

Title

## **The Meaning of the Demarcation Line after Riboflavin-UVA Corneal Collagen Crosslinking**

Authors

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Authors declare no financial interests

Nothing to disclose

No funding/support

## Abstract

Since at least two decades, corneal cross-linking by riboflavin and UV-A irradiation stands as an established treatment option of progressive corneal ectasia. More than ten years ago, the histological evaluation of animal corneas and later of human corneas by means of *in vivo* bio-microscopy and scanning laser confocal microscopy demonstrated in corneas treated with cross-linking procedure the occurrence of a demarcation line running almost parallel to the corneal surface. The feature is a direct consequence of the cross-linking-induced photo-oxidative modification of the stromal tissue due to riboflavin stromal concentration, light distribution within the corneal stroma, riboflavin activation, oxygen availability and diffusion in the corneal stroma, radicals release, and UV-A exposure time. In this study, the general role of the demarcation line observed after a corneal cross-linking process, in particular its mechanical relevance, clinical consequences, and other various findings in the numerous nowadays available modalities of this treatment, are reviewed objectively.

## Introduction

Riboflavin-UVA Cross-linking (CXL) represents an innovative therapy changing the paradigm of the “*conservative*” management of progressive Keratoconus (KC) and iatrogenic corneal ectasia.<sup>1-3</sup> By combined action of 0.1% Riboflavin (photosensitizing agent) and UV-A irradiation, this treatment induces release of singlet oxygen that photo-polymerizes stromal collagen, reduces the lytic effect of collagenase and increases corneal resistance to deformation,<sup>1</sup> counteracting certain major pathophysiological mechanisms of KC degeneration.<sup>4</sup> Long-term clinical studies have shown that CXL treatment with epithelium removal (Epi-Off) slows and in over 80% of cases blocks KC progression, with variable refractive improvement.<sup>5-9</sup> Standard Epi-Off CXL (S-CXL) reduced the need of corneal transplants in patients affected by progressive KC or secondary ectasia by 25-30% up to 50%.<sup>10,11</sup> According to unpublished data of the Veneto Italian Eye Bank the advent of CXL reduced the percentage of KC requiring a corneal transplant from 5% to 20%, in any case delaying the necessity of corneal transplant surgery of at least 5 years. Various modifications of S-CXL as originally described by Wollensak et al.<sup>1</sup> in 2003 have been proposed in the last fifteen years. The so-called demarcation line (DL) visible at the posterior edge of the cross-linked stroma has been used as a convenient tool for the assessment of the efficacy and safety of the new cross-linking procedures. Recently, the role of the DL as an indicator for cross-linking efficacy has been questioned, opinion which we do not support and consider to be unjustified.

## Demarcation Line in Epi-Off Standard Fluence CXL

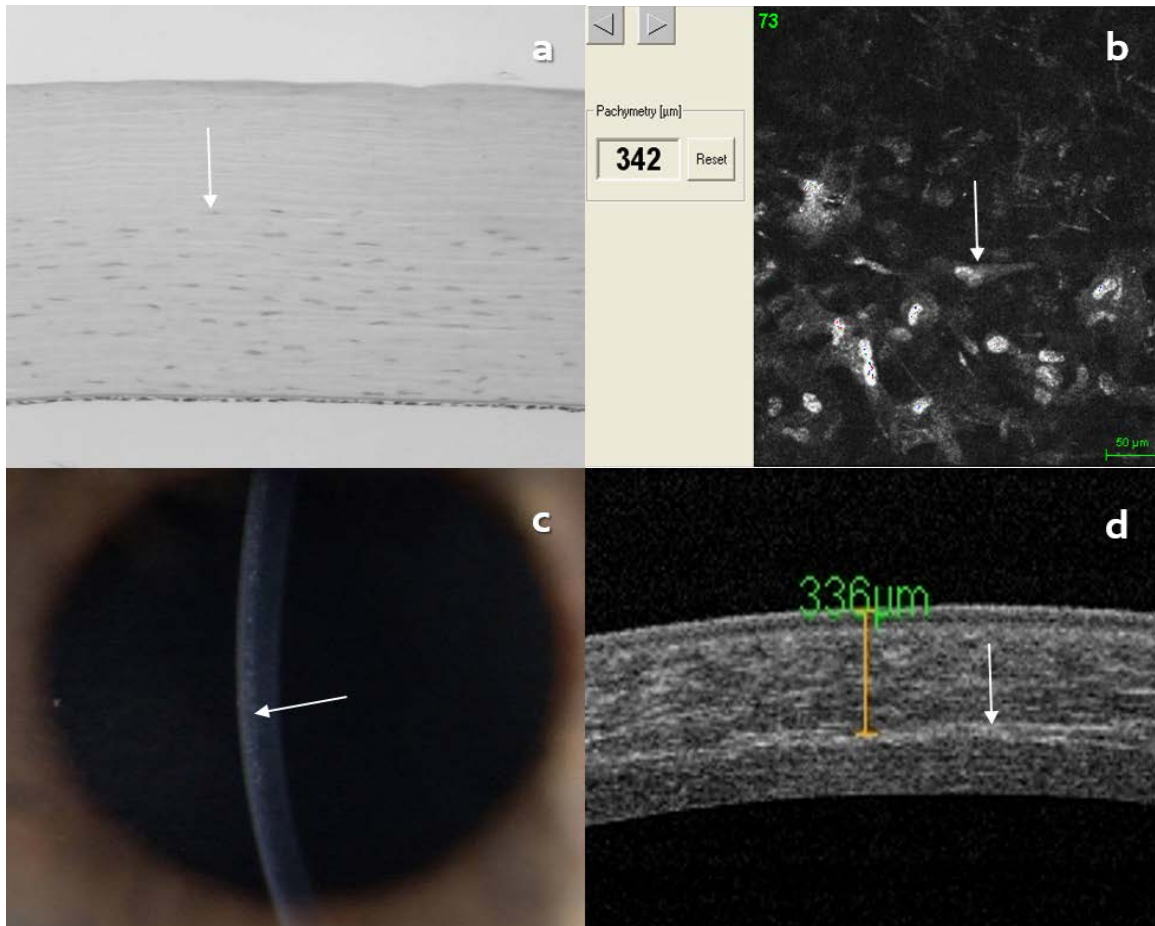
Since the beginning of CXL experience,<sup>1,2</sup> a sort of “*cutting edge*” between the cross-linked (CX-Linked) and not CX-Linked corneal stroma was demonstrated by histological evaluation in rabbit corneas by Wollensak et al.<sup>12</sup> The boundary was first established in post-CXL *in vivo* animal models corneas by a clear confirmation of keratocytes disappearance

(apoptosis) due to photo-oxidative damage driven by reactive oxygen species (ROS), particularly singlet oxygen, delivered during the CXL process, being expression of the “*correct interaction*” between UV-A at 370 nm wavelength, activated 0.1% Riboflavin molecules, Oxygen and Collagen-Proteoglycans Complex (CPC) of the corneal stromal extracellular matrix (ECM).<sup>13</sup> The pivotal histological studies by Wollensak et al<sup>12,13</sup> described a clear separation between the anterior-mid stroma with lacunar edema and apoptosis of keratocytes and the posterior stroma regularly populated by cells nuclei at an average depth of 300  $\mu\text{m}$  according to the S-CXL parameters set in the Dresden protocol.<sup>1</sup> **Figure 1 a.** The stromal depth of the keratocyte apoptosis was clearly correlated with the applied surface UV-A irradiance,<sup>14</sup> **Figure 2.**

Seiler et al<sup>15</sup> and Mazzotta et al<sup>16,17,18</sup> demonstrated, for the first time in vivo in humans by means of biomicroscopy and *In Vivo* Scanning Laser Confocal microscopy (IVCM), respectively, in the first 15 days after S-CXL protocol, the presence of a clear transition between an edematous hypo-reflective anterior mid-stroma depleted of keratocytes nuclei and a normo-reflective posterior corneal stroma without edema and regularly populated by cells, **Figure 1 b.** Usually the DL gets shallower in the periphery and approaches the corneal surface because of the UVA beam profile.<sup>19</sup>

The Vertical Transition Area (VTA) between 270 and 330  $\mu\text{m}$  of stromal depth described by Mazzotta at IVCM in humans<sup>18</sup> was exactly correspondent to the keratocytes apoptosis depth described by Wollensak et al<sup>12</sup> approximately at 300  $\mu\text{m}$  in *in vivo* rabbit studies, documenting stromal cell apoptosis and lacunar edema with corneal reflectivity changes. IVCM pivotal studies<sup>16-18</sup> after S-CXL documented that the microstructural changes were initially characterized by the presence of a “lacunar” stromal edema associated with keratocytes loss, disappearance of sub-epithelial and anterior-mid stromal nerve fibres. Moreover, bright micro-particles described as “*apoptotic bodies*” were also found in a dense network of hyper-reflective “trabecular patterned stroma” extracellular matrix (ECM) surrounding edematous lacunae.<sup>16</sup> Later, between the third and the sixth month after S-CXL, “*needle-shaped*” hyper-reflective micro-bands were documented at IVCM analysis with progressive disappearance of corneal edema, associated with gradual keratocytes repopulation, sub-epithelial and stromal nerves regeneration with increasing in ECM reflectivity.

