Optical Coherence Tomography and Confocal Microscopy Following Three Different Protocols of Corneal Collagen-Crosslinking in Keratoconus

Nacim Bouheraoua,[1–4] Lea Jouve,[1] Mohamed El Sanharawi,[1] Otman Sandali,[1] Cyrille Temstet,[1] Patrick Loriaut,[1] Elena Basli,[1–4] Vincent Borderie,[1–4] and Laurent Laroche[1–4]

[1]Quinze-Vingts National Ophthalmology Hospital, Sorbonne Universités, UPMC Univ Paris 06, Paris, France
[2]INSERM UMR S 968, Institut de la Vision, Paris, France
[3]Sorbonne Universités, UPMC Univ Paris 06, UMR S 968, Institut de la Vision, Paris, France
[4]CNRS, UMR 7210, Paris, France

Purpose. We compared the efficacy and early morphological changes in the cornea following conventional (C-CXL), transepithelial by iontophoresis (I-CXL), and accelerated (A-CXL) collagen cross-linking in keratoconus.

Methods. A total of 45 eyes of 45 patients with progressive keratoconus who underwent corneal collagen crosslinking (CXL) was divided into three groups: C-CXL ($n = 15$), A-CXL ($n = 15$), and I-CXL ($n = 15$). Patients were examined before surgery and at 1-, 3-, and 6-month intervals following surgery. Density of corneal sub-basal nerves, anterior and posterior keratocytes, corneal endothelium, demarcation line depth, and maximal simulated keratometry values (Kmax) were all assessed.

Results. Compared to preoperative values, the mean corneal sub-basal nerve and anterior stromal keratocyte densities were significantly lower at 6 months in the C-CXL and A-CXL groups ($P < 0.001$), whereas they returned to preoperative values in the I-CXL group ($P = 0.083$ and $P = 0.909$, respectively). The corneal demarcation line was visible 1 month after surgery in 93% of cases (mean depth, 302.8 ± 74.6 μm) in the C-CXL group, 87.5% (mean depth, 184.2 ± 38.9 μm) in the A-CXL group, and 47.7% (mean depth, 212 ± 36.5 μm) in the I-CXL group ($P = 0.006$). There were no significant differences between confocal microscopy and optical coherence tomography measurements of the corneal demarcation line depth ($P > 0.05$). The Kmax, corneal central thickness, and BSCVA remained stable during the whole study period.

Conclusions. Iontophoresis was associated with weaker damage of corneal sub-basal nerves and anterior keratocytes compared to conventional procedures, but the demarcation line was present in less than 50% of cases and was more superficial than with the traditional procedure.

Keywords: keratoconus, confocal microscopy, optical coherence tomography, cross-linking

Keratoconus is a bilateral, progressive, and noninflammatory corneal ectasia that affects 1 in 2000 in the younger working-age population.[1] It usually leads to progressive corneal deformation and decreased vision.[2] Corneal collagen cross-linking (CXL) is a surgical treatment used to increase corneal strength and to stabilize the ectatic cornea.[3–8] Even if conventional CXL (C-CXL) is well-vetted and has an excellent safety profile, the major drawbacks of the procedure are related to the time necessary to remove the epithelium and complete the procedure. A variety of protocols have been suggested to reduce operative time, increase patient comfort, and decrease the likelihood of complications, such as infectious keratitis and stromal haze.[9] One of those protocols is accelerated CXL (A-CXL), where a higher irradiance is delivered to the cornea; thus. reducing the required light exposure time.[10] Several transepithelial protocols also have been tried to avoid the necessity for epithelial debridement. Unfortunately, at this time, none of these has come close to reaching the efficacy of the standard protocol.[11] The most recent transepithelial method for stromal riboflavin delivery before CXL is iontophoresis (I-CXL).[12]

Because collagen cross-linking has become more widely adopted, in vivo confocal microscopy (IVCM) has been used to identify the microstructural changes in the keratoconic cornea associated with this treatment.[13–16] The corneal stromal demarcation line was first described as a thin line detectable on slit-lamp examination two weeks following C-CXL.[17] This corneal line also can be identified using confocal microscopy and anterior segment optical coherence tomography (AS-OCT).[18] The depth of this acellular zone has been correlated with the effectiveness of the CXL treatment.[19,20]

The aim of this study was to compare the efficacy of three different protocols of CXL (C-CXL, A-CXL, and I-CXL) by measuring the corneal stromal demarcation line using AS-OCT and confocal microscopy, and to use IVCM to quantitatively analyze the microstructural corneal changes occurring over time.

METHODS

This prospective observational nonrandomized comparative study was performed at the Quinze-Vingts National Ophthal-

TABLE 1. Patient Demographics and Ocular Characteristics for All Subject Included in the Study

	Conventional CXL, $n = 15$	Accelerated CXL, $n = 15$	Iontophoresis CXL, $n = 15$	All Patients, $n = 45$	P Value
Age, mean y (SD)	25.4 (5.6)	26.7 (6.2)	32.4 (6.6)	28.2 (6.1)	0.02*
Sex, male female, n (%)	12 (80) 3 (20)	9 (60) 6 (40)	11 (73) 4 (27)	32 (71) 13 (29)	0.33†
Side, OD OG, n (%)	9 (60) 6 (40)	9 (60) 6 (40)	11 (73) 4 (27)	29 (64) 16 (36)	0.72†
Mean stage keratoconus, (SD)	2.3 (0.6)	2.3 (0.5)	2.3 (0.5)	2.3 (0.5)	0.81*
Mean maximum k-value, (SD)	50.3 (5.4)	47.1 (5.5)	47.7 (5.0)	48.4 (5.3)	0.23*
Mean CCT, (SD)	445.1 (46.7)	468.7 (36)	466.5 (25.3)	460.1 (36)	0.24*
Mean BSCVA, (SD)	0.33 (0.24)	0.12 (0.3)	0.25 (0.2)	0.22 (0.2)	0.08*

