UNIVERSITY OF MEDICINE AND PHARMACY

“CAROL DAVILA” BUCHAREST

DOCTORAL SCHOOL

FACULTY OF MEDICINE

RESEARCH ON SPECULAR MICROSCOPY ANALYSIS IN THE DIAGNOSIS AND FOLLOW-UP OF PATIENTS WITH KERATOCONUS

PHD THESIS SUMMARY

PhD supervisor:

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PhD student:

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List of published scientific papers

Articles *in extenso* published in ISI indexed journals

1. **Impact of corneal crosslinking on endothelial and biomechanical parameters in keratoconus**, Maria-Silvia Dina, Maria-Cristina Marinescu, Catalina-Gabriela Corbu, Mihaela-Monica Constantin, Catalina-Ioana Tataru, Calin-Petru Tataru, **Journal of Clinical Medicine**, Impact factor at publication **3.0** (Chapter II.5).

Articles *in extenso published in journals indexed in international databases (IDB)*

2. **Corneal topography - review of available methods of investigation and impact in the diagnosis and follow-up of keratoconus**, **Maria-Silvia Dina,** Maria-Cristina Marinescu, Catalina-Gabriela Corbu, Mihaela-Monica Constantin, Catalina-Ioana Tataru, Calin-Petru Tataru - **Maedica**, Volume 20, No. 2, 2025, indexat Pubmed indexed (Chapter I.2.4)

3. **Standard and accelerated crosslinking protocols in keratoconus - differences and evolution at one year - Romanian Journal of Ophthalmology**, Volume 69 Issue 2, 2025 -Pubmed indexed (Chapter II.5)

Introduction

Keratoconus (KC) is an ectatic corneal disease characterized by progressive corneal thinning, leading to myopia, astigmatism, and a decrease in visual acuity that cannot be corrected by air-assisted optical correction [1]. The condition is bilateral, asymmetric with an insidious evolution that appears in the young population during puberty, evolving in the second and third decades and stabilizing around the age of 40. Slit-lamp examination findings in the early stages of keratoconus may appear normal, becoming apparent only in the advanced stages. Visual acuity is reduced, leading to a lower quality of life in young adults. The incidence is estimated at 1.5 - 25 cases per 100,000 people/year, with a higher rate in patients from the Middle East and Asia [1].

Corneal topography is essential in the early diagnosis of the disease, analyzing the corneal geometry and thus helping to establish the treatment method and follow the evolution of patients. This investigation allows the staging of keratoconus, providing specific markers of this pathology and supporting clinicians in better management of this underdiagnosed condition that affects young people.[2]

Corneal biomechanical properties are increasingly important in ocular pathology, being useful both in the progression of glaucoma, in refractive errors and in the pathology of keratoconus.[3][4][5] Corneal hysteresis (CH) and corneal resistance factor (CRF) can be measured using the Ocular Response Analyzer (ORA), with studies showing that the values of these biomechanical parameters are statistically significantly lower in keratoconus compared to healthy individuals. In addition, as keratoconus progresses, corneal biomechanics appear to be more affected. [18]

A significant step in the treatment of keratoconus has been represented by corneal collagen cross-linking (CXL), introduced into clinical practice in the 2000s, which stops the progression of ectasia[6]. CXL aims to stabilize corneal ectasia, being a surgical procedure used to increase the biomechanical strength of the cornea by forming chemical bonds between collagen fibers, which leads to inhibition of keratoconus progression, thus maintaining visual acuity over time and preventing complications [7][8][9].

The CXL technique of epithelial removal was first applied to human keratoconus corneas in 1998 and consists of epithelial removal, after which the corneal stroma is saturated with riboflavin (vitamin B2), a photosensitizer, for 30 minutes. Then, ultraviolet A radiation (UVA, 370 nm) is applied, which produces reactive oxygen species, inducing

interfibrillar collagen crosslinks. The reason for epithelial removal has been described as allowing adequate penetration of riboflavin into the stromal tissue, where it absorbs UVA light and produces effective crosslinks between the collagen fibers in the corneal stroma. Numerous corneal crosslinking techniques have been created over the years in an attempt to find a method that combats the ocular discomfort created by corneal deepithelialization and the long time of the procedure and that provides the same result of stopping the progression of the disease. [10][11][12]

Specular microscopy is a noninvasive technique that allows us to visualize and analyze the corneal endothelium. Corneal endotheliopathy is a broad term used to classify several diseases and clinical conditions that affect the structure and function of the corneal endothelium. One of the physiological functions of the endothelium is to secrete a collagen matrix that forms Descemet's membrane, but the main physiological function of the corneal endothelium is to maintain the health and transparency of the corneal stroma. [13].

