to CXL, an increase in the stiffness of the stroma of over 300% is reported, with an increase in the diameter of the collagen fibers by 12.2% and the formation of cross-linked bonds in the collagen structure[13-15].

Conventional Dresden Protocol The first cross-linking method, called the Dresden Protocol (standard technique) from the place of birth, was developed in 1998[1].

The standard protocol involves the removal of the epithelium, followed by 30min of imbibition of the corneal parenchyma with a riboflavin-dextran solution. The second phase consists of 6 steps of 5min each of UV-A irradiation (370 nm), associated with the instillation of two drops of riboflavin every 5min, with a fixed intensity of the power heuristically defined at 3 mW/cm^2 for a total energy of 5.4 J/cm^2[11-13].

The removal of the epithelium (EPI-OFF technique) is motivated by the need for rapid and suitable absorption of riboflavin in the corneal parenchyma. The basal layer cells of the corneal epithelium, joined by tight junctions, constitute the main barrier to the diffusion of hydrophilic molecules through the epithelial barrier, while the diffusion of lipophilic solutes can occur through lipid cell membranes[16]. Hydrophilic molecules traverse the paracellular spaces and it is known that the molecular weight of 500 daltons represents the critical threshold of diffusion through the intact corneal epithelium[17].

For a long time, it was believed that the riboflavin molecule was too large to cross the corneal epithelium (PM=514.36)[18-19], but the major obstacle to permeation of riboflavin through the corneal epithelium is due to excessive hydrophilicity, having a very high water/octanol partition coefficient (logP=1.85). Furthermore, the presence of a negative charge due to the phosphate group of riboflavin causes a repulsive effect with the net charge, of the same sign, present at the level of corneal glycosaminoglycans[20].

The removal of the epithelium allows to achieve a sufficient imbibition of the stroma, but it is invasive and exposes to limitations from the point of view of safety and risks of adverse events[21]. The application of a therapeutic corneal lens promotes the healing and re-epithelialization process; nevertheless, in the first months following treatment, the new stratification of the corneal epithelium leads to an initial worsening of the initial refractive, topographic, and aberrometric measurements[22]. It has been documented that, only from about 6mo after treatment and only in 40% of patients, there is a progressive, although modest, improvement of the clinical and instrumental picture[23-25]. The cross-linking effect affects the anterior 200-250 μm of the corneal stroma. To avoid damage to the endothelium, the treatment can only be carried out on corneas with a stromal thickness of more than 400 μm[26]; this excludes several patients with very thin corneas from the procedure. To artificially increase its thickness, it has been tried to soak the corneal stroma of thin corneas with hypo-osmolar solutions of riboflavin, but there has been no statistical evidence of greater efficacy or safety of this variant compared to the traditional protocol[26-28]. In addition, the

literature reports up to 10% of corneal complications, such as haze and scarring, following CXL EPI-OFF treatment[29-31].

Less Invasiveness: EPI-ON The limitations of the standard technique introduced the need to develop protocols capable of ensuring high stromal absorption and homogeneous distribution of riboflavin without removal of the epithelium, defined as EPI-ON transepithelial cross-linking (te-CXL)[26,30-33]. These procedures are based on the use of ocular penetration enhancers: substances that increase the trans-corneal passage of riboflavin, such as ethyl alcohol[34], mixtures of benzalkonium chloride[35-36], trometamol, EDTA[28,32] and local anesthetics[35]; the role of these substances was to cause partial or complete disruption of the epithelial barrier by damaging the desmosomal junctions, to allow riboflavin to spread into the corneal stroma[37].

We went from a mechanical EPI-OFF to a chemical one, with two immediate consequences: less effective removal with lower performance than the standard treatment and an increase in unwanted effects[17,34,38]. For these reasons, the EPI-ON treatments did not initially meet the hoped-for success, except for iontophoresis, in which electrodes are applied near the eyeball and a weak current is applied to the surface of the cornea which facilitates, during the phase of imbibition, the passage of a solution of positively charged riboflavin through the epithelium[39-40]. At present, there are long-term clinical follow-ups for iontophoresis, which show its effectiveness and safety[41-42].

Faster: Accelerated CXL At the same time, so-called accelerated UV treatment protocols have been developed to reduce UV irradiation times[43]. These procedures are based on Bunsen Roscoe's law of reciprocity[44], according to which the irradiation intensity is increased, up to 45 mW/cm^2, to reduce the time of the irradiation phase, keeping the total energy applied constant[43]. Various authors have expressed doubts about the safety of these protocols, both for possible damage related to the instantaneous UV intensity applied and for the rapid oxygen consumption that occurs[45-49].