* Kruskal-Wallis test.
† Cochran χ^2 test.

mology Hospital with ethics approval granted by French Ethics Committee (IRB 00008855) and adhered to the tenets of the Declaration of Helsinki. A total of 45 eyes of 45 patients with progressive keratoconus who underwent CXL between September 2011 and September 2013 was included based on inclusion/exclusion criteria. Informed consent was obtained before surgery. Of the eyes, 15 treated with C-CXL, 15 treated with A-CXL, and 15 eyes treated with I-CXL were selected among all patients who underwent CXL in the study period. Patients were included consecutively in the study and treated according to the protocol available at the time of inclusion. The three protocols were performed successively at our hospital between September 2011 and September 2013. There was no randomization among the three groups. The diagnosis of keratoconus was based on corneal topography data (Orbscan IIz; Bausch & Lomb Surgical, Rochester, NY, USA) and stromal thinning. Inclusion criteria were the following: patients aged at least 18 years with progressive keratoconus, maximal keratometry $\leq$ 60 diopters (D), minimal corneal thickness $\geq$ 400 µm, best spectacle-corrected visual acuity (BSCVA) $\geq$ 20/80, and clear cornea without visible scar on slit-lamp examination. Exclusion criteria were previous ocular trauma or surgery, ocular disease (other than keratoconus), or systemic disease that may affect the cornea, and stable keratoconus. Progression was defined as an increase in maximal keratometry $\geq$ 0.75 D in the last 3 months, a change in refractive astigmatism $\geq$ 0.75 D in the last 12 months, or a decrease in corneal thickness by 30 µm or more in the last 6 months.

Patients were examined before surgery, and at 1-, 3-, and 6-month intervals following corneal CXL treatment. The following were recorded at each visit: findings on slit-lamp examination, BSCVA using a conventional Snellen chart, central corneal thicknesses (CCT) measured by Fourier-domain optical coherence tomography (RTVue; Optovue, Inc., Fremont, CA, USA), highest K value (Kmax) in the 3-mm central zone measured with corneal topography (Orbscan IIz; Bausch & Lomb Surgical). Laser scanning IVCM was performed using the Heidelberg Retina Tomograph II with Rostock Corneal Module (Heidelberg Engineering GmBH, Heidelberg, Germany). As corneal endothelial cell density has been shown to be significantly overestimated with the Rostock Corneal Module laser scanning system,[21] corneal endothelial cell densities were assessed by means of specular microscopy (SP-3000P; Topcon Canada, Inc., Waterloo, Ontario, Canada). The demarcation line depth was measured centrally with AS-OCT and confocal microscopy 1 month following the CXL procedure. A Fourier-domain OCT system with a corneal adaptor module was used in this study. Two independent examiners (LJ and NB) measured the depth of the demarcation line centrally and 2 mm nasally and temporally from the center. The visibility of the demarcation line was scored (0, line not visible; 1, line visible

but measurement not very accurate; 2, line clearly visible). A cornea-specific in vivo laser scanning confocal microscope was used for this study. After topical anesthesia with tetracaine 1% eye drops (Novartis Laboratories, Inc., New York, NY, USA) and instillation of eye high-viscosity gel (carbomer 3.0 mg/g, Thilogel; Alcon laboratories, Inc., Ft. Worth, TX, USA), patients were asked to fixate using an external fixation target. The central corneal depth of the transition area between the acellular and cellular zones was assessed using the software provided by Heidelberg Engineering by the same 2 independent observers (LJ and NB). For each time point, 2 of the clearest images from each layer were selected. The sub-basal nerve plexus images were defined as the first clear images of the nerves at the level of Bowman's layer. The anterior stromal images were defined as the first clear images immediately posterior to Bowman's layer, whereas the posterior stromal images were defined as the first clear images immediately anterior to the endothelium. All selected images were
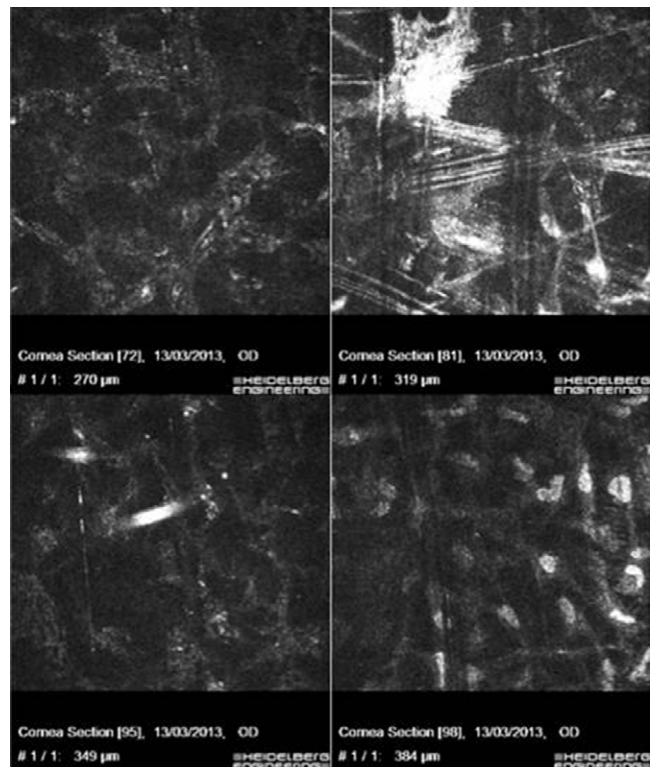