Corneal crosslinking techniques that aim to interrupt the ectatic progression of keratoconus were created in such a way as not to damage the corneal endothelium, whose regeneration capacity is limited. However, over the years, studies have not been able to determine whether endothelial changes occur in this pathology, the research being contradictory.

The pathology is still not fully understood, which makes this paper address a topical issue in corneal ectatic diseases and aims to evaluate the corneal endothelium, correlating the parameters provided by specular microscopy with the topographic and biomechanical parameters of the cornea. By studying the two groups of patients, I aimed to follow the corneal endothelial parameters according to the stage of the corneal disease and to investigate whether there are any changes after the crosslinking intervention. Considering that the corneal demarcation line can be followed after the procedure to observe the degree of depth of the corneal reticulation, I will analyze whether there are differences between the two epi-off CXL techniques (standard and accelerated) that raise the issue of endothelial damage or biomechanical and topographic parameters.

Study Hypothesis and general objectives

The purpose of this research is to explore the evolution of endothelial, biomechanical and corneal topographic parameters in patients with keratoconus, before and after the intervention to stop the progression - corneal crosslinking, either the standard protocol or the accelerated protocol.

The objectives of the first study aim to deepen a series of main ideas and correlational analyses, based on the group of patients enrolled in the study:

- Evaluation of the existence of correlations between the staging of the condition and the parameters measured using specular microscopy, Ocular response analyzer and corneal topography;
- Evaluation of topographic parameters (minimum, maximum, average keratometry, corneal astigmatism), endothelial (cell density, average cell surface, variability and hexagonality of cells, both central and paracentral), biomechanical (corneal hysteresis and corneal resistance factor) and corneal thickness before the epi-off corneal crosslinking procedure and one month and 6 months after the procedure;

In the second study, a series of hypotheses related to the comparison of the two corneal crosslinking procedures, the standard protocol and the accelerated protocol, were evaluated:

- Evaluation of corneal topographic parameters (minimum, maximum, average keratometry, corneal astigmatism), central corneal thickness, corneal biomechanics (corneal hysteresis and corneal resistance factor) before the standard protocol corneal crosslinking procedure and 12 months after the procedure and evaluation of the demarcation line 12 months after the procedure, both standard and accelerated, to observe whether there are differences between the two procedures applied and whether the depth of the demarcation line differs between the two procedures such that endothelial damage occurs.

Study I: Effect of crosslinking on the corneal endothelium in keratoconus

Materials and methods

This study is designed as a retrospective, non-randomised, interventional study. All patients who presented at the Oftaclinic Clinic in Bucharest between 2019 and 2022 were screened for inclusion to the study. The study cohort was formed by applying the inclusion and exclusion criteria. All patients, and, in the case of minors, their legal representatives, offered informed consent. The study was approved by the Oftaclinic Ethics Committee.

The inclusion criterion was the diagnosis of progressive keratoconus. Keratoconus was diagnosed and staged according to the Amsler-Krumeich staging, and progression was defined as an increase of at least 1 diopter (D) within a year of follow-up of the steepest corneal meridian (Kmax), decreased visual acuity or thinning of the cornea by more than 5% of the corneal thickness [14].

Patients were excluded from the study if they presented other ocular pathologies (vitreoretinal pathology, glaucoma, ocular hypertension, cataract, history of corneal surgery or corneal disease - particularly Fuch's endothelial dystrophy), if they were pregnant or breastfeeding, or if they were not compliant with the investigations needed (for example, low waveform score in Ocular Response Analyser testing), if they presented any of the known CXL contraindications: known sensitivity against ingredients used during the procedure, corneal thickness under 400 microns, history of corneal pathology [15].

All patients underwent a complete ophthalmological examination, which included visual acuity (without and with correction), biomicroscopy, manifest refraction, corneal topography (Topcon, Tokyo, Japan), corneal biomechanics examination (Ocular Response Analyser), central corneal thickness, and specular microscopy (Nidek, Gamagori, Japan).

The data analyzed within this study originates from the pre-intervention consultation and from the consultation performed 1 month and 6 months after the CXL intervention.

The data analyzed in this article was tested with Levene's Test, followed by the dependent t Test, in order to identify significant differences between examinations (same patient - before the intervention, one month and 6 months after the intervention). The 4 keratoconus stages were compared using the One-way ANOVA test, followed by the post-hoc analysis Tukey test.