The role of oxygen in CXL is still the subject of heated scientific debate. For some researchers, it is the third actor necessary for a clinically optimal procedure, together with riboflavin and ultraviolet radiation[50-52].

The reason for this position lies in the existence of two different photodegradation mechanisms of riboflavin: type I and type II[53-54]. Type I mechanism, which develops in hypoxic conditions or with low oxygen tension, consists of three subsequent reactions, where starting from the excited state of riboflavin (^{3}Rib*), anion radical (RibH*) is first formed, then riboflavin in reduced form (RibH$_2$) and, finally, oxidized riboflavin phosphate (RibOx) and hydrogen peroxide (H_2O_2) are obtained as final products, probably less effective in determining the formation of cross-linking bonds and potentially cytotoxic. In type II mechanism, which develops in an aerobic environment, ^{3}Rib* determines the formation of singlet oxygen ($^1O_2$), which is involved in the oxidation of substrate molecules contained in the corneal parenchyma and guarantees the possibility of forming cross-linking bonds in an effective, controlled, and safe manner[55]. The availability of oxygen began to gain importance following a series of *in vitro* experiments that used sodium azide as a singlet oxygen extinguisher and heavy water (deuterium oxide) to increase its half-life in the reaction environment[56-58]. Instead, several authors believe that the interaction between triplet riboflavin and stromal proteins plays a fundamental role in CXL[59-60], relegating oxygen to a subordinate role[61-63].

Considering the hypothesis of the importance of oxygen in CXL procedures to be true, the most plausible mechanism is that during continuous exposure to UV-A, the aerobic conditions are unable to persist for more than one minute of irradiation with consequent passage towards anaerobic conditions, *i.e.* towards the type I mechanism, with the production of (toxic) hydrogen peroxide[55,64]. These steps could justify the failure of accelerated CXL procedures that use high energies for short times (>20 mW/cm^2), resulting in rapid oxygen depletion that does not have time to re-diffuse at the stromal level[65-67]. Some experimental data demonstrate the futility of operating under positive oxygen tension to increase the success of CXL procedures[67].

Safety: Pulsed CXL The proposed clinical solution, to address these problems, is represented by the use of pulsed light: instead of administering the dosage of UV-A continuously, irradiation involves alternating phases on/off. These protocols have shown good efficacy and tolerability, at least as regards some parameters, mainly represented by the depth of the demarcation line (DL) and by the reduced toxicity[68-70]. The problem is that, even from a clinical point of view, the results collected are contradictory. The apparent greater safety of pulsed light procedures has been questioned by a work that identified a greater apoptotic effect due to pulsed light, compared to a high intensity protocol[71-72].

Standardized and Experimental Bases: Custom Fast Protocol A useful system to meet clinical needs and, at the same time, overcome the discordant information from the literature regarding the importance of oxygen in the CXL, is represented by the use of customized methods. If the problem is to be able to avoid the excessively rapid consumption of riboflavin and oxygen and the shifting of the reaction towards a type I mechanism, the answer could be to create modular irradiation systems, able to take into account both individual morphological characteristics of the patient[71-74], and of the photodegradation kinetics of riboflavin. These protocols are

based on experimental knowledge tested with mathematical models and provide for the use of low and adjustable intensities, dependent on pachymetry and corneal curvature, throughout the procedure, with very promising clinical results[75-76].

In CXL treatment, the riboflavin-soaked cornea is irradiated with UV-A at a frequency of 370 nm. The parameters of the Dresden protocol (intensity and duration of UV irradiation) were established heuristically considering total energy 5.4 J/cm^2, the maximum fluence value that the cornea can tolerate[77-79]. Through the Bunsen-Roscoe law of reciprocity (year 1839)[44] it is possible to calculate the intensity to be delivered and the time of exposition to UV ray by knowing the fluence received by the biological target (Figure 1).

Starting from the fluence of 5.4 J/cm^2, in the Dresden protocol the treatment parameters were set at 3 mW/cm^2 of UV-A intensity for a total time of 30 minutes. In all CXL protocols subsequent to the standard technique, such as trans-epithelial and accelerated, the theoretical basis always remains governed by the law of reciprocity and by 5.4 J/cm^2 of total energy. In summary, these are therefore modified Dresden protocols from which they do not differ in the fluence delivered (5.4 J/cm^2) but only in the intensity and duration of the irradiation time, also in these cases, established heuristically (Figure 2).