FIGURE 1. In vivo real-time confocal microscopy scans of the corneal stroma obtained 1 month after C-CXL. Consecutive scans at different corneal depths: 270, 319, 349, and 384 µm. Transition zone from acellular (treated) to cellular (untreated) corneal stroma is at 384 µm.
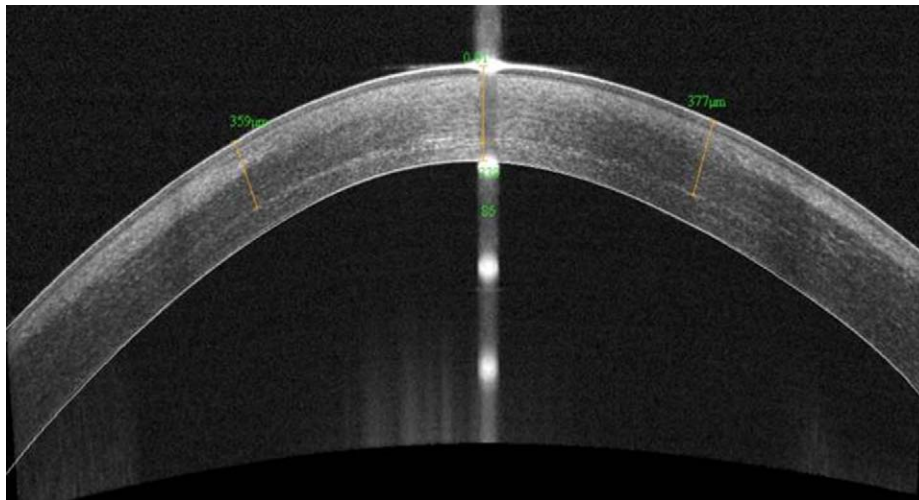
FIGURE 2. High-resolution corneal AS-OCT scan visualizing the corneal stromal demarcation line at a mean depth of 365 μm, 1 month after C-CXL.

deidentified and randomized by an examiner (LJ). Quantitative analysis subsequently was performed by a single masked examiner (NB) using ImageJ software (National Institutes of Health [NIH], Bethesda, MD, USA) for keratocyte density and NeuronJ software (NIH) for corneal sub-basal nerve density. Sub-basal nerve density was calculated by measuring the total length of nerves per image.

All patients received topical anesthesia (tetracaine 1% drops), 1% pilocarpine eye drops before CXL. All procedures were performed using the manufacturers' protocol as follows.

C-CXL Protocol

Calibration was performed preoperatively for an intended irradiance of 3 mW/cm2 (5.4 J/cm2 surface dose). The central 7.0 to 9.0 mm of corneal epithelium was removed by mechanical debridement using a blunt spatula. After abrasion, photosensitizing riboflavin 0.1% in 20% dextran (Ricrolin; Sooft SPA, Montegiorgio, Italy) was applied on the cornea every two minutes for 20 minutes. The central cornea then was irradiated for 30 minutes with 370 nm wavelength UVA light (X-Vega; Sooft SPA) at a 5-cm working distance. Riboflavin eyedrops were applied every 5 minutes during the UVA irradiation. A soft bandage contact lens was placed at the end of surgery until complete re-epithelialization.

A-CXL Protocol

The corneal epithelium was removed in the same way as for C-CXL. After abrasion, photosensitizing riboflavin 0.1% in 20% dextran (VibeX; Avedro, Inc., Waltham, MA, USA) was applied on the cornea every minute for 10 minutes. The central cornea then was irradiated for 3 minutes with UVA light (KXL System; Avedro, Inc.) for an intended irradiance of 30 mW/cm^2 (5.4 J/cm^2 surface dose) at a 5-cm working distance. A soft bandage contact lens was placed at the end of surgery as for the standard protocol.

I-CXL Protocol

The epithelium was not removed. Impregnation of the cornea with a riboflavin 0.1% hypotonic solution (Ricrolin+; Sooft SPA) was performed using the iontophoreris device (I-ON CXLr; Iacer, Veneto, Italy). After the suction ring was taped to the cornea, it was filled with riboflavin 0.1%. The electric intensity was initially 0.2 mA and gradually increased to 1.0 mA. Total iontophoresis time was 5 minutes. The central cornea then was irradiated for 9 minutes with UVA light (X-Vega; Sooft, SPA) for an intended irradiance of 10 mW/cm^2 (5.4 J/cm^2 surface dose) at a working distance of 5 cm.

Postoperatively, patients were instructed to instill topical tobramycin four times daily for 1 week, topical dexamethasone and hyaluronate eye drops four times a day for 1 month, and to take oral analgesics as required.
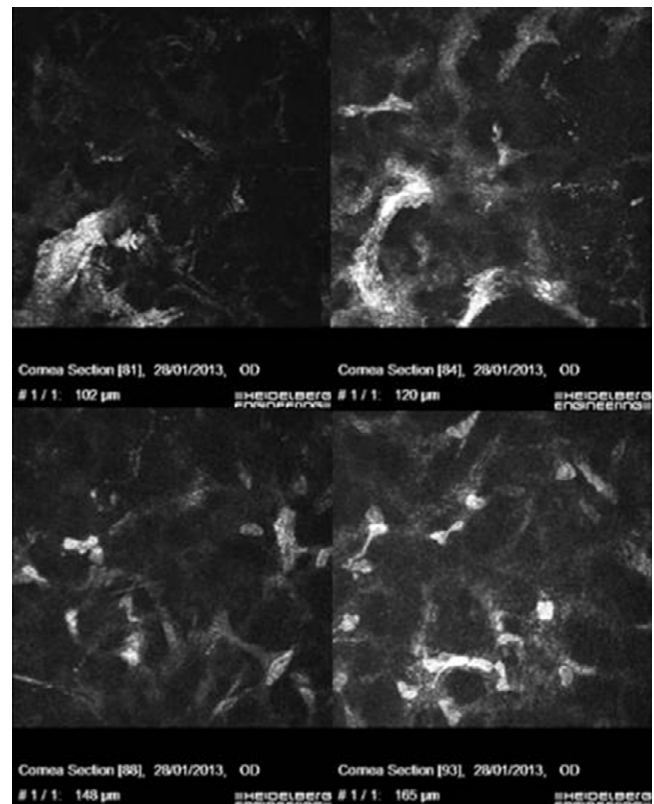