Pearson's correlation coefficient ("Pearson's r") was calculated to determine the degree of correlation between variables (weak correlation: Pearson's r between 0.3 and −0.3, moderate correlation: 0.3 - 0.5 or −0.3 - −0.5, strong correlation: over 0.5, under −0.5). The p value of 0.05 was considered the threshold for statistical significance. Statistical analysis was performed using the Statistical Package IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA).

Results

The study includes 47 patients, which provided 66 eyes for analysis (diagnosed with progressive keratoconus and recommended corneal crosslinking). 17.02% of patients were female, and 82.98% were male. The average age was 25.43 years old (SD 6.68). According to the Amsler-Krumeich classification, 48.48% of eyes were stage I Keratoconus (32 eyes), 33.33% stage II (22 eyes), 12.12% stage III (8 eyes) and 6.06% stage IV (4 eyes).

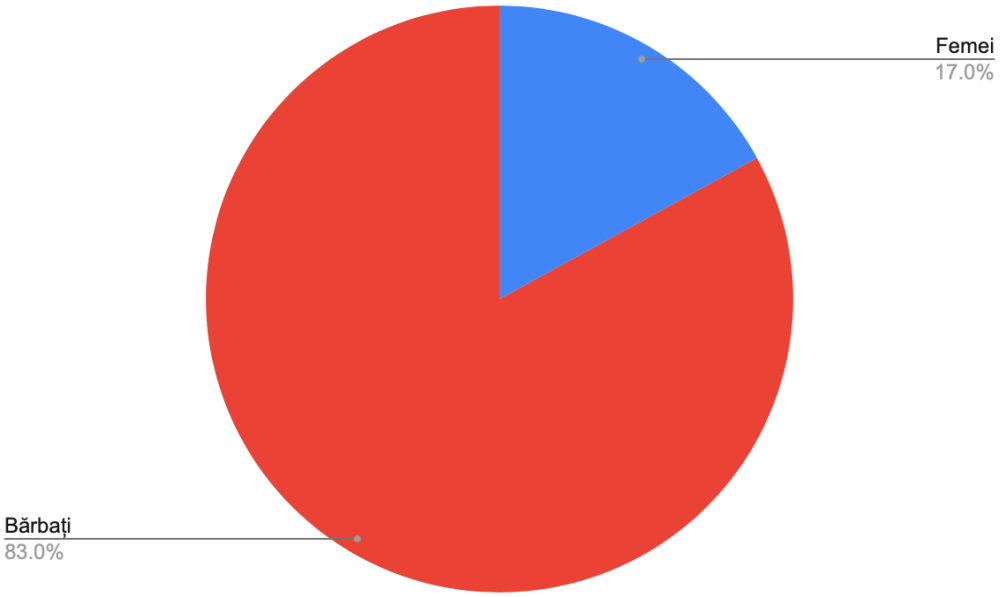


Figure 1. Distribution of patients with keratoconus by gender

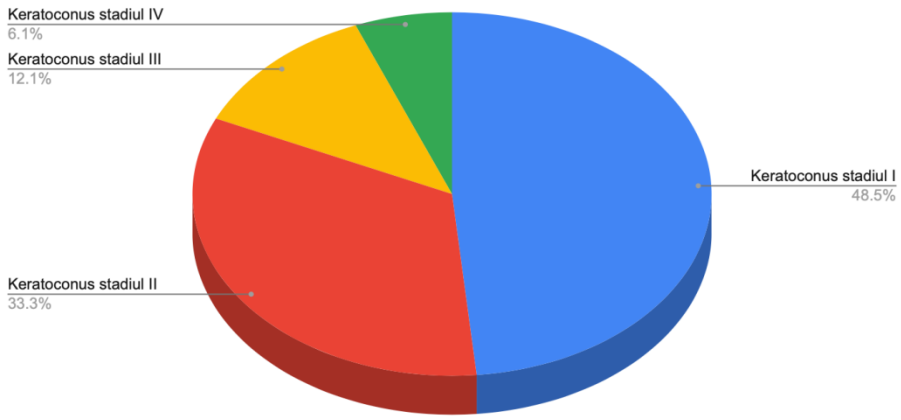


Figure 2. Distribution of patients according to the stage of Keratoconus

The endothelial parameters did not differ significantly between the different KC stages: as the ectasia was more advanced, the cell variability was increased (p value 0.033) (mean value of 24.81% in stage I, 24.77% in stage II, 26.00% in stage III and 28.75% in stage IV), however hexagonality was not significantly modified (67.19% in stage I, 69.96% in stage II, 65.50% in stage III and 71.25% in stage IV). However, other parameters differed significantly: CRF was smaller (p value 0.006), and the central cornea was thinner (p <0.001). Also, topographical parameters were more advanced, with higher minimum, maximum and average keratometry and astigmatism (< 0.001) in more advanced keratoconus.