The first clinical observation of a “demarcation line” (DL) was reported by Seiler et al at corneal bio-microscopy<sup>15</sup> as showed in **Figure 1 c.** Authors observed this clinical feature at slit lamp examination of the cornea two weeks after a CXL procedure, representing a “*direct clinical mark*”, additionally to corneal topography, of the morphological changes induced by CXL to the cornea.<sup>15</sup>



**Figure 1. Demarcation Line 15 days after standard irradiance  $3\text{mW}/\text{cm}^2$  and  $5.4\text{ J}/\text{cm}^2$  of energy dose for 30 minutes of UV-A exposure (Dresden Protocol): histology (a white arrow), in vivo confocal microscopy (b white arrow), biomicroscopy (c white arrow) and corneal OCT (d white arrow). All the morphological (histological, confocal, biomicroscopic and OCT) studies agreed that the stromal DL after CXL represents the “boundary” between CX-linked and untreated cornea, corresponding to penetration of CXL-induced keratocytes apoptosis depth at ultrastructural level, to keratocytes apoptosis and stromal edema at in vivo confocal microscopy (IVCM) microstructural level, to changes in the refraction index and optical reflection properties of treated versus untreated corneal stroma at biomicroscopic examination, to optical interface line due to reflectivity changes, different tissue density and light-scattering at OCT.**

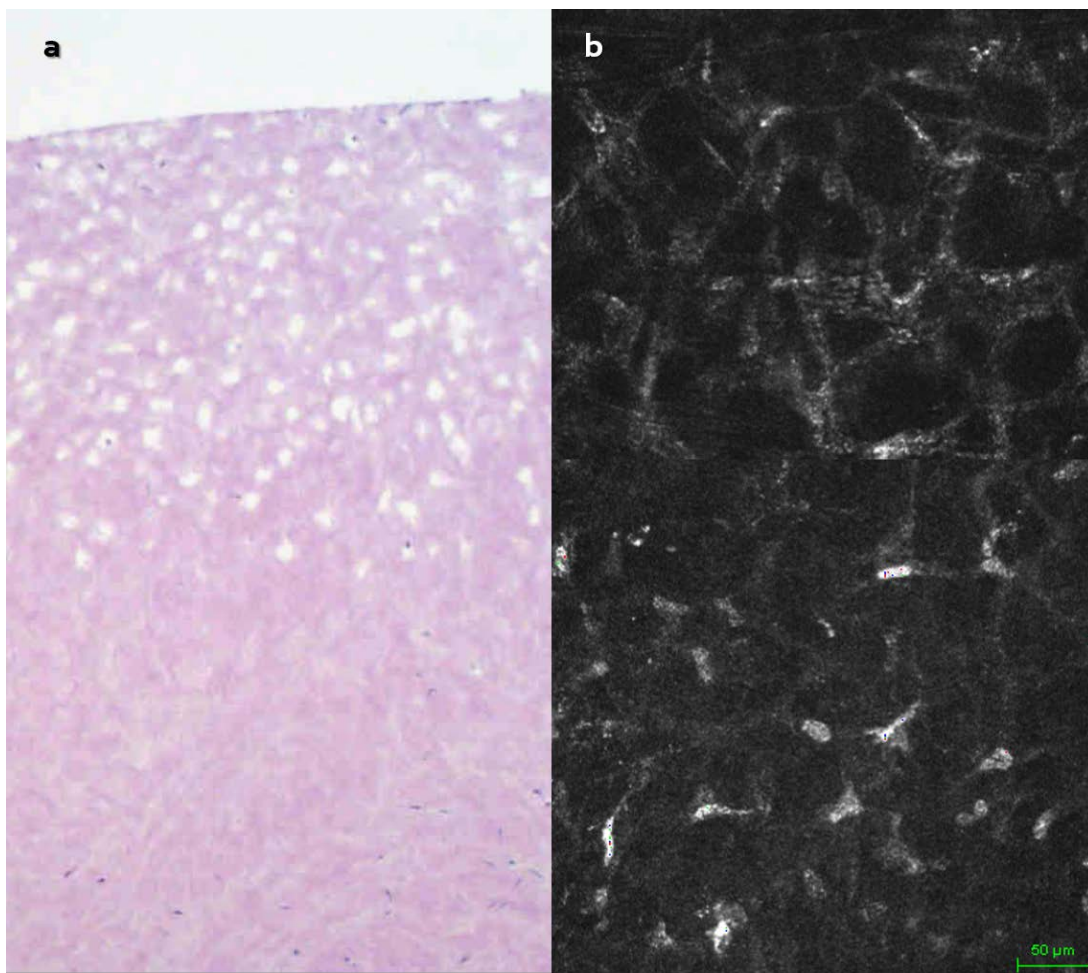
Early IVCM analysis after S-CXL procedure<sup>16</sup> confirmed in vivo in humans that the cytotoxic effect of corneal collagen CXL with the standard irradiance of  $3\text{mW}/\text{cm}^2$  and  $5.4\text{ J}/\text{cm}^2$  of energy dose “fluence” delivered for 30 minutes of UV-A exposure (Dresden Protocol) was confined in the upper  $300\text{ }\mu\text{m}$  of the corneal stroma (about  $350\text{ }\mu\text{m}$  from the epithelial surface). The keratocytes apoptosis observed in in vivo confocal scans confirmed the preclinical in vivo rabbit studies<sup>12</sup> showing that apoptosis was associated with post-CXL stromal edema<sup>13,14</sup> persisting from 1 to 3 months and reducing progressively its intensity in time by application of topical steroid therapy.<sup>17,18,20</sup>

Mazzotta et al<sup>16,17</sup> described *in vivo* the CXL-induced microstructural changes characterized by the presence of a “lacunar” or “spongy” stromal edema associated with keratocytes loss, disappearance of sub-epithelial, and anterior-mid stromal nerve fibres. Bright micro-particles

“apoptotic bodies” were also found in a dense network of hyper-reflective “trabecular patterned stroma” ECM. Moreover “*needle-shaped*” hyper-reflective micro-bands were documented at IVCN analysis after S-CXL. A clear transition between photo-oxidative damage and not CX-Linked stroma without edema, with nerves and regularly populated by vital cells nuclei was documented.<sup>20</sup>

The postoperative microstructural transition constituted a DL between CX-Linked and not CX-Linked stroma as an expression of the *CXL penetration* was correlated with its physical and biochemical impact leading to its well-known biomechanical changes.<sup>20</sup>

There was a “*perfect match*” between the first histological observation by Wollensak et al<sup>12</sup>, the first clinical DL documented by Seiler et al<sup>15</sup> and the first IVCN studies by Mazzotta et al<sup>16</sup>, that were also confirmed by corneal optical coherence tomography (OCT) records by Kymionis et al<sup>21</sup>, **Figure 1 d**.



**Figure 2. Comparative view of the lacunar edema and keratocytes apoptosis in the first two weeks after S-CXL by means of optical microscopy (a) of a rabbit Cornea (Wollensak’s first observation)<sup>12,13</sup> and IVCN (b) of human cornea (Mazzotta’s first observation).<sup>16,17</sup>**

The authors of the morphological (histological, biomicroscopic confocal, and OCT scans<sup>21</sup>) studies agreed that the meaning of stromal DL after CXL represented a “*boundary line*” between CX-linked and untreated cornea, corresponding to penetration of CXL-induced keratocytes apoptosis depth at ultrastructural level, to keratocytes apoptosis and stromal

edema at in vivo confocal microscopy (IVCM) microstructural level, to changes in the refraction index and optical reflection properties of treated versus untreated corneal stroma at biomicroscopic examination, to optical interface line due to reflectivity changes, different tissue density and light-scattering at OCT.<sup>12,15,16,21</sup> Interestingly, a similar DL has been reported after chemical burns, in which case it is also associated to keratocyte damage and stromal edema dependent on the penetration depth of the chemical burn.<sup>22</sup>

Biomicroscopic and corneal OCT identification of the DL, represent simple and effective non-invasive clinical-instrumental options easily to monitor the “*effective depth*” of Epi-Off CXL treatment in the daily clinical practice, more accurate than Scheimpflug cameras,<sup>23</sup> while IVCM is the most powerful diagnostic tool identifying the penetration of CXL treatment directly *in vivo* at cellular level.<sup>20</sup>

The DL is undoubtedly the “*morphological sign*” of CXL stromal penetration generated by the CXL-induced photo-oxidative damage itself (e.g. keratocytes apoptosis and corneal edema), it is visible at slit lamp due to changes in tissue reflectivity and/or different refraction index of the CX-Linked stroma vs not CX-Linked stroma, it is recordable at AS-SD OCT as typical expression of the post-CXL stromal light-scattering induced by the different tissue density.

The detection of DL represents also a “*therapeutic milestone*” in terms of safety, demonstrating that S-CXL spared the corneal endothelium, and efficacy, showing that the biomechanical effects and increase in tissue stiffness were mainly confined in the anterior two-thirds of the corneal stromal layer.<sup>18,21,22</sup>

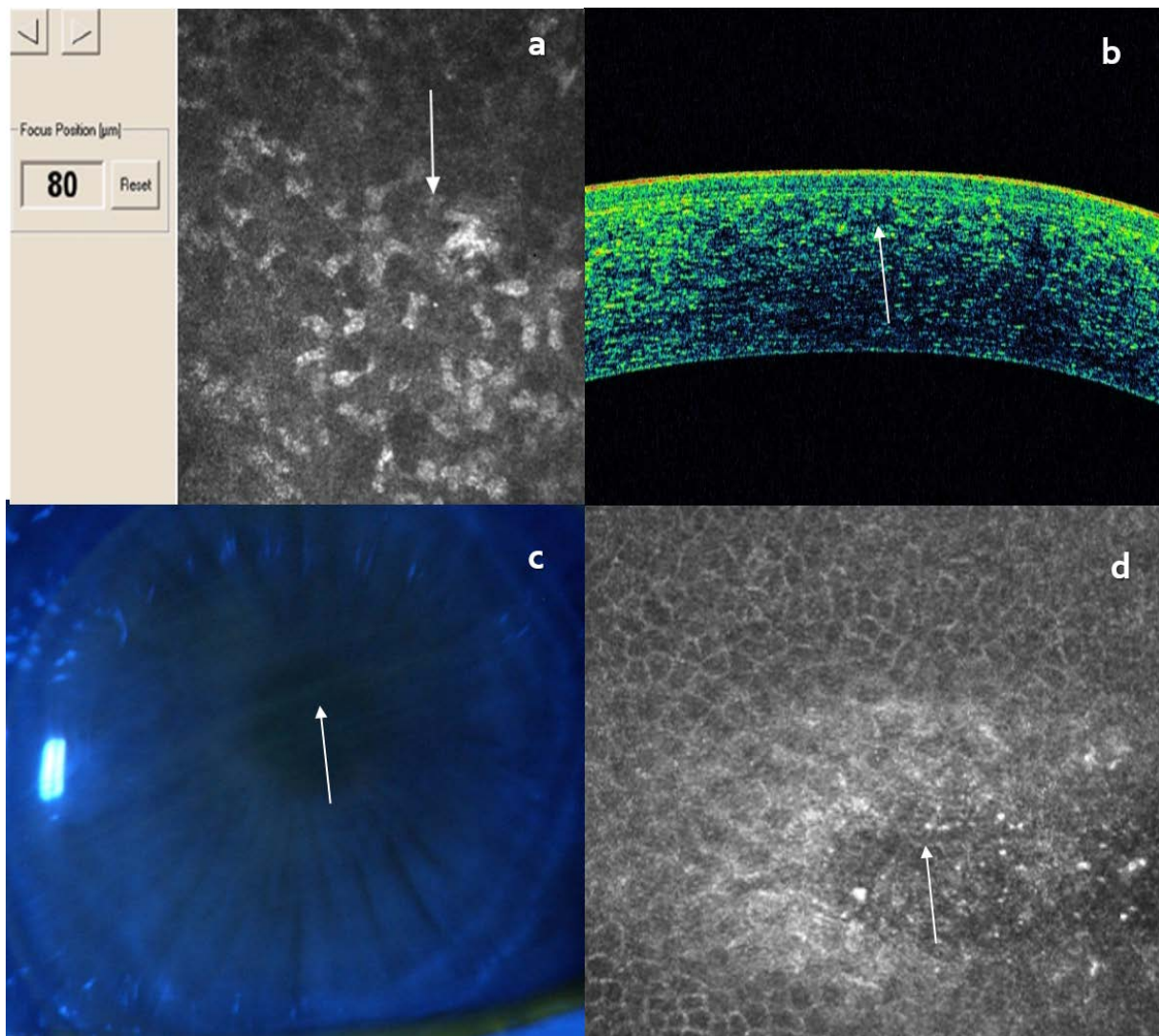
## **The Demarcation Line in Trans-Epithelial Standard Fluence CXL**