FIGURE 3. In vivo real-time confocal microscopy scans of the corneal stroma obtained 1 month after A-CXL. Consecutive scans at different corneal depths: 102, 120, 148, and 165 μm. Transition zone from acellular (treated) to cellular (untreated) corneal stroma is at 148 μm.
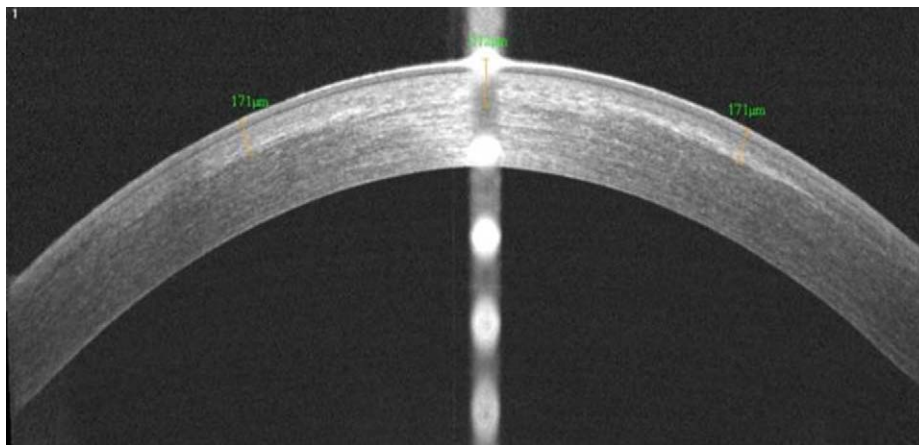
FIGURE 4. High-resolution corneal AS-OCT scan visualizing the corneal stromal demarcation line at a mean depth of 184 μm, 1 month after A-CXL.

Results are presented as mean ± SD for continuous variables and as proportions (%) for categorical variables. For binary outcomes, the stratified Cochran χ^2 test was used for intergroup comparisons of proportions. The Kruskal-Wallis test and the Mann-Whitney U test were used to compare continuous data as appropriate. Wilcoxon signed rank test and paired Student *t*-test were used to statistically evaluate comparisons between preoperative and postoperative continuous data. Spearman rank correlation analysis was used to detect the relationship between the CXL demarcation line depth and the change in BSCVA, CCT, and Kmax at 6 months after each procedure. The Snellen BSCVA was converted to logMAR units for analysis. Corrected *P* values < 0.05 were considered statistically significant. Statistical analysis was done using SPSS for Windows version 20.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

Patients in the three treatment groups were comparable in terms of most of the baseline characteristics (Table 1). There was no statistically significant difference between confocal microscopy and AS-OCT measurements of the corneal demarcation line for both observers. Indeed, the measurement of the corneal demarcation line with AS-OCT was comparable with the measurement of the line with confocal microscopy at 81% (Spearman rank correlation analysis $r^2 = 0.81$, $P < 0.0001$).

The corneal demarcation line visible in AS-OCT and IVCM after C-CXL was measured in 93% at a mean depth of 302.8 μm (SD, 74.6; Figs. 1, 2). For A-CXL it was seen in 87.5% and at a mean depth of 184.2 μm (SD, 38.9; Figs. 3, 4). Finally, for the I-CXL, the corneal demarcation line was seen only in 47.7% at a mean depth of 212 μm (SD, 36.5; Figs. 5, 6). The corneal demarcation line was significantly deeper after C-CXL than after A-CXL ($P < 0.001$). The corneal demarcation line also was significantly deeper after C-CXL than after I-CXL ($P = 0.0101$, Table 2). The demarcation line was significantly more visible with C-CXL and A-CXL than for I-CXL ($P = 0.0057$).

On AS-OCT there was a significant difference in the visibility score of the demarcation line between C-CXL and I-CXL, and between A-CXL and I-CXL ($P = 0.0057$, Table 2).

At 6 months postoperatively, with all protocols, the Kmax, CCT, and BSCVA showed stability (Table 3).

There were no significant correlations of the CXL demarcation line depth with the change in BSCVA ($r^2 = 0.09$, $P = 0.63$), change of Kmax ($r^2 = 0.21$, $P = 0.24$), or change of CCT ($r^2 = 0.08$, $P = 0.63$) at 6 months.

No intraoperative or postoperative complications were observed in any group of patients. Indeed, there were no statistically significant differences between the mean endothelial cell counts before and after CXL (6-month follow-up) for all of the protocols (Table 3).

Compared to baselines values, the mean corneal sub-basal nerve density had significantly decreased at 1, 3, and 6 months postoperatively. The sub-basal nerve plexus was essentially obliterated immediately following C-CXL and A-CXL compared to I-CXL. However, there was no significant difference in sub-
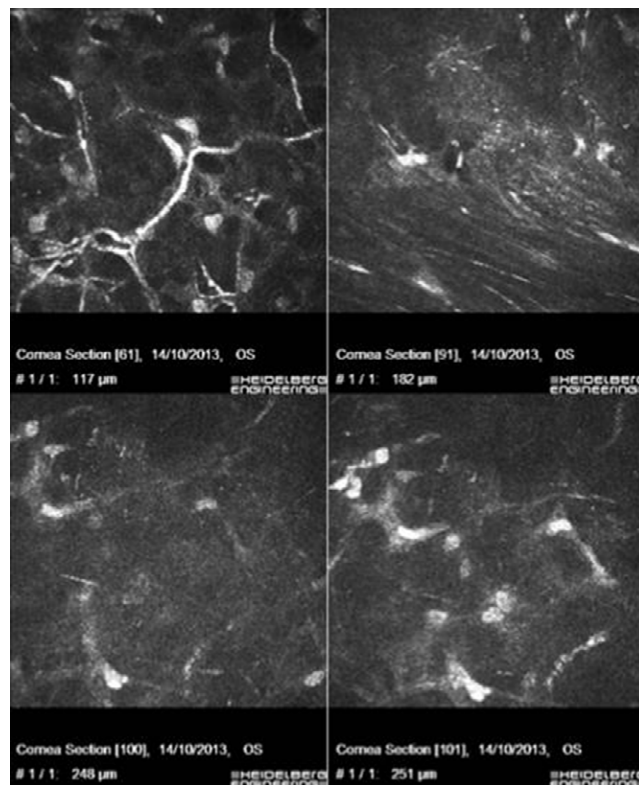