In addition, the Dresden protocol provides for the administration of drops of riboflavin solution at regular intervals, in order to attenuate as much intensity of the UV-A beam as possible that crosses the cornea and shield the internal structures of the eye (corneal endothelium, lens, retina)[17,80] (Figure 3).

As UV-A passes through the cornea, it is absorbed by its layers, by the riboflavin with which it is imbued, and by the photolysis products of riboflavin, including lumichrome. The intensity of the beam is reduced following the Lambert-Beer law[81] in a manner directly proportional to the length of the optical path, the concentration of substances that absorb ultraviolet radiation and their molar absorbance. This law allows to calculate the spatial distribution of the attenuation of the UV-A beam in the corneal thickness, once the distribution of riboflavin in the corneal tissue and the absorption characteristics of the tissues and of the riboflavin itself are known. The intensity of a UV-A beam crossing the cornea should not exceed the maximum threshold of 0.35 mW/cm^2, in order not to cause damage to the internal structures of the eye[82].

The limits of the standard technique, knowing the endothelial cytotoxic threshold of 0.35 mW/cm^2[83], are: 1) the rate of consumption of riboflavin is not known, 2) the mean intrastromal concentration of riboflavin penetrated into the cornea is unknown.

Not knowing the rate of consumption of riboflavin during CXL, nor its average amount in stroma at the end of imbibition, Wollensak *et al*[80] proposed the application of a

Figure 1 Equation of the Bunsen-Roscoe Law.

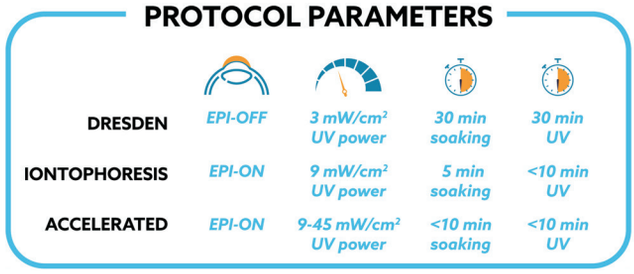

Figure 2 Different no customized protocols of corneal irradiation.

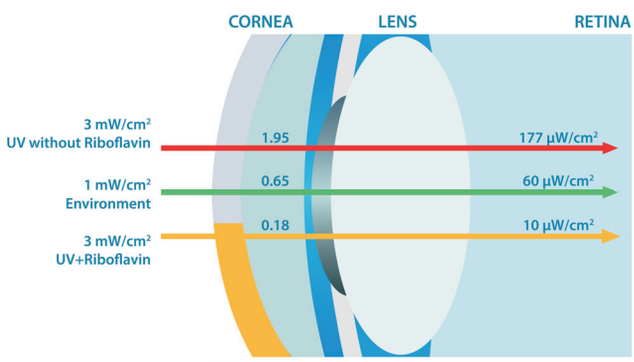

Figure 3 Intensity of the UV-A ray at the internal structures of the eye UV: Ultraviolet.

pre-corneal film of riboflavin to protect the endothelium during the Dresden treatment, at in order not to exceed this limit.

Subsequently, it was shown that the instillation of riboflavin solution during irradiation causes a barrier effect that reduces the passage of UV rays through the riboflavin precorneal film by up to 85 times and which fades with its dilution[80]. The addition of riboflavin, therefore, determines a greater variability of the results, as it does not allow to accurately establish the intensity of UV rays reaching the corneal stroma.

In fact, the Lambert-Beer law alone is not sufficient to explain the entire physical process that occurs in a CXL procedure as the UV intensity that passes through the cornea increases over time[84].

To try to mathematically describe the progressive increase in the intensity of the UV-A beam that crosses the cornea during the cross-linking treatment, it was hypothesized that this was due to the progressive diffusion of riboflavin in the corneal thickness according to Fick's Second Law[85]. More appropriately, the variations in riboflavin concentration have been attributed to the phenomenon of photolysis during the CXL procedure, which causes the reduction of riboflavin available in the cornea according to a time law of consumption[86].