Various laboratory studies<sup>24-29</sup> confirmed the necessity of epithelium removal to favour sufficient homogeneous concentration of Riboflavin into the stroma and thus to guarantee the efficacy and safety of the CXL method.<sup>25,26,27</sup> Laboratory studies by Wollensak et al<sup>28</sup> on Trans-Epithelial Crosslinking (TE-CXL) have shown 20% of the biomechanical efficacy of the S-CXL method involving epithelial removal (e.g. 80% less efficient). Scarcelli et al<sup>29</sup> Brillouin microscopy studies also documented that after TE-CXL the biomechanical stiffening of the corneal stroma was superficial and 70% less than Epi-Off S-CXL. The penetration of the (hydrophobic) corneal epithelium by riboflavin (a high molecular-weight hydrophilic substance)<sup>25</sup> was “*facilitated*” by different compounds that alter the epithelial barrier integrity: preservatives (benzalkonium chloride), surfactants and amino-alcohols such as trometamol (TRIS) and anaesthetics like tetracaine.<sup>30,31</sup> Nevertheless, the presence of epithelium *in situ* represents a physical barrier for both Riboflavin and UV-A<sup>25,26</sup> inducing a partial penetration, that for riboflavin results *in vivo* at 1/4 of the standard riboflavin concentration achieved after passive diffusion in Epi-Off S-CXL<sup>32</sup> and for the UV-A a 30% photo-absorption according to photobiology measurements using the standard 370 nm wavelength.<sup>33</sup> Moreover, an additional main limitation of the presence of epithelium *in situ* is oxygen consumption, estimated to be at least of 40% of the total corneal oxygen consumption,<sup>34,35</sup> limiting its diffusion and concentration, thus reducing the biomechanical efficacy of CXL.<sup>36</sup>

Caporossi et al<sup>37,38</sup> proved as a first in IVCM studies after TE-CXL with enhanced Riboflavin solutions such as Riboflavin 0.1% plus Dextran 15%, EDTA and TRIS (Ricrolin TE®, Sooft, Montegiorgio, Italy) at 3mw/cm<sup>2</sup> for 30 minutes, *in vivo* a superficial impact of the treatment showing that apoptosis of keratocytes after TE-CXL was superficial, variable and unevenly distributed in the anterior sub-Bowman corneal stroma.<sup>35</sup> The typical rarefaction of cell nuclei mixed with lacunar edema found in S-CXL was often inhomogeneous, moreover the maximum depth of the apoptotic effect was recorded at maximum  $100 \pm 20 \mu\text{m}$  depth measured from the epithelial surface, **Figure 3 a**. OCT after TE-CXL detected no DL in the majority of cases or a shallower and inhomogeneous DL in 20% of patients, **Figure 3 b**.

A systematic IVCM review analysis after CXL<sup>20</sup> documented that the most significant aspect differentiating TE-CXL and Epi-Off S-CXL on the qualitative confocal microstructural plane was that after TE-CXL the apoptosis of stromal keratocytes *in vivo* in humans was superficial, variable and unevenly distributed in the anterior stroma under the Bowman's lamina with a maximum penetration of about  $100 \mu\text{m}$  (measured from the surface of corneal epithelium), whereas after Epi-Off S-CXL it was deeper ( $300 \mu\text{m}$  under the Bowman's lamina) and more homogeneous. According to Mazzotta et al<sup>20</sup> the depth of keratocytes apoptosis correlates linearly with CXL penetration DL depth and the biomechanical effect of CXL. Moreover, TE-CXL leads to transitory alteration of corneal epithelial cells as showed in **Figure 3**. The toxic effects on the epithelium are related to chemical toxicity of enhanced Riboflavin solutions and the associated UV-A photodynamic damage itself causing a diffuse superficial punctate keratitis in the first months after treatment as showed in **Figure 3 c**, with variable discomfort for the patient induced by epithelial-cells apoptosis **Figure 3 d**.





**Figure 3: IVCM (a) and OCT (b) after TE-CXL. IVCM after TE-CXL with chemically enhanced Riboflavin solutions showing that apoptosis of keratocytes is superficial, variable and unevenly distributed in the anterior sub-Bowman corneal stroma with a maximum depth of the apoptotic effect recorded at  $100 \pm 20 \mu\text{m}$  depth measured from the epithelial surface (a white arrow). OCT after TE-CXL detected no clear optical DL in the majority of cases or a shallower and poorly visible DL in 20% of patients with inhomogeneous increased reflectivity (b white arrow). Diffuse superficial punctate or striate keratitis in the first months after treatment was documented at biomicroscopy (c white arrow) and basal corneal epithelium apoptosis at IVCM (d white arrow).**

The TE-CXL largely showed unsatisfactory mid to long-term clinical results.<sup>30,31,38,39</sup> Leccisotti et al<sup>30</sup> documented a limited effect of TE-CXL compared with S-CXL with epithelium removal. Koppen et al<sup>31</sup> in a cohort study evaluating the efficacy of TE-CXL by using proparacaine drops 0.5% plus preserved with BAC 0.005% showed that despite no complications or haze, the TE-CXL was not effective in stabilizing progressive keratoconus, documenting a statistically significant continuous maximum K increasing and thinnest point decreasing (i.e. KC progression) throughout the study. Caporossi et al<sup>38</sup> reported an unacceptable 50% of retreatments 24-months after TE-CXL in paediatric patients that required an additional S-CXL with epithelium removal.



In a recent prospective, interventional multicenter cohort study, assessing the efficacy of an enhanced Riboflavin solution containing benzalkonium chloride 0.01% for TE-CXL, Gatzoufas et al<sup>39</sup> confirmed the clinical inefficacy of the of TE-CXL documenting that K max, uncorrected and corrected distance visual acuity did not change significantly after 12-months follow-up and a progression of KC (defined by an increase in K max greater than 1.00 dioptre) occurring in 46% of treated eyes. Moreover, in analogy with IVCN studies, marked punctate corneal epitheliopathy/loose epithelium were observed in 23% of the patients in the immediate postoperative period. Even if no adverse events (corneal infection, sterile infiltrates, or haze) were observed after this procedure, the 46% of KC progression was disconcerting.<sup>39</sup>

The factors leading to TE-CXL high percentage of failure ranging from 46% to 100%<sup>31,38,39</sup> in a follow-up between 12 and 24 months are not correlated to its refractive impact but reasonably (if not obviously) to its incapacity to sufficiently penetrate in the stroma<sup>25</sup> allowing a consistent amount of crosslinks distribution, sufficient and deep crosslinks saturation thus stabilizing KC progression in the long-term follow-up like the S-CXL. In the order of importance there are three main limiting factors in the TE-CXL low efficacy: the UV-A photo-absorption by antioxidant systems of epithelium,<sup>33</sup> the oxygen consumption by the epithelium (tenfold higher than stroma),<sup>34,35</sup> the halved and inhomogeneous intra-stromal riboflavin concentration<sup>32</sup> due to the uneven epithelial barrier alteration provided by all the chemically enhanced riboflavin solutions available on the market.<sup>38,39</sup> Due to its marginal impact in the anterior sub-Bowman corneal stroma, the above mentioned causes explain why after TE-CXL the DL is invisible in the majority of patients, shallower and poorly visible as documented by IVCN keratocytes apoptosis confined in the anterior 100 µm of corneal stroma and OCT.<sup>20</sup> In this contest, a correlation between DL depth and failure rate of TE-CXL remains unquestionable.

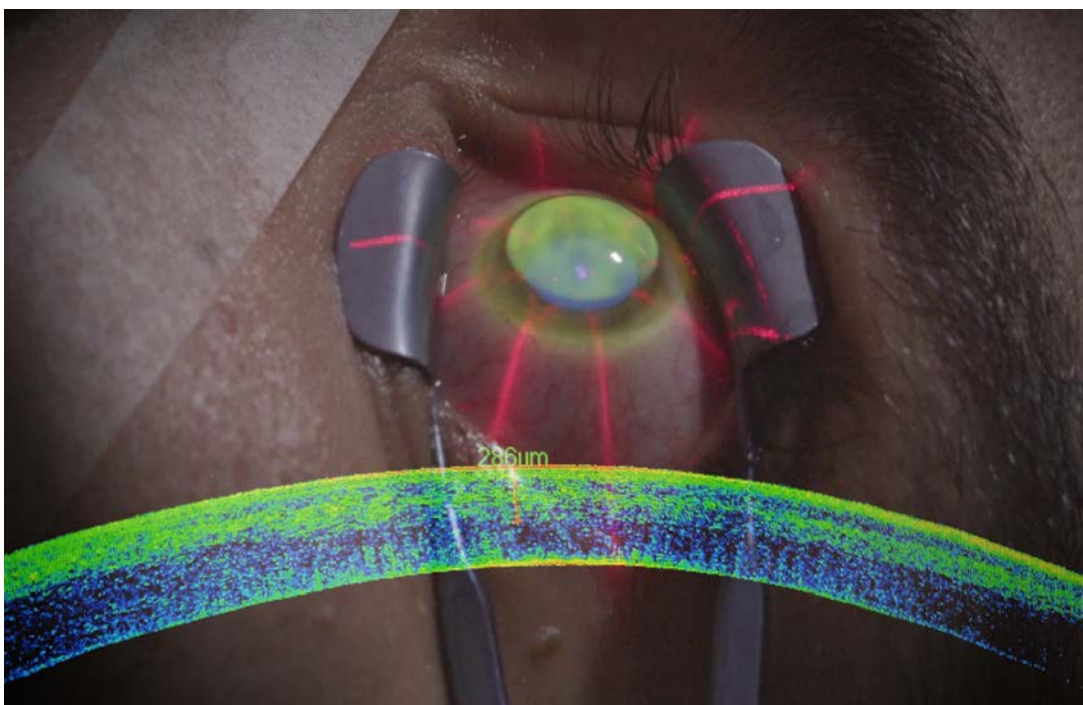
In order to overcome the limitations of the TE-CXL techniques based on chemical disruption and uneven alteration of the epithelial barrier, an electricity-assisted methodology of riboflavin transport with epithelium in situ, so called Iontophoresis-assisted CXL (I-CXL), was developed with promising results with respect to standard CXL.<sup>40-46</sup> The purposes of Epi-On CXL remain the prevention of pain, risk of infectious keratitis and wound healing complications related to epithelial removal, with faster recovery and reduced glare disability, thus avoiding the necessity to perform CXL necessarily in an operating theatre.