FIGURE 5. In vivo real-time confocal microscopy scans of the corneal stroma obtained 1 month after iontophoresis. Consecutive scans at different corneal depths: 117 (activated keratocytes), 182, 248, and 251 μm. The transition zone from acellular (treated) to cellular (untreated) corneal stroma is at 248 μm.
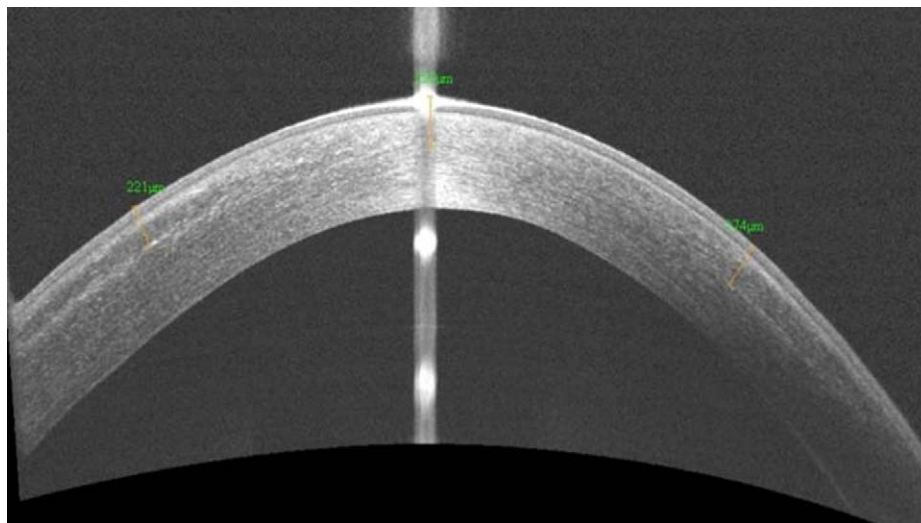
FIGURE 6. High-resolution corneal AS-OCT scan visualizing the corneal stromal demarcation line at a mean depth of 247.5 μm, 1 month after iontophoresis.

basal nerve density from preoperative values at 6 months with I-CXL (Table 4, Fig. 7). During regeneration, the sub-basal nerve plexus exhibited a fragmented appearance before forming an interconnected network.

Quantitative analysis confirmed a significant decrease in the mean anterior keratocyte density 1, 3, and 6 months postoperatively when compared to preoperative values (Table 5, Fig. 8). The C-CXL and A-CXL resulted in significant obliteration of stromal keratocytes compared to I-CXL. Moreover, there was no significant difference in anterior keratocyte density from preoperative values at 6 months with I-CXL.

The demarcation between treated and untreated corneal stroma appeared as a region where normal keratocytes transitioned into elongated, hyper-reflective, structures and then into an area of large hyper-reflective stromal bands. There was no significant change in posterior keratocyte density or endothelial density at any postoperative time point when compared to baseline values.

DISCUSSION

The CXL treatment of progressive keratoconus is based on a photochemical reaction between riboflavin (vitamin B_2) and UVA, and was introduced successfully by Wollensak et al.[3-8] in 2003. To our knowledge, the current study is the first to quantify and compare the microstructural changes that occur in the cornea over time after 3 different collagen cross-linking procedures.

One of the indirect clinical outcomes of CXL efficacy is the corneal demarcation line.[17] This line can be detected using confocal microscopy and AS-OCT.[18] The average treatment depth is approximately 320 μ for a standard CXL with the best

visibility noted 1 month after treatment.[18,19] In our study, we confirmed those findings; we found that absence of keratocytes was evident 1 month after a standard CXL up to 292.3 μm (SD, 74.7) and that there was a stromal demarcation line clearly visible on AS-OCT at a depth of 302.8 μm (SD, 74.6). We also noted stabilization of Kmax and CCT 6 months following the procedure. Our findings confirmed those of previous reports[3-8] demonstrating the effectiveness of standard CXL in stabilizing keratoconus. Recently, Kymionis et al.[20] evaluated the correlation of the corneal demarcation line using confocal microscopy and AS-OCT in keratoconic patients 1 month after a standard CXL. They showed that there was no statistically significant difference between the two measurements. Our results confirmed theirs, since we found a statistically significant correlation between the measurement of the demarcation line with AS-OCT and with confocal microscopy ($r^2 = 0.81$, $P < 0.0001$). Furthermore, previous reports concerning repeatability and reproducibility of the corneal depth measurements with the flap tool show that AS-OCT revealed good correlation among measurements and observers.[22] Nonetheless, as for Yam et al.,[23] our results failed to demonstrate a correlation between the CXL demarcation line depth with change of visual acuity ($r^2 = 0.09$, $P = 0.63$) and change of Kmax ($r^2 = 0.21$, $P = 0.24$) at 6 months postoperatively in all groups.