Using the Lambert-Beer law in combination with the temporal law of the rate of consumption of riboflavin, and knowing the

average intrastromal concentration penetrated into the cornea, through high performance liquid chromatography (HPLC) tests, it was possible to build the mathematical model[87] that allowed the Protocol Custom Fast, unique and exclusive of its kind, to calculate the treatment parameters no longer in a heuristic way (Figure 4), as in all the other protocols.

Promoting Penetration: Vitamin E TPGS The HPLC analysis shows how in 15min the average accumulation in the cornea of riboflavin associated with vitamin E TPGS is equal to that obtained with a standard solution in EPI-OFF (Figure 5). Furthermore, it has been shown that the amount of "riboflavin corneal accumulation" varies between 15 and 40 μg[87-89].

This confirms that the TPGS vitamin E formulation of riboflavin solution is ideal for soaking corneas in EPI-ON, the reason why it was adopted in the Custom Fast protocol (CF-CXL).

The test showed that, at a certain point, for both solutions, an intrastromal saturation of riboflavin equal to 0.45 μg/cm^3 is reached[88]. This shows that: 1) There is no dependence on the concentration of the solution; 2) The saturation parameter, together with the discovery of the consumption rate of riboflavin, were the two reliable laboratory data on which the mathematical model of CF-CXL was built.

In addition to its role as a corneal penetration enhancer, vitamin E TPGS also plays an antioxidant action during treatment, protecting tissues from the photo-oxidative stress of UV irradiation. This action was confirmed by ultrastructural analysis with scanning electron microscopy (SEM) of cornea epithelia treated with CXL and formulation of riboflavin with vitamin E TPGS, which have greater morphological integrity than epithelia treated with CXL and standard solutions of riboflavin[88] (Figure 6).

The common cross-linking protocols regulate the treatment parameters based on the Bunsen-Roscoe Law and an incomplete interpretation of the Lambert-Beer law, as they do not take into account the complex biological, absorption, and kinetic processes that take place in the corneal tissue. After the absorption of riboflavin and its consumption under the photo-oxidative effects of UV-A irradiation[90]. Knowing the rate of consumption of riboflavin and its average amount present in the cornea allows you to correctly set the intensity and duration of the UV beam to be delivered. It is precisely in this scenario that the research project that led to the definition of the Custom Fast customized protocol was developed[91].

The data obtained experimentally, object of numerous publications, have therefore allowed to define the equation of the rate of consumption of riboflavin and its mean value present in the cornea. To calculate the rate of consumption of riboflavin, the first studies focused on the measurement of

$$\frac{\partial R_A(z,t)}{\partial t} = -\frac{\varepsilon \times P}{\gamma} \times I(z,t) \times R_A(z,t)$$

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Figure 4 Protocol Custom Fast: the mathematical model[87].

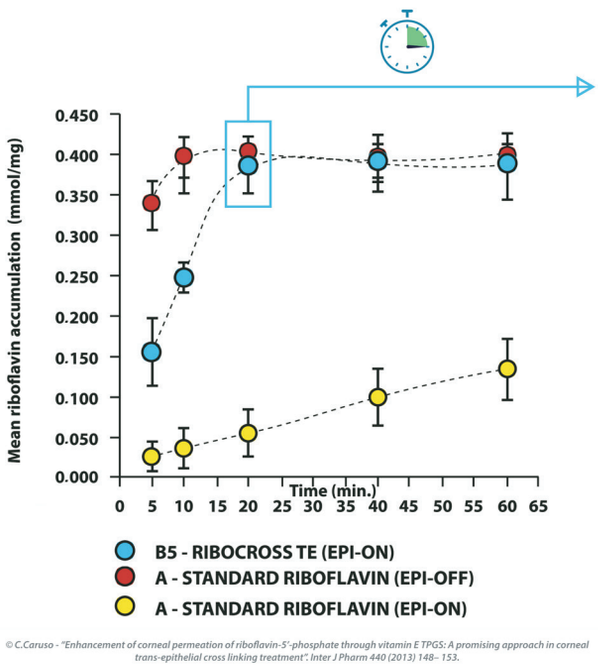

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Figure 5 Accumulation in the cornea of riboflavin solution in EPI-OFF (red), riboflavin solution in EPI-ON (yellow) and riboflavin with vitamin E TPGS in EPI-ON (blue)[88].

energy and intensity of UV-A passing through the cornea at different thicknesses, with and without epithelium, before and after imbibition with riboflavin, and the variation over time of the intensity of the post-corneal beam to derive the rate of consumption of riboflavin within the corneal stroma itself[75-76].