Early clinical data on I-CXL have shown an increased permeation of the riboflavin into the stroma compared to previously mentioned trans-epithelial techniques.<sup>32</sup> Clinical data reported in literature showed the efficacy of I-CXL in stabilizing KC in the short term follow-up.<sup>40-42</sup> The 24-months follow-up evaluation concluded that I-CXL halted progression of keratoconus better than the pharmacological TE-CXL but less efficiently than the standard Epi-Off CXL.<sup>43,44</sup> Nevertheless, the DL assessed by OCT and IVCN after I-CXL was visible in less than 50% of the cases and it was more superficial (150-200µm measured with epithelium) than the one of the traditional procedure.<sup>45</sup>

However, the percentage of DL observation in I-CXL is twofold the one in TE-CXL (50% instead of 25%) as well as the penetration of the photo-oxidative effect (200 instead of 100µm) even though non homogeneous and still inferior to S-CXL.<sup>44,45</sup>

In this setting, Vinciguerra et al<sup>46</sup> removed also the epithelium and after I-CXL imbibition improved the efficacy of this CXL technique in both functional and DL detection, regrettably losing the overall advantages of the Epi-On CXL: less pain, reduced corneal infectious risk and CXL-wound related complications.

Mazzotta et al<sup>45</sup> recently published a new Iontophoresis protocol called Enhanced Fluence Pulsed-light Iontophoresis (EF-ICXL) showing promising 12-months follow-up reduced maximum keratometry, corneal surface asymmetry indices and higher-order aberrations. This new protocol improved the visibility of DL in over 80% at  $285 \pm 20 \mu\text{m}$  depth on average, increased the penetration of photo-oxidative damage improving the photochemical kinetic of the original I-CXL technique by increasing of 30% the treatment fluence (from  $5.4\text{J}/\text{cm}^2$  to  $7\text{J}/\text{cm}^2$ ) compensating the UV-A energy photo-attenuation provided by the corneal epithelium and Bowman's layer antioxidants systems, **Figure 3**.



**Figure 3. Demarcation Line after Mazzotta's Enhanced Fluence Pulsed Light Iontophoresis (EF I-CXL). Increasing of 30% the treatment fluence (from  $5.4\text{J}/\text{cm}^2$  to  $7\text{J}/\text{cm}^2$ ) thus compensating the UV-A energy photo-attenuation provided by the corneal epithelium and Bowman's layer antioxidants system and adding the pulsed light partially compensating the 40% estimated oxygen consumption by the corneal epithelium in situ, the DL is detectable in over 80% at  $280 \pm 20 \mu\text{m}$  depth on average, increasing the penetration of photo-oxidative damage improving the photochemical kinetic of the original I-CXL technique.**

The variability and unpredictability of collagen and ECM collagen-proteoglycans redistribution after CXL may partially explain the non-linear correlation between DL depth and functional results.<sup>47</sup> Indeed, there is no linear correlation between the depth of DL and the functional results after CXL but there are incontrovertible evidences that the best postoperative clinical response in the sense of long-term ectasia stabilization was statistically and practically achieved after S-CXL with epithelium removal where the DL was deeper and detectable in over 90% of patients.<sup>48</sup> Moreover, though the CXL is not a refractive procedure

but it has long-acting unpredictable refractive impact on the cornea, the treatment's goal is the stiffening of the corneal structure achieving a long-term, possibly irreversible biomechanical stabilization of the progressive ectatic diseases. The fundamental ex vivo and in vivo CXL studies clearly demonstrated that the best biomechanical stability can be achieved if the photo-oxidative process includes a sufficient volume of corneal stroma of at least 2/3 of the baseline stromal thickness.<sup>48-50</sup>

### **The Demarcation Line in Standard Fluence A CXL Protocols**

Since the Epi-Off S-CXL procedure required long treatment time (1 hour approximately)<sup>24</sup>, accelerated crosslinking (A-CXL) protocols have been proposed to shorten the duration of the procedure improving patient's comfort.<sup>51-63</sup>

According to equal dose principles stated in the BunsenRoscoe's Law of reciprocity<sup>64</sup>, by setting the UV-A power at  $9\text{mW/cm}^2 \times 10\text{ min}$ ,  $30\text{mW/cm}^2 \times 3\text{ min}$ ,  $18\text{mW/cm}^2 \times 5\text{ min}$ ,  $45\text{mW/cm}^2 \times 2\text{ min}$  while maintaining a constant fluence of  $5.4\text{J/cm}^2$  the same photochemical effect as the conventional Dresden protocol at  $3\text{mW/cm}^2$  for 30 min could be theoretically achievable.<sup>65</sup>

As an adjunctive enhancement, facilitating the CXL photodynamic reaction in A-CXL, recent laboratory studies by Kamaev et al<sup>51</sup> highlighted the importance of intraoperative oxygen diffusion into the corneal stroma demonstrating that by pulsing the ultraviolet-A (UV-A) light radiation light (1 second ON/1 second OFF), CXL efficiency may be improved allowing partial oxygen re-diffusion during the UV-OFF pauses. This kinetic mechanism was also described as "*dark phase amplification*".

Touboul et al<sup>66</sup> demonstrated that by using a continuous light UV-A exposure in A-CXL with  $30\text{mW/cm}^2$  the average DL depth was found at  $150 \pm 20\ \mu\text{m}$ . Mazzotta et al<sup>67</sup> confirmed this finding in vivo by means of IVCN after  $30\text{mW/cm}^2$  continuous light irradiation A-CXL, **Figure 4 a - b**, and documented for the first time that fractionating the UV-A exposure by pulsing the light (1 second on/1 second off) an enhancement of CXL penetration can be achievable increasing the depth of DL by a mean of  $50 \pm 20\ \mu\text{m}$  of depth as documented by corneal OCT and IVCN photo-oxidative keratocytes damage, **Figure 4 c - d**.

This finding was also confirmed by Moramarco et al<sup>68</sup> and Peyman et al<sup>69</sup> evaluating corneal OCT scans in the same A-CXL protocol. These results proved the consistency of the kinetic models provided by Kamaev et al<sup>51</sup> demonstrating that by pulsing the UV effectively increases intraoperative oxygen diffusion and the penetration of CXL apoptotic effect reasonably due to the enhanced amount of singlet oxygen formation.

Jiang et al<sup>70</sup> documented that the DL depth in the pulsed light  $30\text{mW/cm}^2$  A-CXL group was  $201 \pm 27\ \mu\text{m}$  at 1 month postoperatively confirming Mazzotta's first observation<sup>67</sup> as showed in **Figure 4 c - d**. The study revealed keratocyte apoptosis and stromal edema at 1 month postoperatively, which gradually recovered towards the normal status with no changes in the posterior stroma and endothelium. Pulsed light A-CXL<sup>71</sup> was safe and effective procedure in stabilizing the progression of keratoconus and by comparing the S-CXL, where the DL depth was  $284.94 \pm 33.29\ \mu\text{m}$ , authors obtained more effective visual and topographic outcomes than

with pulsed light 30mW/cm<sup>2</sup> A-CXL group. A correlation between DL depth and clinical efficacy was documented.<sup>70,71</sup> The pulsed light A-CXL ensured a shorter treatment time, increased the average penetration of the DL from 150 μm of the continuous light to 200 μm and reduced postoperative microstructural damage thus limiting stromal wound healing reactivity.<sup>67,70,71</sup>

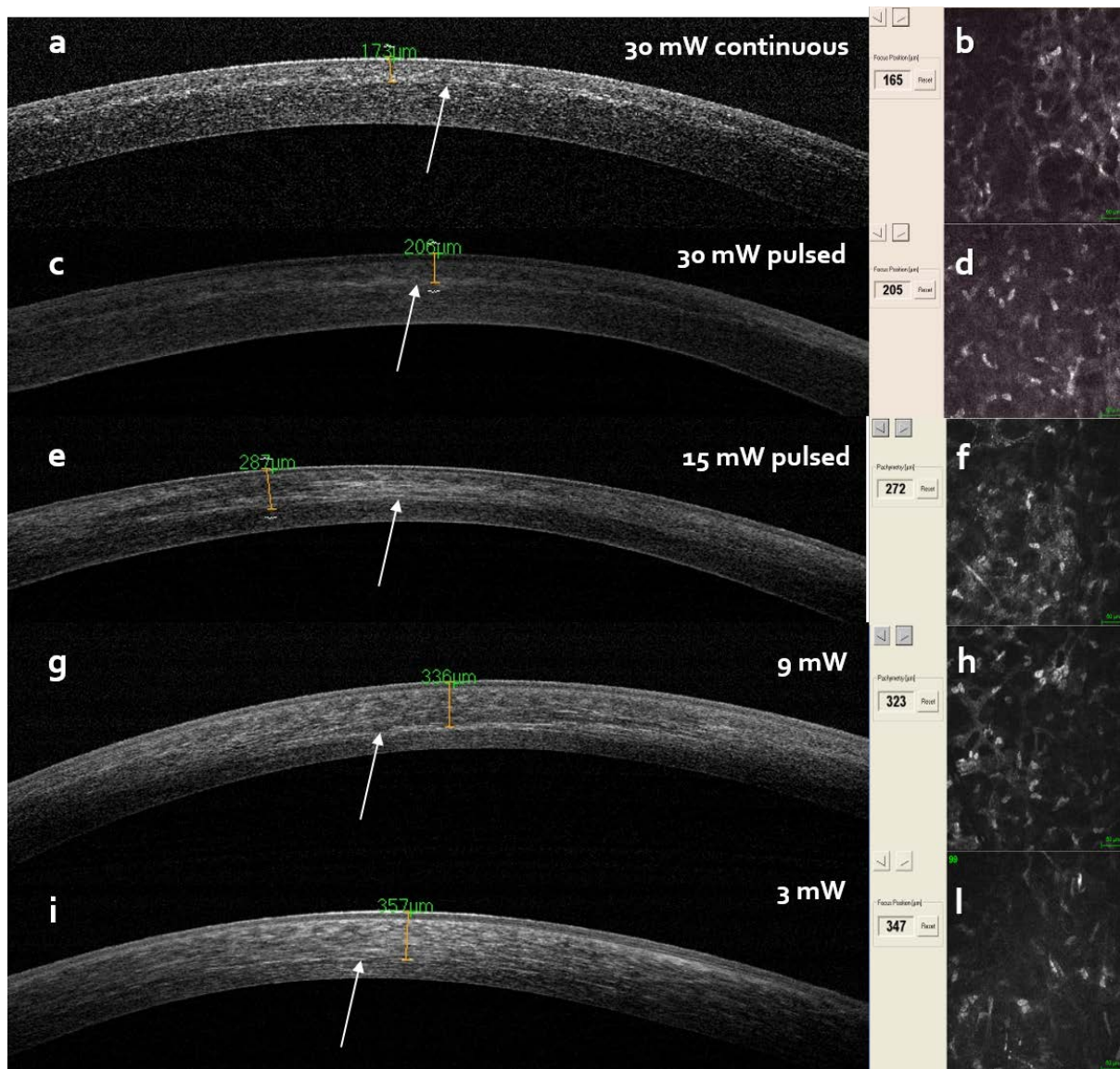
Substantially, the kinetic models after S-CXL and A-CXL protocols confirmed that there is a faster oxygen depletion and a slow oxygen replenishment shifting the CXL photodynamic reaction predominantly in the type I. It happens also in the standard protocol with continuous light UV-A exposure because of the fast oxygen consumption (10-15 sec) during the UV-ON phase.<sup>51</sup>