A recent quantitative study reveals that the mean corneal nerve and mean anterior stromal keratocyte densities were significantly reduced in the first 6 months, returning to normal levels at 12 months following C-CXL.[24] This current quantitative study reveals that the mean anterior stromal keratocyte and mean corneal nerves densities were significantly reduced in the first 6 months following C-CXL and A-CXL, but return to

TABLE 2. Cross-Linking Demarcation Line Mean Depth ± SD Measured at 1 Month Postoperatively With AS-OCT and HRT Following C-CXL, A-CXL, and I-CXL Cross-Linking

CXL Demarcation Line Depth	C-CXL, $n = 15$	A-CXL, $n = 15$	I-CXL, $n = 15$	*P* Value			
				Kruskal-Wallis Test	C-CXL vs. A-CXL	C-CXL vs. I-CXL	A-CXL vs. I-CXL
OCT, mean (SD)	303 (75)	184 (39)	212 (36)	0.0005	$P < 0.0001*$	$P = 0.01*$	$P = 0.113*$
HRT, mean (SD)	292 (75)	148 (46)	183 (81)	0.0004	$P < 0.0001*$	$P < 0.0001*$	$P = 0.396*$

* Mann-Whitney *U* test.

TABLE 3. Evolution of the Kmax (D), CCT, BSCVA, and Endothelial Density Following C-CXL, A-CXL, and I-CXL Cross-Linking

	C-CXL, *n* = 15	A-CXL, *n* = 15	I-CXL, *n* = 15
Evolution of the highest K-value, D			
Preoperative, mean (SD)	52.7 (5.9)	48.9 (6.6)	49.8 (5.6)
6 mo, mean (SD)	50.9 (5.5)	49.4 (6.8)	50.2 (5.0)
P Wilcoxon signed rank test	0.09	0.06	0.28
Evolution of the CCT, μm			
Preoperative, mean (SD)	411 (52)	441 (32)	445 (23)
6 mo, mean (SD)	410 (47)	438 (37)	442 (29)
P Wilcoxon signed rank test	0.55	0.15	0.23
Evolution of BSCVA, logMAR			
Preoperative, mean (SD)	0.33 (0.2)	0.12 (0.3)	0.25 (0.2)
6 mo, mean (SD)	0.28 (0.16)	0.11 (0.4)	0.19 (0.15)
P Wilcoxon signed rank test	0.63	0.91	0.5
Evolution of specular microscopy			
Preoperative, mean (SD)	2764 (251)	2592 (433)	2520 (336)
6 mo, mean (SD)	2760 (336)	2612 (353)	2433 (274)
P Wilcoxon signed rank test	0.46	0.85	0.15

the preoperative status after 6 months with I-CXL. Touboul et al.[25] described confocal microscopy in patients who had undergone A-CXL for keratoconus in a qualitative study. They believed that the extent of keratocyte and corneal nerve apoptosis appeared greater with A-CXL than with the standard technique. In our study, we were able to confirm a significant quantitative difference in anterior keratocyte and corneal nerves densities between C-CXL and A-CXL; anterior keratocyte and corneal nerves densities after A-CXL were significantly lower than after C-CXL 3 months postoperatively, but were similar 6 months postoperatively. Our study further confirmed their findings, since the UVA-riboflavin stiffening effect seems to be concentrated in the anterior 150 to 200 μm of the cornea with greater keratocyte apoptosis and increased stromal tissue reflectivity than with the standard protocol. In the current study, posterior stromal keratocyte and endothelial cell densities were unaffected by the treatment. This agrees with previous studies that generally report the posterior stroma to remain qualitatively unaffected after cross-linking.[24,25] These changes also mirror the observation of a considerable decrease in corneal sensitivity immediately after collagen cross-linking, followed by a gradual recovery in corneal sensitivity over at least a 6-month period.[26] In regard to other refractive surgery procedures, studies report variable periods of time for the restoration of sensory function and innervation following the procedure.[27–30] Corneal denervation has a causative role in dry eye, one of the recognized complications of refractive surgery procedures. An important aspect is that corneal innervation,

sensitivity, and tear function already are initially disturbed in patients with keratoconus.[31–34] Our results demonstrated that the corneal sub-basal nerve plexus in keratoconus is able to return to the preoperative status after almost complete denervation caused by CXL, and is able to return to the preoperative status after 6 months with I-CXL.

Our main findings were that the corneal demarcation line was significantly deeper after a standard CXL protocol than after an accelerated one ($P < 0.001$) or following iontophoresis ($P = 0.0101$). Our results allowed us to discuss the indication and effectiveness of these two new procedures. The accelerated protocol initially was proposed as an alternative treatment to reduce procedure time by delivering a higher irradiance to the cornea.[35] As riboflavin acts as a photosensitizer, the deeper corneal demarcation line with standard CXL may be a consequence of this difference in soaking duration. Although the same number of photons interact with the fibrils in both protocols, since irradiance is 10 times higher with A-CXL, it is conceivable that this may result in endothelial injuries, though this, including in this study, has not been noted to date.[36,37] Thus, A-CXL appears to be a CXL modality that is safe. It also appears to be effective, since we noted stability of the Kmax, CCT, and BSCVA 6 months after the procedure (Table 2). Thus, since A-CXL is effectively limited to a depth of 150 μm, we propose that the A-CXL protocol should be preferentially offered to patients whose minimum corneal thickness is between 350 and 400 μm, as standard CXL requires corneal pachymetry of at least 400 μm to protect the endothelial cells

TABLE 4. Mean Corneal Sub-Basal Nerve Density ± SD (nerve/mm²) Preoperatively and at 1, 3, and 6 Months Postoperatively Following C-CXL, A-CXL, and I-CXL Cross-Linking

	C-CXL	A-CXL	I-CXL	*P* Value C-CXL vs. A-CXL	*P* Value C-CXL vs. I-CXL	*P* Value A-CXL vs. I-CXL
Preoperative	17.75 ± 3.69	17.59 ± 4.23	17.14 ± 2.94	*P* = 0.633*	*P* = 0.548*	*P* = 0.984*
1 mo postoperative	2.58 ± 0.47	1.39 ± 0.53	4.22 ± 1.10	*P* = 0.037*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* < 0.001†			
3 mo postoperative	5.89 ± 1.23	3.89 ± 1.00	8.29 ± 1.27	*P* = 0.029*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* < 0.001†			
6 mo postoperative	10.04 ± 1.82	8.84 ± 0.68	15.48 ± 1.48	*P* = 0.105*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* = 0.083†			