The spectroscopic results obtained show indeed that the intensities of UV-A emerging from the posterior surface of the cornea during standard CXL procedures are not constant over time, as suggested by the Lambert-Beer law, but vary in an increasing sense according to consumption. intra-tissue of riboflavin during irradiation and bridging between collagen lamellae. The average intensity of UV-A emerging from the posterior corneal surface, calculated immediately after the topical application of riboflavin with vitamin E TPGS, is 0.27 mW/cm^2, a value considered safe as it is within the limit of 0.35 mW/cm^2[76].

The safety limit is exceeded in the corneas tested after 10min of UV-A exposure[76]. These results are consistent with what has been reported by several authors[81,85]. Furthermore, the results obtained suggest that riboflavin oxidizes in a shorter time than the standard duration of irradiation, interrupting the shielding effect before the end of the 30min of treatment. Published studies have shown that riboflavin with vitamin E TPGS, penetrated into the cornea, completes the dual shielding

and oxidation effect for collagen crosslinking after 10min. This makes it possible to use a shorter irradiation time, capable of producing an effect similar to the Dresden protocol with lower UV-A intensities and lower risk of exposure of the corneal endothelium to UV-A insult[75,92].

The experimental results achieved were used to develop a mathematical model that would allow to relate precisely all the parameters involved in the CXL, leading to the concept of treatment customization.

Starting from the riboflavin consumption equation and the Lambert-Beer law, it was possible to formulate an algorithm (mathematical model) to calculate the UV-A intensity and fluence based on the pachymetry value of each individual cornea, to achieve a customized and non-heuristic protocol. Specifically, the algorithm uses the thinnest point of the cornea (thinnest point) and the dioptric value of the keratoconus apex (Kmax) to calculate the intensity, duration, and fluence to be delivered in the treatment[86].

To try to mathematically describe the progressive increase in the intensity of the UV-A beam that crosses the cornea during the cross-linking treatment, it was hypothesized that this was due to the progressive diffusion of riboflavin in the corneal thickness according to Fick's Second Law. More appropriately, the variations in riboflavin concentration have been attributed to the phenomenon of photolysis, which causes the reduction of riboflavin available in the cornea according to the temporal law of consumption discovered and identified thanks to the data obtained from the experiments performed and published[87].

Knowing the corneal thickness, the average amount of riboflavin penetrated, the average rate of consumption of riboflavin under irradiation and the endothelial cytotoxic limit (0.35 mW/cm^2), the mathematical model allows to calculate with precision the intensity and duration of the treatment of CXL[87] (Figure 7).

The graph represents the nomogram for the calculation of the model, which highlights the behavior of the individual parameters involved for the individual patient. In this way, a safe, fast, and customized cross-linking protocol is obtained (Figure 8).

The intensity and duration of the treatment are calculated based on the corneal thickness of the eye to be treated, thus making the method customized and not heuristic. This avoids exceeding the maximum endothelial toxicity threshold without having to continuously administer riboflavin during treatment[17,86-87] (Figure 9).

Why not choose high intensities and large diameters of the UV beam? It has been argued, as occurs in the so-called accelerated protocols, that according to the Bunsen-Roscoe law, the cross-linking treatment would produce the