A preclinical laboratory study conducted by Krueger et al<sup>72</sup>, evaluating the biomechanical efficacy of high versus standard irradiance and pulsed light CXL with equivalent energy dose (5.4J/cm<sup>2</sup>), demonstrated that riboflavin 0.1% with 15mW/cm<sup>2</sup> UV-A exposure was as effective as conventional 3mW/cm<sup>2</sup> CXL and 9mW/cm<sup>2</sup> A-CXL in biomechanical strengthening of the cornea. Also this study established that pulsed UV-A delivery should improve the degree of cross-linking, especially with the faster higher-irradiance exposures where oxygen is more quickly consumed.<sup>51,72</sup>

As for biomechanical model studies<sup>49,50</sup>, the CXL treatment should cover at least 200 μm of corneal stroma to be stronger conferring a durable strength to corneal stroma. Contradictory results are reported in the literature after different A-CXL protocols by using 9mW/cm<sup>2</sup>, 18mW/cm<sup>2</sup> and 30mW/cm<sup>2</sup> UV-A power. However, according to Lang et al,<sup>63</sup> with respect to either accelerated protocols the standard protocol (S-CXL) showed at 12 months significant improvements in a larger number of parameters.<sup>52-62</sup> A-CXL with irradiance of 9mW/cm<sup>2</sup> for 10 min<sup>52</sup> was demonstrated to be effective in stabilizing topographic parameters after 12-month of follow-up in mild-moderate keratoconus-affected corneas. Improvement in the UDVA and stabilization of all tested corneal parameters were noted after the treatment. Moreover, the 9mW/cm<sup>2</sup> A-CXL was safe for corneal endothelium, stabilizing the progression of keratoconus and iatrogenic ectasia with a significant reduction in topographic keratometry values and a significant increase in CDVA, comparable with conventional 3mW/cm<sup>2</sup> CXL in a mid-term (two-years) follow-up.<sup>63</sup>

Mazzotta et al<sup>73</sup> documented the clinical and microstructural IVCN and OCT results of accelerated 15mW/cm<sup>2</sup> pulsed-light corneal crosslinking (CXL) in progressive KC showing a distinct DL at 280 ±30 μm depth on average. The 15mW/cm<sup>2</sup> pulsed-light epithelium-off A-CXL confirmed the laboratory data of Krueger et al<sup>72</sup> demonstrating the safety and the clinical efficacy of this protocol in stabilizing KC progression through 2 years of follow-up. However, none of these protocols have long-term follow-up, **Figure 4 e - f**.

Mazzotta et al<sup>74</sup> demonstrated by means of comparative corneal OCT and IVCN scans that the DL depth achievable after the 9mW/cm<sup>2</sup> A-CXL protocol with 5.4J/cm<sup>2</sup> fluence and 10 minutes of continuous light UV-A exposure was at 310 ± 30 μm depth on average measured from the epithelial surface, **Figure 4 g - h**, nearer to S-CXL 3mW/cm<sup>2</sup> Dresden protocol (350 μm measured from the epithelial surface), **Figure 4 i - l**.



**Figure 4.** DL depth determined by spectral domain corneal OCT and comparative photo-oxidative damage documented by scanning laser IVCN after different Accelerated CXL protocols at standard fluence of  $5.4\text{J}/\text{cm}^2$ .  $30\text{mW}/\text{cm}^2 \times 3\text{ min}$  UV-A continuous light,  $150 \pm 20\mu\text{m}$  (a-b);  $30\text{mW}/\text{cm}^2 \times 6\text{ min}$  UV-A pulsed light (1 sec on 1 sec off),  $200 \pm 30\mu\text{m}$  (b-c),  $15\text{mW}/\text{cm}^2 \times 12\text{ min}$  UV-A pulsed light (1 sec on 1 sec off),  $280 \pm 30\mu\text{m}$  (e-f);  $9\text{mW}/\text{cm}^2 \times 10\text{ min}$  UV-A continuous light, DL  $310 \pm 30\mu\text{m}$  (g-h);  $3\text{mW}/\text{cm}^2 \times 30\text{ min}$  continuous light,  $350 \pm 30\mu\text{m}$  (i-l).

In a recent study Hashemi et al<sup>60</sup> documented that A-CXL with  $18\text{mW}/\text{cm}^2$  UV-A power showed less topographic flattening compared with conventional CXL. Refractive and visual results of the A-CXL by using continuous light and pulsed light treatment at  $30\text{mW}/\text{cm}^2$  gave similar results compared to conventional  $3\text{mW}/\text{cm}^2$  CXL after 12-months follow-up. All the protocols giving similar results compared to  $3\text{mW}/\text{cm}^2$  were using a power in the range between  $9\text{mW}/\text{cm}^2$  and  $18\text{mW}/\text{cm}^2$ , demonstrating similar clinical outcomes, sometimes less flattening compared to standard  $3\text{mW}/\text{cm}^2$ .<sup>63</sup>

### The Demarcation Line in Enhanced Fluence A-CXL Protocols

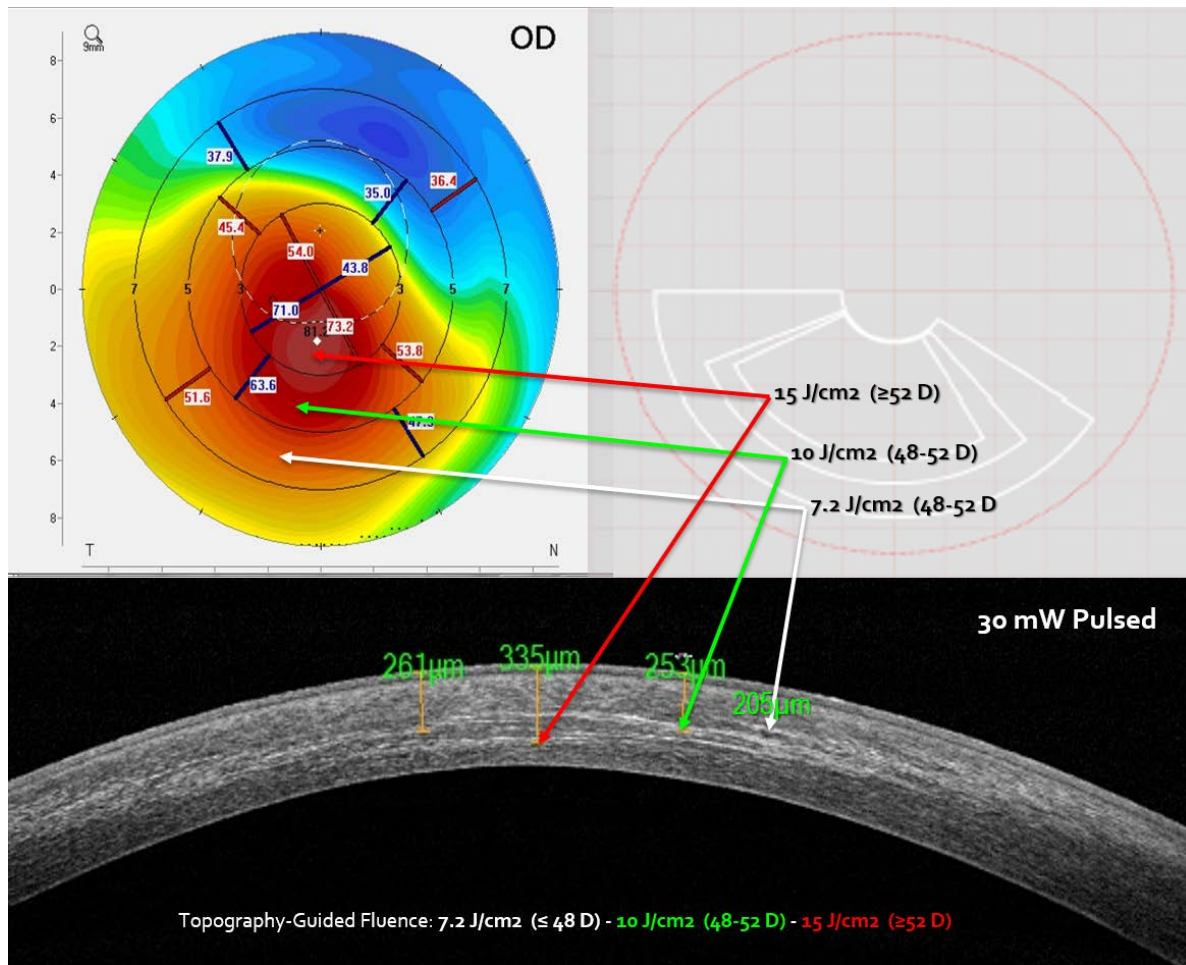


Roy et al<sup>75</sup> firstly proposed Topography guided A-CXL as a potential approach to improve the optical predictability of CXL and maximize the corneal regularization using a computational patient specific model of keratoconus progression and differential responses to CXL. In simulations comparing broad-zone CXL treatments and focal, cone-localized treatments, for a variety of patients cone-localized patterns presented in tomography examinations larger reductions in the cone curvature and higher order aberrations (HOA).

According to Roberts et al<sup>76</sup>, given that corneal ectasia is driven by “focal” rather than generalized weakness, focal stiffening of the cone region may promote a more favourable material property redistribution with compensatory steepening of surrounding areas, thereby enhancing topographic normalization.