* Mann-Whitney *U* test.
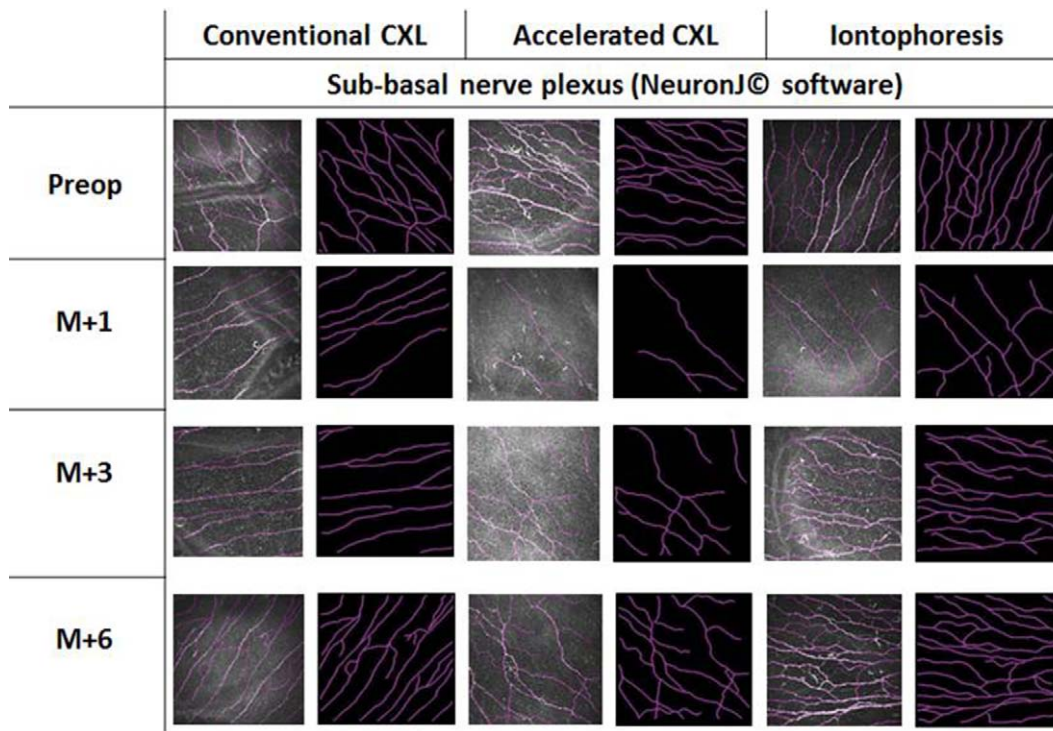† Wilcoxon signed rank test.

FIGURE 7. Corneal confocal microscopy images of the sub-basal nerve plexus before CXL and following C-CXL, A-CXL, and I-CXL cross-linking, preoperatively (preop), and at 1 (M ± 1), 3 (M ± 3), and 6 (M ± 6) months postoperatively. the HRT images are 400×400 µm. Quantitative analysis was performed using NeuronJ software for sub-basal nerve density.

unless hypo-osmolar riboflavin also is used.[38] Intensification of the photochemical effect in the anterior part of the stroma with A-CXL also could lead to subsequent differences in tissue biomechanics, and may have implications in terms of tissue healing response and long-term clinical results. Nonetheless, A-CXL should be used as a less penetrating treatment to stabilize the progression of keratoconus in thinner corneas. Longer follow-up after A-CXL will be necessary to determine whether any subsequent tissue changes occur late in the recovery phase, to confirm the efficacy and safety of this procedure, and to correlate the depth of the demarcation line to the effect on corneal biomechanics. Iontophoresis is one of several trans-epithelial protocols developed to avoid the necessity for epithelial debridement.[39] Bikbova et al.[12] examined 22 eyes that underwent iontophoresis in a prospective study. They found a corneal demarcation line clearly visible on OCT and Heidelberg retinal tomography (HRT) at 1 month following treatment at a depth of 200 to 250 µ in all of their patients. Thus, they concluded that iontophoresis might be an effective method for riboflavin impregnation of the corneal stroma. However, their protocol for iontophoresis differed from ours. They used another iontophoresis device, the "galvanizator" (Potok-1; Russian Federation, Moscow, Russia) where riboflavin is administered over 10 minutes and the cornea then irradiated with a standard UVA light (370 nm, 3 mW/cm²; Ufalink, Russian Federation, Ufa, Russia) for 30 minutes. The differences in these two techniques of iontophoresis, may explain the differences between our results and theirs. The reduced time of riboflavin exposure (5 minutes) in our protocol may not allow a sufficient penetration of riboflavin into the cornea. We found the corneal demarcation line with AS-OCT in half (47.7%) of the patients at a mean depth of 212 µm. Indeed, Caporossi et al.[11,40] investigated transepithelial crosslinking using modified riboflavin (Ricrolin TE) and confirmed that Epi-ON protocol resulted in keratoconus instability after 24 months of follow-up, especially in pediatric patients for whom this disease is known to have a more aggressive course. This lack of efficacy can be explained by

TABLE 5. Mean Anterior Stromal Keratocyte Density ± SD (cells/mm²) Preoperatively and at 1, 3, and 6 Months Postoperatively Following C-CXL, A-CXL, and I-CXL Cross-Linking