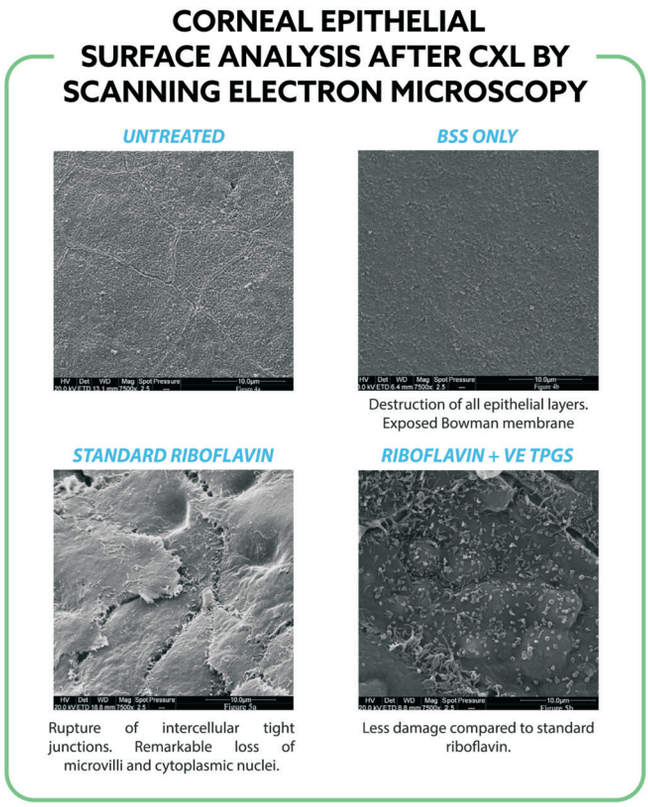

Figure 6 Corneal epithelial surface analysis (SEM) in untreated cornea and after UV-A treatment with different solutions[88] SEM: Scanning electron microscopy; UV: Ultraviolet.

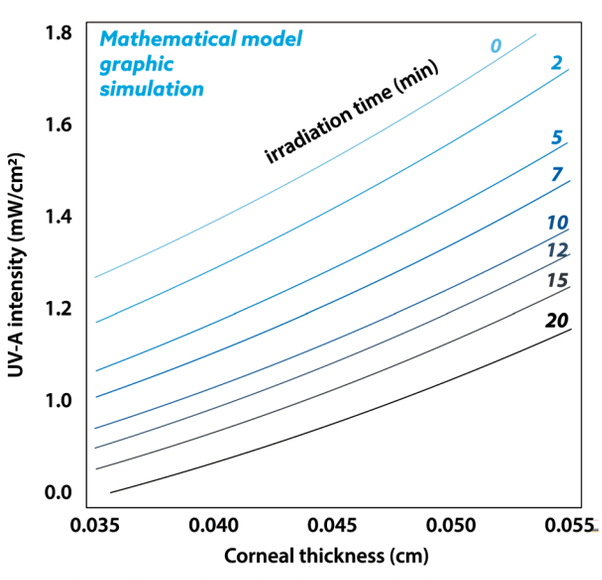

Figure 7 UV-A irradiation time and intensity based on corneal thickness and average rate of consumption of riboflavin[87] UV: Ultraviolet.

Figure 8 Custom Fast Protocol: 15min of EPI-ON soaking+customized irradiation (<3 mW/cm^2 for <15min).

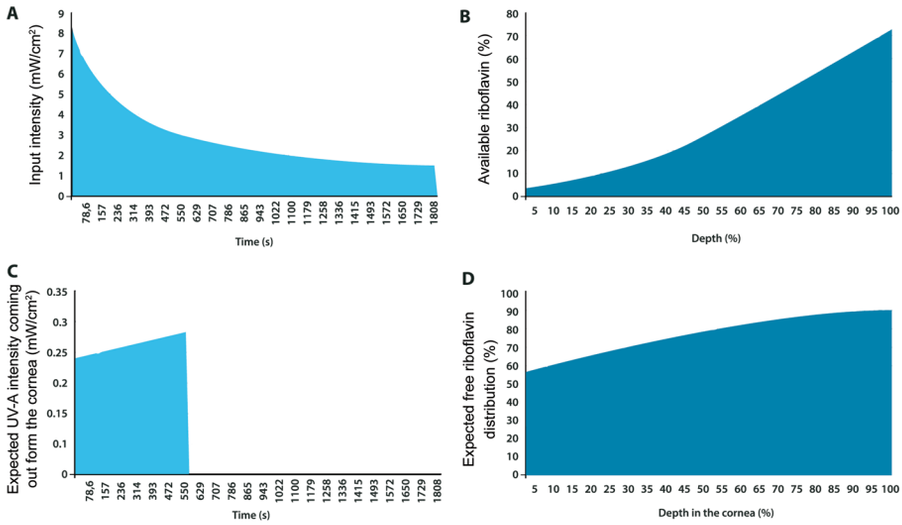

Figure 9 Parameters calculated on basis of the experimental results and the mathematical model.