In 2016 Seiler et al<sup>77</sup> and Mazzotta et al<sup>78</sup> carried out for the first time in Europe the Topography-Guided A-CXL with the KXL II™ UVA illuminator (Avedro, Waltham, MS, USA), using a 30 mW/cm<sup>2</sup> UV-A power with pulsed light emission (1 second on/ 1 second off) and enhanced fluence (7.2 J/cm<sup>2</sup>, 10 J/cm<sup>2</sup> and 15 J/cm<sup>2</sup>) associated to the maximum corneal curvature. Experimental KC treatments were planned individually by means of a dedicated software (Avedro’s Mosaic System version 1.0, Avedro Inc., Waltham, MS, USA), as suggested by the preoperative topography data, **Figure 5**. The 30 mW/cm<sup>2</sup> topography based A-CXL treatments consisted in a differentiated energy dose release according to the corneal curvatures of each keratoconus. The 7.2 J/cm<sup>2</sup> entry level energy dose was delivered as broad beam treatment by using 30mW/cm<sup>2</sup> UVA power and pulsed (1sec on/1 sec off) light illumination for 8 min exposure time. Then, KC areas showing a corneal curvature in the range 48 to 52 D were treated with 10 J/cm<sup>2</sup> energy dose, keeping the 30 mW/cm<sup>2</sup> UV-A power, extending the exposure time by 3 min in order to reach the programmed dose of 10 J/cm<sup>2</sup> (reaching 11 min total exposure time), and masking the peripheral area of the KC that just received the 7.2 J/cm<sup>2</sup> energy dose. The paracentral or central steepest area, with a curvature above 52 D, was treated by extending further the UVA exposure time to deliver the 15 J/cm<sup>2</sup> maximum energy dose, reaching 6 min total treatment time. The treatment planning was established by using semi-meridians K values on Pentacam maps. The total treatment time was 8 min for keratoconus with maximum K values not larger than 48 D, 11 min for keratoconus with K values in the steepest area, including simulated, in the range 48 to 52 D, and extended to 16 min for keratoconus showing in the steepest areas K values above 52 D. The treatment started from a baseline broad beam illumination that included the flattest peripheral areas (48 D and below) at 7.2 J/cm<sup>2</sup>. After 8 min, peripheral areas were masked and the illumination was prolonged only in the steepest zones to deliver a final **energy** dose of 10 J or 15 J/cm<sup>2</sup>, depending on the maximum curvature values. The thinnest point and the area of major posterior elevation were included within the highest dose treatment zone. The irradiation patterns shapes included arc, circular, oval and combined patterns, according to keratoconus tomography and shape. The irradiation pattern was aligned by using a direct real time visualization of the cornea, maintaining a perfect centration with the eye-tracking system provided by the machine. Corneal OCT scans of the same cornea showed multiple local DL, dependent on the irradiance delivered over the corneal tissue and the exposure time. The studies on Topography-Guided ACXL by Seiler et al<sup>77</sup> and Mazzotta et al<sup>78</sup> demonstrated that the DL is also function of the irradiance, the treatment time, the concentration of riboflavin, the depletion of riboflavin, and the oxygen.



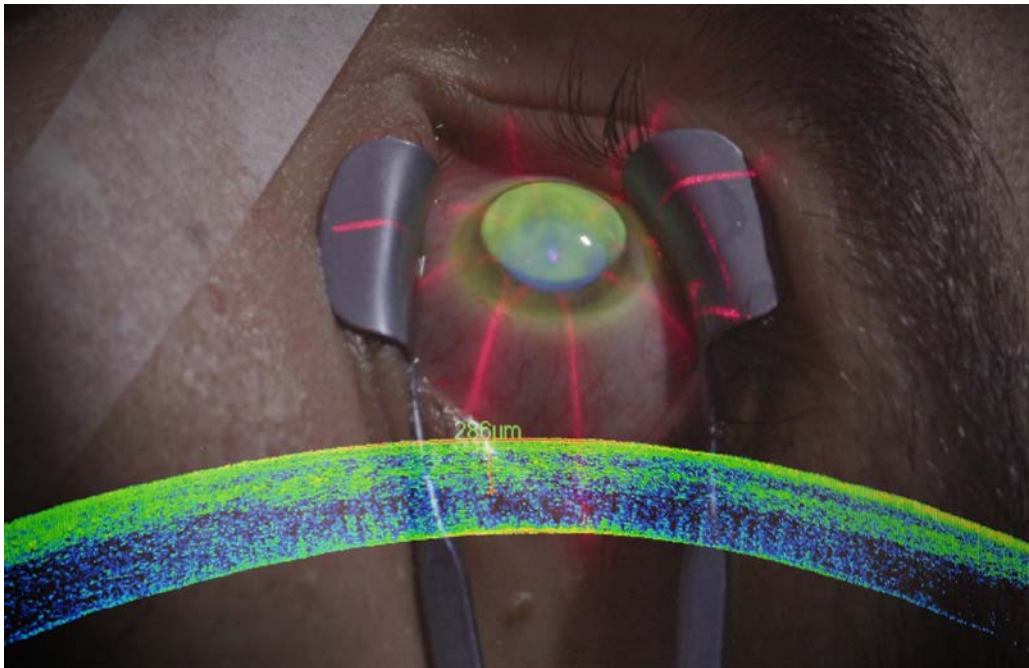


**Figure 5. Topography Guided A-CXL showing the depth of DL depending on Enhanced Fluence and exposure time. After 8 min of UV-A exposure time at 30mW/cm<sup>2</sup> with pulsed light illumination (1 sec on/1sec off) delivering 7.2 J/cm<sup>2</sup> in a broad beam spot of 9 mm, including the peripheral flattest area of the cone, the depth of DL measured from epithelial surface was 200 ± 20 μm (white arrows) in the flatter corneal area; 250 ± 20 μm in the intermediate curvature paracentral area between 48 and 52 D undergoing 10J/cm<sup>2</sup> fluence and 11 minutes of UV-A exposure time (green arrow); 330± 20 μm (red arrow) in the central steeper cone area undergoing 15J/cm<sup>2</sup> for 16 minutes of UV-A exposure. The increased depth of DL in the paracentral and central areas was correlated with the prolonged exposure times (11 and 16 min respectively) necessary to deliver 10 and 15 J/cm<sup>2</sup> fluence.**

After 30 mW/cm<sup>2</sup> with 7.2 J/cm<sup>2</sup> enhanced fluence pulsed light (1 sec on 1 sec off) A-CXL, Böhm et al<sup>79</sup> observed a mean stromal DL at a depth of 203.00 μm ± 13.53 (SD) and concluded that, by increasing the fluence and using the pulsed light while increasing the exposure time from 3 to 4 minutes (to 8 minutes in pulsed light enhanced fluence protocol), the depth of DL increases, confirming the pivotal OCT and IVCN studies on A-CXL.<sup>67,80</sup>

Mazzotta et al<sup>81</sup> published recently a new Iontophoresis protocol with an Enhanced Fluence Pulsed-light Iontophoresis (EF-ICXL) showing a promising 12-months follow-up characterized by reduced maximum keratometry, corneal surface asymmetry indices, and higher-order aberrations. The new protocol improved the visibility of DL in over 80% at 285 ± 20 μm depth on average and increased the penetration of photo-oxidative damage, by

enhancing the photochemical kinetic of the original I-CXL technique with a 30% increase of the treatment fluence (from  $5.4\text{J}/\text{cm}^2$  to  $7\text{J}/\text{cm}^2$ ) in order to compensate the UV-A energy photo-attenuation provided by the corneal epithelium and Bowman's layer antioxidants systems, **Figure 6**.



**Figure 6. Demarcation Line after Mazzotta's Enhanced Fluence Pulsed Light Iontophoresis (EF I-CXL). Increasing of 30% the treatment fluence (from  $5.4\text{J}/\text{cm}^2$  to  $7\text{J}/\text{cm}^2$ ) thus compensating the UV-A energy photo-attenuation provided by the corneal epithelium and Bowman's layer antioxidants system and adding the pulsed light partially compensating the 40% estimated oxygen consumption by the corneal epithelium in situ, the DL is detectable in over 80% at  $280 \pm 20 \mu\text{m}$  depth on average, increasing the penetration of photo-oxidative damage improving the photochemical kinetic of the original I-CXL technique.**

The pulsed-light irradiation was added to partially increase the intraoperative oxygen diffusion and treatment penetration according to literature data.<sup>51</sup> These two modifications of the original I-CXL protocol allowed a superior and repeatable visualization of the DL that was considered an advance in the recent view on trans-epithelial CXL. The preliminary data observed in the study were closer to S-CXL evidences, in absence of wound related complications (haze) and/or endothelial damage. Of course long-term data in larger cohorts of patients will clarify if the crosslinks amount, distribution and saturation will be sufficient to maintain a long-term stability similar to Epi-Off S-CXL.<sup>81</sup>

Interestingly, Kannelopoulos et al<sup>82</sup> showed a DL depth closer to  $250 \mu\text{m}$  from the epithelial surface by calibrating a fluence of  $6.3 \text{J}/\text{cm}^2$  at the corneal surface setting a UV-A power of  $7 \text{mW}/\text{cm}^2$  for 15 minutes.

In analogy with the same principles of the Enhanced Fluence I-CXL protocol, Kymionis et al<sup>53</sup>, in evaluating the stromal DL depth following a modified accelerated (A-CXL) procedure by using  $18\text{mW}/\text{cm}^2$  UV-A power, with an enhanced fluence of  $7.5 \text{J}/\text{cm}^2$ <sup>27</sup> for 7 minutes of UV-A exposure time instead of standard  $5.4\text{J}/\text{cm}^2$  for 5 minutes, demonstrated that increasing the fluence and consequently the UV-A exposure time by 30-40%, the depth of DL was

similar to the one of S-CXL. However, whether accelerated CXL procedures warrant a sufficient crosslinks amount, distribution and saturation to maintain long-term stability similar to Epi-Off S-CXL remains an open question.