	C-CXL	A-CXL	I-CXL	*P* Value C-CXL vs. A-CXL	*P* Value C-CXL vs. I-CXL	*P* Value A-CXL vs. I-CXL
Preoperative	377.5 ± 30.39	375 ± 38.18	364.1 ± 46.53	*P* = 0.678	*P* = 0.290	*P* = 0.534
1 mo postoperative	106.1 ± 18.65	91.40 ± 20.34	176.7 ± 21.75	*P* = 0.042*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* < 0.001†			
3 mo postoperative	239.7 ± 30.99	195.6 ± 21.32	306.1 ± 19.47	*P* = 0.006*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* < 0.001†			
6 mo postoperative	298 ± 33.00	275.5 ± 30.83	356.9 ± 29.63	*P* = 0.089*	*P* < 0.001*	*P* < 0.001*
	P < 0.001†	*P* < 0.001†	*P* = 0.909†			

* Mann-Whitney *U* test.
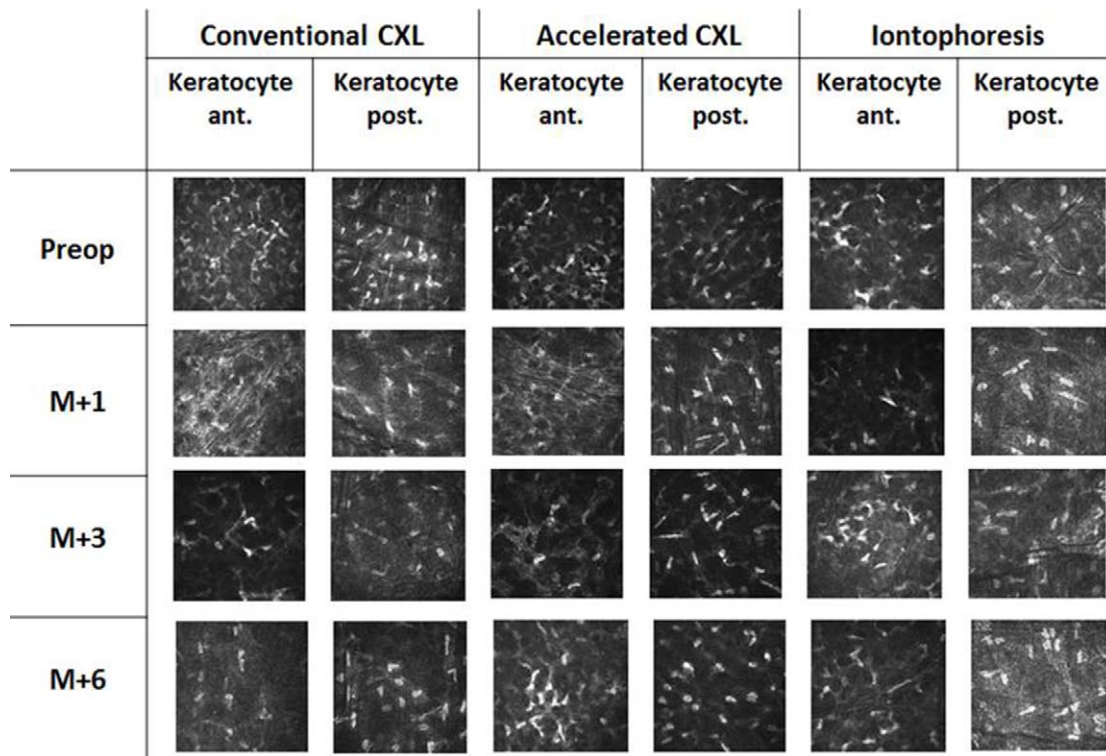† Wilcoxon signed rank test.

FIGURE 8. Corneal confocal microscopy images of anterior and posterior keratocytes before CXL, and following C-CXL, A-CXL, and I-CXL cross-linking, preoperatively (preop), and at 1 (M ± 1), 3 (M ± 3), and 6 (M ± 6) months postoperatively. At 1 month postoperatively, anterior stromal edema with hyper-reflective cytoplasm and extracellular lacunae, a honeycomb-like appearance, was observed and persisted at 3 months for each procedure. At 6 months, repopulation of the anterior stroma with keratocyte nuclei was apparent. The HRT images are 400×400 µm. Quantitative analysis was performed using ImageJ software for keratocyte density.

limited UVA stromal penetration and inhomogeneous character of riboflavin penetration with the epithelium in situ.[11,41,42] Indeed, the presence of epithelium in situ is a physical barrier for riboflavin and also for UVA penetration, limiting the depth of apoptotic effect and the corneal biomechanical strength.[11] In addition, during UV exposure, riboflavin serves as a photosensitizer and as a UV light blocker.[37] Consequently, we could hypothesize that if insufficient riboflavin penetrates the cornea during iontophoresis, not only this will limit the efficacy of the procedure, but it also could damage the endothelial cells. Nonetheless, in our study, we did not find any endothelial loss after the I-CXL protocol and we found that the Kmax, CCT, and BSCVA all seemed stable 6 months after the procedure. However, a longer follow-up is necessary to conclude on the efficacy and safety of this new procedure and we must remain cautious with use of iontophoresis, as with the other Epi-ON protocols. Nonetheless, the enthusiasm for transepithelial CXL is easily understood, due to the reduction of CXL complications.[9] We believe that, at present, I-CXL should be used with extreme caution in pediatric patients, and preferentially proposed to patients with thin corneas and with slowly progressive keratoconus. Limitations of our study include the small sample size. A longer follow-up with a larger cohort of patients in different age groups is necessary to understand the actual impact of these different CXL protocols on stabilization of keratoconus and to determine which protocol is best suited to each patient.

In conclusion, iontophoresis was associated with reduced damage of corneal sub-basal nerves and anterior keratocytes compared to conventional procedures, but the demarcation line was present in less than 50% of cases and was more superficial than with the former procedures. In the I-CXL, a small part of the UV light is absorbed by the epithelium (15%–20%) and this reduction should be compensated by increasing the treatment time by approximately 20%.[43] The use of iontophoresis still is under investigation and should be considered with greater caution.

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