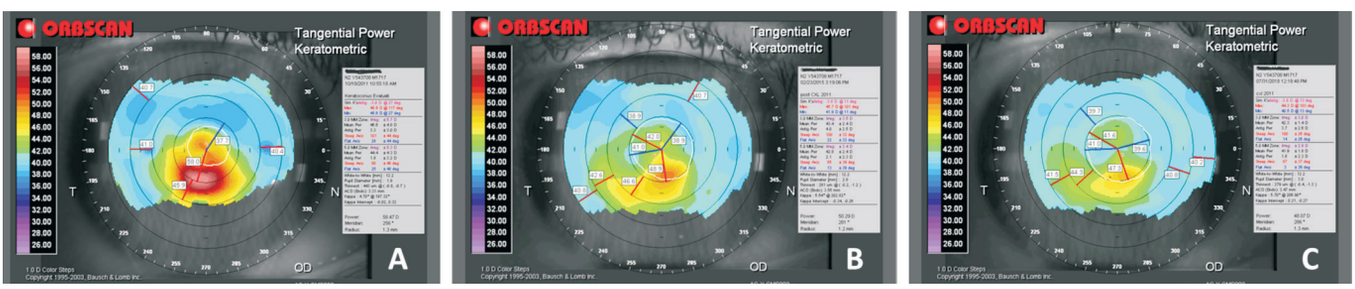

Figure 10 Right eye: topographic examination (Orbscan) of progressive keratoconus before (A), 4y (B), and 7y (C) after Custom Fast-CXL treatment CXL: Corneal collagen crosslinking.

same effects by increasing the intensity of the UV-A beam and proportionally reducing the duration of the treatment to keep the energy administered. Recent experiments have shown smaller topographic flattening and reduced interfibrillar spacing in the anterior 50 μm of the corneal stroma than the conventional CXL irradiation[90-91,93]. The *ex vivo* results of CXL performed in 100 porcine corneas with constant irradiation dose of 5.4 J/cm^2 and different intensities and illumination times, show that the Bunsen-Roscoe reciprocity law is only valid for illumination intensities up to 40 to 50 mW/cm^2 and illumination times of more than 2min[92]. A reduction in treatment time can be achieved by removing the superficial layer of riboflavin that remains in the tear film before starting irradiation[75].

Beam diameter: why reduce it? UV-A irradiation of the riboflavin-soaked cornea causes stiffening of the cornea but is not necessarily accompanied by a correction of the curvature. Also with the Custom Fast, it was proposed to reduce the diameter of the UV-A beam during the cross-linking treatment between 3-7 mm, focusing it only on the most curved part of the cornea. Mathematical considerations lead us to believe that localized stiffening only on the most curved part leads to a reduction in corneal curvature which can be observed as early as 1mo after cross-linking treatment[94-96].

Custom Fast-CXL *vs* Dresden Thanks to the maintenance of the endothelial safety threshold and to the perfect impregnation with its specific riboflavin RIBOCROSS te/RIBOFAST® (Fidia Farmaceutici S.p.A., Abano Terme, Italy), the Custom Fast-CXL allows to treat ectatic corneal pathologies with thicknesses below 400 μm.

The software of CF X-linker (SERVImed Industrial S.p.A., Naples, Italy), the device used for Custom Fast-CXL, provides the diameter of the UV-A beam profile which can vary from 3 to 7 mm depending on the characteristics and severity of the ectasia[75,96]. Compared to the standard cross-linking protocol, the Custom Fast-CXL guarantees the same stabilization effect over time as the ectatic disease, but also a faster visual rehabilitation[95] (Figure 10).

The main differences between the Dresden and Custom fast protocol are summarized in Table 1.

New Customized Protocols Several protocols defined as customized have been proposed in recent years, which should be more correctly named topoguided CXL (TG-CXL), as centering of the treatment is carried out on the apex of the cone according to the topographic corneal curvature, using uniform or personalized energy levels, based on the distance from the apex of the cone[97-101], in continuous or pulsed mode, with or oxygen supplementation[102]. On top of the cone, TG-CXL gives

Table 1 Parameters used by Custom-Fast and Dresden Protocols

Parameters	Custom-Fast (EPI-ON)	Dresden (EPI-OFF)
Corneal thickness	Considered	Not considered
Kmax	Considered	Not considered
Soaking time	15min	30min
Irradiation time	<15min	30min
UV-A intensity	Low/variable (1-3 mW/cm^2)	Standard (3 mW/cm^2)
Riboflavin consumption	Calculated	Not considered
Endothelial cytotoxic limit	Considered (0.35 mW/cm^2)	Uncertain
UV-A irradiation modality	Variable	Continuous
Diameter of UV-A beam	Variable (3-7 mm)	Standard (9 mm)
Fluence	Personalized (<2 J/cm^2)	Standard (5.4 J)

EPI-ON: Without removal of epithelium; EPI-OFF: Removal of epithelium; UV-A: Ultraviolet A.

similar biological effects as conventional EPI-OFF treatment (deep demarcation line, keratocytes activation, decrease of nerve density), inducing lower modifications on the surrounding area, resulting in significant reduction of Kmax, reduced astigmatism, and improved visual acuity[102]. Limits of these studies are short time of follow-up, usually one year.