### Relationship between depth of DL and CXL biomechanical efficacy

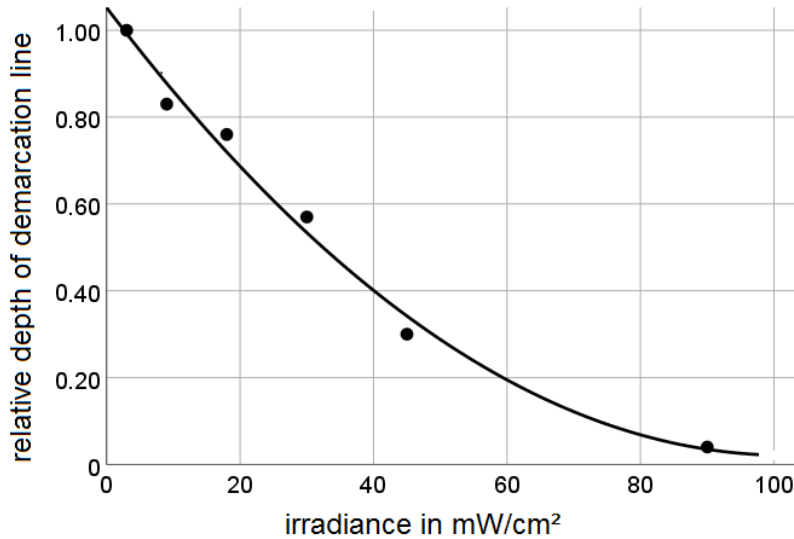
Author (First Name-Journal-Year)	DL Depth ( $\mu\text{m}$ ) no epi	Irradiance ( $\text{mW}/\text{cm}^2$ )	Time (min)	E-modulus ( $\text{MPa}$ ) <sup>84</sup>	Rel E
Yam-JRS-2013	262	3	30		
Yam-JRS-2012	241	3	30		
Thorsrud-JCRS-2017	237	3	30		
Ng-Cornea-2015	295	3	30		
Asgari-JCurrOphthalmol-2018	315	3	30		
Awwad-AJO-2019	282	3	30		
Doors-AJO-2009	273	3	30		
Brittingham-IOVS-2014	283	3	30		
Bouheraoua- IOVS-2014	263	3	30		
Mesen-Cornea-2018	295	3	30		
Mazzotta-AJO-2008	260	3	30		
Kymionis-JCRS-2014	310	3	30		
Kymionis-Cornea-2013	270	3	30		
Kymionis-AJO-2014	298	3	30		
Knutsson-BJO-2018	209	3	30		
Cerman-JCRS-2015	227	3	30		
Shetty-AJO-2015	240	3	30		
<b>Average</b>	<b>268</b>			<b>1.18</b>	<b>1.00</b>
Ng-Cornea-2015	203	9	10		
Kymionis-JCRS-2014	248	9	10		
Brittingham-IOVS-2014	205	9	10		
Pircher-Graefe-2018	200	9	10		
Shetty-AJO-2015	252	9	10		
<b>Average</b>	<b>221</b>			<b>1.14</b>	<b>0.93</b>
Mesen-Cornea-2018	265	18	5		
Asgari-JCurrOphthalmol-2018	183	18	5		
Shetty-AJO-2015	163	18	5		
<b>Average</b>	<b>204</b>			<b>1.03</b>	<b>0.75</b>
Bouheraoua- J Vis Exp-2015	144	30	3		
Shetty-AJO-2015	161	30	3		
	153			0.78	0.34
Celik-JCRS-2012	80	45	2	0.70	0.21
Estimated	15	90	1	0.62	0.08

**Table 1: Compilation of depths of the DL for several irradiances by a fluence of  $5.4\text{J}/\text{cm}^2$  and of riboflavin 0.1 % concentration. The depth of the DL does not include the thickness of the epithelium.**

Asgari et al<sup>83</sup> reported a lot of values for the depth of DL for several irradiances, see **Table 1**. From these DL depths the relative DL was calculated as

$$\text{Rel DL} = \frac{DL}{DL_3}$$

where  $DL$  is the depth at a specific irradiance, and  $DL_3$  is the depth of  $DL$  at  $3 \text{ mW/cm}^2$  for 30 min (standard CXL with a fixed dose of  $5400 \text{ mJ/cm}^2$ ) according to the Dresden protocol  $DL$  outcome assumed as benchmark<sup>12-16</sup>. **Figure 8** shows the dependence of the average values of the relative depth on the irradiance.

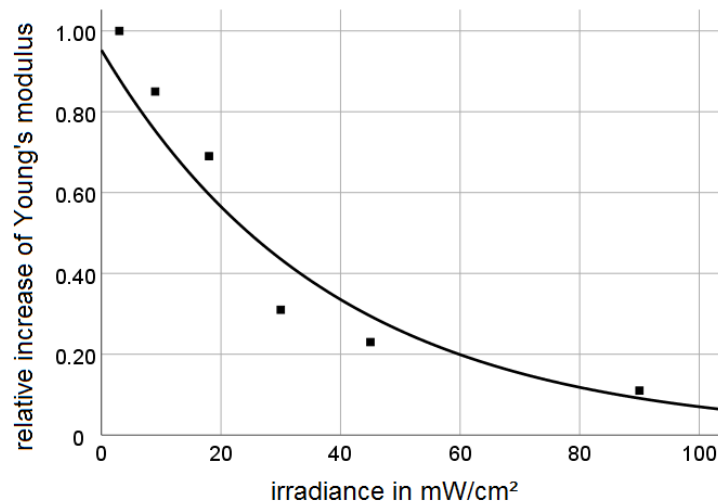


**Figure 8: Mean values of relative depth of  $DL$  as a function of the irradiance, according to experimental data from the literature, see Table 1.**

By means of ex-vivo inflation tests on rabbit corneas, Bao's<sup>84</sup> measured the average elastic modulus (E-modulus) for irradiances within the range  $3$  to  $90 \text{ mW/cm}^2$ . Starting from these results, the relative increase of the E-modulus due to CXL was estimated according to the following expression

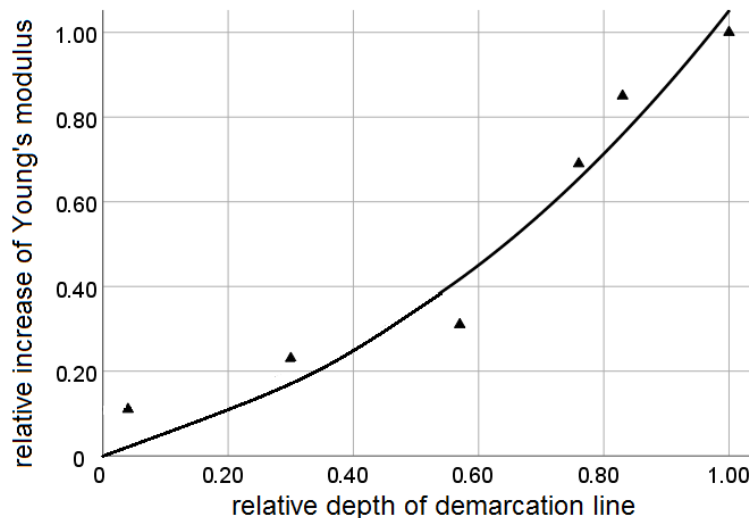
$$\text{Rel } E = \frac{E - E_0}{E_3 - E_0}$$

where  $E_0 = 0.57 \text{ MPa}$ <sup>84</sup> is the average elastic modulus of untreated corneas,  $E_3$  the average elastic modulus of corneas treated with  $3 \text{ mW/cm}^2$  irradiance for 30 min (standard CXL with a fixed dose of  $5400 \text{ mJ/cm}^2$ ) and  $E$  the E-modulus at the generic irradiance. The relative increase of the elastic modulus as a function of the irradiance power is shown in **Figure 9**.



**Figure 9: Relative increase of the corneal elastic modulus as a function of irradiance (values determined according to experimental measurements of Bao et al<sup>76</sup>). The Bunsen-Roscoe law is not valid, otherwise the relative increase of Young's modulus would be 1 for all equivalent UVA doses.**

**Figure 10** shows the dependence of the relative value of the elastic modulus on the relative DL depth, characterized by a strong nonlinear dependence of the relative stiffness increase of the corneal material induced by CXL and the depth of the demarcation line.



**Figure 10: Overall CXL biomechanical effect in terms of relative increase of the elastic modulus E on the relative depth of D driven by the irradiance.**

The diagram is a clear visualization of the biomechanical effects of the CXL. **The result of Scarcelli et al<sup>27</sup>, i.e., TE-CXL with an DL = 100µm (Rel D L= 0.37)** shows about 70% lower efficacy than standard CXL, is confirmed here. From this curve it is also possible to estimate the biomechanical efficacy of the TE-CXL or enhanced fluence CXL methods.

## Discussion

Starting from the original S-CXL protocol described by Wollensak et al. in 2003<sup>1</sup>, the so-called DL occurring at the posterior edge of the CX-Linked stroma has been universally accepted as “*therapeutic milestone*” and has been considered an appropriate tool for the assessment of the effectiveness and safety of CXL procedures.

Critical reviews of basic laboratory and clinical CXL studies about the DL, in terms of relative DL depth and the relative CXL-induced change in the average corneal E-modulus, show that a deeper DL is associated with a higher amount and saturation level of crosslinks and a more marked stiffening effect (Figures 8, 9, 10). Furthermore, clinical studies report that, at a deeper DL, a more durable ectasia stability occurs, often accompanied with a stronger corneal flattening.

By taking the irradiance at **3 mW/cm<sup>2</sup> for 30 min (5400 mJ/cm<sup>2</sup> dose)** as reference, the relative DL depth and the relative increase of the corneal E-modulus are asymptotic functions of the irradiance (Figures 8, 9). A saturation occurs in the relative DL depth, showing that the best penetration and corneal stiffening is achieved at low irradiance and long times.

By expressing the dependence of the relative change in E-modulus on the relative depth of the DL, the resulting non-linear function confirms the existence of a marked correlation between the depth of the DL and the biomechanical behaviour of the stromal tissue, see Figure 10, although a correlation with post-CLX functional results cannot be directly established.

The deeper the apoptosis at IVCN and optical interface line at bio-microscopy and OCT, the higher is the volume of CX-linked stroma, the higher is the amount (%) of crosslinks, the higher the increase of biomechanical stiffness conferred to the corneal stroma. The non-linear biomechanical correlation can be explained by the saturation and homogeneity of crosslinks distribution into the stroma, that depends on many factors other than the DL depth.

The strong motivation that promoted the DL concept is that it can be used to assess the performance of CXL procedures in terms of biomechanical efficacy and safety without the need of conducting extensive invasive experiments. This feature represents also the main advantage of the DL concept itself.

It is well known that CXL is an oxygen dependent reaction. The amount of singlet oxygen depends on the energy transfer from the activated riboflavin to the oxygen and, therefore, on the oxygen concentration in corneal stroma available for this transfer. The epithelium in situ and the presence of antioxidants systems (radical absorbers) decrease the CXL amount by diminishing the availability of oxygen and absorbing a portion of the UV-A light. With no oxygen, no stiffening effects are possible, although no direct correlation between the percentage of oxygen reduction and percentage of stiffening reduction has been established yet.

The occurrence of DL is a direct morphological sign of performed CXL and is a fact confirmed by histology, IVCN, bio-microscopy and OCT. This feature is validated in the majority of CXL protocols, and depends on the protocol used and accuracy of the CXL technique application. The DL is the clear evidence of the CXL-induced photo-oxidative tissue modification derived from riboflavin stromal concentration and gradient of concentration, light distribution within the stroma and riboflavin activation, oxygen availability and diffusion, radical release, and UV-A exposure time **(the longer the deeper DL)**. Moreover, the DL is a function of irradiance, treatment time, concentration of riboflavin,



depletion of riboflavin, and oxygen. If, after CXL, the DL is detected, it can be taken as a positive indicator that the interaction among UV, riboflavin and oxygen was successful.

Not fortuitously, with respect to the conventional Dresden CXL protocol, the DL measured through of higher-resolution spectral domain (SD) corneal OCT was shallower, or less detectable, after Epi-On treatments and A-CXL protocol.

The variability of the complex distribution of collagen and ECM collagen-proteoglycans redistribution and the uneven collagen compaction and rearrangement associated with the changes in the refraction index of the cornea after CXL protocols may explain the non-linear functional correlation between DL depth and changes in visual acuity documented in recent studies by Pircher et al<sup>77</sup> and Mesen et al<sup>78</sup>.

Records of topographic changes in KC after CXL are more complex and depend on cone localization or eccentricity, age, baseline visual acuity and baseline thinnest pachymetry, status of epithelium, and lacrimal fluid on the corneal surface, together with accuracy of the instrumental measurements, and consistency and comparability of collected data.<sup>79-81</sup>

Indeed, no statistically significant differences in topographic/refractive results between the different CXL protocols, A-CXL protocols and Epi-On protocols are observed. Literature reports on various CXL protocols have been presenting comparable results with no statistically significant differences, even though corneal flattening measurements achieved after Epi-On CXL, I-CXL and A-CXL were often inferior to K max flattening and visual acuity recorded after Epi-Off S-CXL.<sup>50</sup>

According to Uysal et al<sup>82</sup>, the optical performance of the cornea following CXL gained an improvement in visual, refractive, topographic, and most corneal higher-order aberrations (HOAs) outcomes. In addition to the maximum Keratometry flattening, not appropriate to reflect the global corneal changes induced by CXL because is a local parameter relative to a single point, significant improvements in mean Root Mean Square (RMS) error values for corneal total HOAs, vertical coma and vertical trefoil following CXL were reported. Such results, though, cannot be considered predictable in CXL. The only functional predictability of CLX confirmed by literature data is restricted to the preoperative baseline thinnest pachymetry (less than 450 $\mu$ m), which was found to be significantly associated with higher flattening in maximum keratometry.

Recent studies by Mathews et al<sup>83</sup>, correlating the corneal densitometry after CXL to visual acuity, demonstrated that, although the greatest and most durable post-CXL densitometry change was in the anterior layer, the degree of increased densitometry haze in the mid-stromal layer was mostly associated with (possibly predictive factor) improvement in CDVA, maximum K, and HOAs. The persistence of corneal haze at 6 months, measured by increased densitometry, might be a prognostic marker for CXL effectiveness. This could imply a potential refractive implication of DL depth if associated with higher densitometry values of the mid stroma.

Clearly, in addition to the important morphological meaning as “*boundary line*” between CX-linked and non-CX-linked stroma and the potential refractive impact, the depth of the DL has important implications on the biomechanical changes induced by CXL.

Wollensak et al<sup>85</sup> *ex vivo* stress strain tests on CXL corneas demonstrated a correlation DL depth and corneal stiffening. As previously mentioned, *ex vivo* tests are not able to quantify

the level of stiffening and strengthening, but they provide an indication of biomechanical changes. Non-destructive Brillouin microscopy demonstrated a biomechanical stiffening of the stroma after TE-CXL for cases where the DL was invisible, poorly visible or located below 100µm (measured with epithelium), about 70% less than the conventional epithelium-off Dresden protocol.<sup>27</sup>

The biomechanical implication of the DL depth can be explained by considering that ectasia originates in almost all cases from the posterior float of the cornea.<sup>86</sup> The well-known structure of the human corneal stroma has been revealed by many studies based on X ray diffraction<sup>87-91</sup>. The anisotropy, the orientation, the intersection and the distribution of corneal lamellar structure and collagen fibrils interweaves are not uniform across the thickness of the stroma, but the reinforcing structure characterizes a stronger cornea in the anterior third (160-180µm) and a progressively weaker stroma in the posterior part (over 160-180). The particular structure explains the fact that most of ectatic corneas treated with Epi-Off S-CXL were stable in the long term follow-up<sup>5</sup>, even in paediatrics patients 18 years and younger<sup>6</sup>, whereas after Epi-On treatments showing a superficial DL, with a depth from the epithelial surface less than 200 µm and unevenly detectable, had almost 50% of unstable cases with the need to repeat CXL procedures.<sup>25,18,35,36</sup>

A supporting prove was provided by the stress-strain tests performed by Kohlhaas et al<sup>92</sup> at different depths, showing that the CXL treatment of the cornea with riboflavin and UVA significantly stiffened the cornea only in the anterior 200µm. This depth-dependent stiffening effect may be explained by the absorption behaviour for UV-A in the riboflavin-treated cornea: 70% percent of UV-A irradiation was absorbed within the anterior 200 µm and only 30% reached the deeper layers.

Clearly, the goal of a CXL procedure is the halt of the progression of keratectasia. The refractive improvement represents an eventual additional gain. There is general consensus regarding to the fact that the efficacy of S-CXL is reflected by a deep DL which implies better clinical results. The deeper the DL the stronger the overall biomechanical effect. By accounting that the posterior stroma beyond 160 µm depth is the weakest portion and that ectasia originates from the posterior side of the cornea, optimization models of CXL and clinical results recommend to stiffen the cornea by reaching at least a stromal DL depth of 200 µm or more.

It has been observed that the biomechanical effect does not necessarily correlates with the UV-A dose when the treatment time or UV-A dose exceed certain limits. For example, the biomechanical effect obtained after 60 min irradiation is equivalent and not significantly increased with respect to the effect obtained after 30 min irradiation as described in the Wollensak's protocol<sup>1</sup>. Moreover, the photo-oxidative damage would not diminish between 30 and 60 minutes UV-A irradiation, because cells that become apoptotic after 30 min irradiation will not recover, and a higher cumulative UV-A dose is delivered to the endothelium.

Interestingly, on AS-OCT immediately after hydration, Wollensak et al<sup>13</sup> observed at the depth of 540 µm in the treated portion of the anterior stroma a pronounced line, correlating with the combined area of the anterior intensely and less intensely CX-Linked intermediate layers. Since hydration is a very sensitive parameter, it seems possible that the line includes the intensely CX-Linked stroma and the less intensely CX-Linked intermediate layers.

Lanchares et al.<sup>93</sup> did not observe CXL effects due to the increase of the cross-linking time to 60 minutes. Nevertheless, they found that for the same UV-A dose of 5.4J/cm<sup>2</sup>, both the CXL efficacy and the increase of the elastic modulus show a reduction at a higher irradiance. This would apparently invalidate the Bunsen-Roscoe law, because the biomechanical effect of CXL reduces at a higher irradiance.

To date, the in-vivo correlation between DL depth and corneal biomechanics cannot be estimated by means of a simple relationship. According to Fuchsluger et al.<sup>94</sup> the biomechanical parameters of the cornea in healthy and KC eyes, CXL treated or not, can be evaluated by means of ultrahigh-speed Scheimpflug measurements (Corvis ST). A newly introduced parameter, namely the difference between inward applanation length and outward applanation length (A1L – A2L), indicated highly significant differences between CX-linked corneas and untreated KC or healthy corneas. Moreover, the velocity at the second applanation (A2V) and the deformation amplitude (DA) were significantly increased in crosslinked KC eyes with respect to both untreated KC and healthy control eyes. Also the radius at the maximum curvature was significant among all groups. Inward applanation length (A1L) was significantly increased in controls, whereas outward applanation length (A2L) was significantly reduced in CX-Linked KC eyes with respect to both untreated KC and healthy control eyes.

CXL essentially improves corneal stiffness by increasing the percentage of links among stromal collagen fibres basically due to a photo-oxidative process followed by reinforcement of CPC and ECM, leading to a local increment of the stromal stiffness. Since the DL is a clear mark of the penetration of the CXL-induced photo-oxidative damage, an evident, although not linear, correlation between the DL depth and the local increase of the stromal stiffness does exist.

The elastic modulus (E-modulus) measures the ability of a material to oppose the deformation, and in biological tissues it is increasing with the level of deformation. In the case of the cornea, it increases with the IOP and, at high deformations, it modifies in time revealing viscosity. The corneal stiffness is a more general term that indicates the propensity of the cornea, thought as a structure, to oppose the change of its geometry under the effect of external actions. The corneal stiffness depends not only on the elastic modulus, i.e. on IOP and time, but on many other geometric parameters, listed as follows, in order of decreasing importance: white-to-white distance (WTWD), mean curvature (K Avg), pachymetry, and scleral connection.

CXL increases the E-modulus only of the anterior portion of the stroma up to the DL, leading to a modification of the overall corneal stiffness. However, the resulting increase of the corneal stiffness is not linearly dependent on the DL depth, because CXL acts only on a portion of the thickness, making the structure inhomogeneous.

It follows that the simple geometrical interpretation of the whole dynamic deformation of the cornea, as induced by a soliciting force by using air-puff tonometry devices<sup>94, 95</sup>, cannot provide a direct estimate of the actual value of the E-modulus of the cornea, for two main reasons. First, the E-modulus across the cornea is not uniform, in particular in pathologic corneas. Second, the motion of the cornea under the dynamic test depends not only on the E-modulus, but also on the geometric parameters listed above. It is therefore hard to try to establish a correlation between the global deformation of the cornea during the air-puff test

and the E-modulus; even harder to find a correlation between the global deformation and the DL depth.

In comparing pre and post-CXL corneas, the parameters of the air puff test based exclusively on the analysis of the geometrical changes of the cornea during the motion can only provide a general indication of the modification of the stiffness, but they will not be able to quantify the change on E-modulus and thus to provide a meaningful estimate of the biomechanical effects of the CXL.

So far, the most reliable approach to the estimate of the biomechanical effects of CXL must be based on numerical analysis using realistic geometrical and material models of the cornea, e.g., the finite element analysis (FEA)<sup>84, 103, 104</sup>, that accounts for the exact geometry, the complex microstructure of the stroma, and furthermore for the presence of the fluids filling the anterior chamber<sup>105, 106, 107</sup>. Numerical approaches must be combined with experimental testing conducted with advanced devices conceived to provide accurate information on the variability of the material parameters of the stroma, such as the ones based on Brillouin microscopy<sup>27</sup>. Such approaches are under development but not available yet for clinical practice.

Up to now, no fast, inexpensive, and patient-friendly method is available to control under clinical conditions the biomechanical efficacy of the CXL treatment but the depth of the DL.

Obviously, DL depth is an immediate indicator unable to predict the long term response to CXL in terms of tissue stabilization, which is strongly affected also by many biological, genetic, and environmental factors.<sup>96-100</sup> Each KC is different from any other, and characterized by different ectasia progression indices related to different genetic penetrance, phenotypic expression and comorbidities,<sup>96</sup> environmental factors such eye-rubbing, patient's age, allergy<sup>97</sup> and presence of hormones (cortisol, thyroxin, estrogens, androgens, progesteron, prolactin).<sup>98-102</sup>

All these factors inevitably have a great influence on the time evolution of KC pathology and also in the clinical response to any CXL treatment. None of them, however, affects the fundamental significance of the DL to assess the evidence of CXL penetration and to quantify the overall CXL biomechanical efficiency.

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