Recently another emerging therapeutic paradigm of personalized medicine has been proposed: the theranostic method. The term refers to the simultaneous integration of therapy and diagnostics. A theranostic medical device is able to measure in real time the concentration of therapeutic molecules into the targeted tissue area and simultaneously treat it, with real-time evaluation of the effectiveness of the treatment[103-104]. Integrating theranostic technology with advanced UV-A device for CXL procedure it is possible to tailor the precise therapeutic dose of riboflavin and its photoactivation with UV-A light to the individual cornea[104-105]. The purpose of this procedure is to improve the predictability of the results, reducing the risks of adverse events. A UV-A theranostic medical device (C4V CHROMO4VIS sw 2.0, Regensight srl, Italy) has recently been made available for the treatment of keratoconus. The main components of the device include a UV-A light LED (365±10 nm), which emits a controlled power density for theranostic imaging and therapy, an RGB camera, which acquires the images emitted by the cornea when illuminated by UV-A light and a single board computer, which manages the correct functioning of the electro-optical components, processes the camera images and calculates two imaging biomarkers estimating the corneal riboflavin concentration and the treatment efficacy in real time during the intervention[105-107]. An ongoing randomized multicenter clinical trial (ARGO) in 50 patients aged between 18 and 40y with progressive keratoconus aims to validate the theranostic score by evaluating the change in the keratometric maximum point value at 1y after surgery, with either EPI-OFF or EPI-ON riboflavin/UV-A CXL protocols[108]. The treatment consists of two phases: the first phase, during the application of riboflavin, involves the photomediated measurement of the corneal concentration of riboflavin, providing the operator with an estimate of this parameter in real time. In the latter phase (UV-A light phototherapy), UV-A light is used for both quantitative imaging and therapy; in that time, the UV-A device calculates a theranostic score, which is related to the corneal stiffening effect induced by CXL; the score takes into account the dose of corneal riboflavin before the start of the UV-A phototherapy phase, the amount of riboflavin photodegraded by UV-A light therapy and the corneal thickness. Once the accuracy of the theranostic score in predicting CXL treatment efficacy is confirmed, the theranostic software module of the UV-A device will be fully activated and ready for assisting surgeons to tailor treatment of keratoconus to individual patients with the high est beneft/safety profile[104-108]. Primary results of the ARGO trial will give information to establish the safety and efficacy of this new customized method.

CONCLUSION

CXL has revolutionized the management of patients suffering from keratoconus, as it has introduced an original etiopathogenetic approach to the disease. Imbibition of the corneal stroma with riboflavin-based solutions and subsequent UV irradiation has been shown to be effective in stiffening the corneal structure by cross-linking and blocking or slowing the progression of the disease. The Dresden protocol, which provides for imbibition of the cornea for 30min after removal of the epithelium and subsequent irradiation for 30min with UV-A 370 nm rays with an intensity of 3 mW/cm^2, still represents the reference point to evaluate the efficacy and safety of other treatment methods. The effort to make the technique less invasive led to look for solutions that avoid the removal of the epithelium (EPI-ON technique), such as the use of enhancers, in particular vitamin E TPGS, or the use of an iontophoretic device, to promote the penetration of riboflavin through the epithelial barrier. At the same time, the need to make the procedure faster has led to an increase in the irradiation power, so as to reduce the times for the same

amount of energy administered; however, intensities higher than 10 mW/cm^2 pose, according to some authors, safety problems, for which a micropulse method of administration of UV has been proposed[109]. The main limitations of the Dresden protocol and of its variants were identified in the lack of standardization, also linked to the use of a riboflavin film by adding the product every 5min, capable of shielding up to 85 times the impact of UV on the cornea, and in the lack of graduation of the treatment based on the minimum thickness and individual characteristics of the cornea. These considerations lead to the conception of standardized and customized methods: the custom fast protocol, thanks to the solid experimental bases and the adoption of a mathematical model developed on the data obtained, appears safe and effective, as also demonstrated by the published clinical experiences[75,94-95].

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