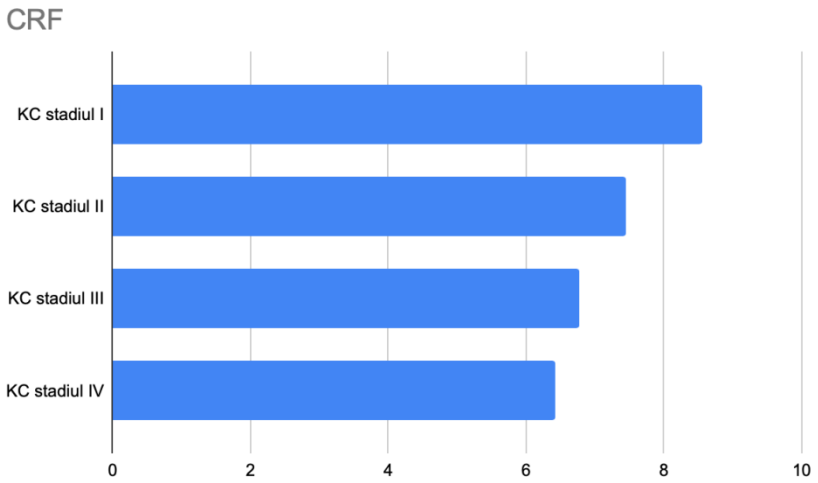


Figure 3. Distribution of the mean value of the corneal resistance factor between the stages (before CXL)

The Stage I Keratoconus group was composed of 32 eyes. The paired T-test, comparing pre-CXL variables with post-CXL values at one and six months, reveals that very few parameters are modified after the procedure. The number of cells (central and paracentral), the hexagonality and paracentral cell density are significantly decreased at 6 months compared to baseline, cell variability is significantly increased both at one and at 6 months compared to baseline.

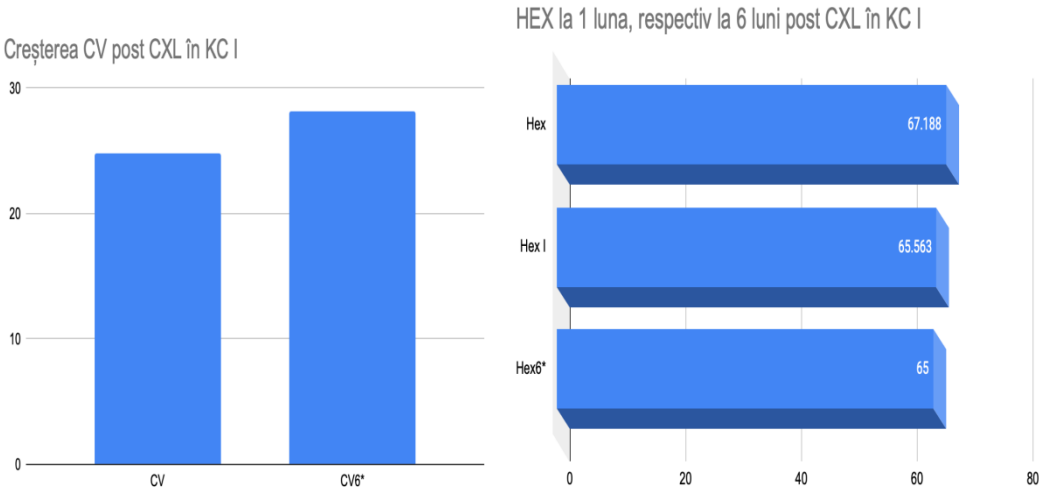


Figure 4. Increase in the mean value of the coefficient of variation at 6 months post CXL in KC I and **Figure 5.** Decrease in the mean of hexagonal cells post CXL in stage I KC

The Stage II KC group was composed of 22 eyes. The T-test, comparing pre-CXL parameters with post-CXL parameters at 1 and 6 months, revealed small changes after the procedure, but the differences became significant after 6 months. Cell density decreased significantly at 6 months, variability increased significantly compared to the baseline group. Also, both Kmax and K average decreased significantly at 6 months post-CXL.

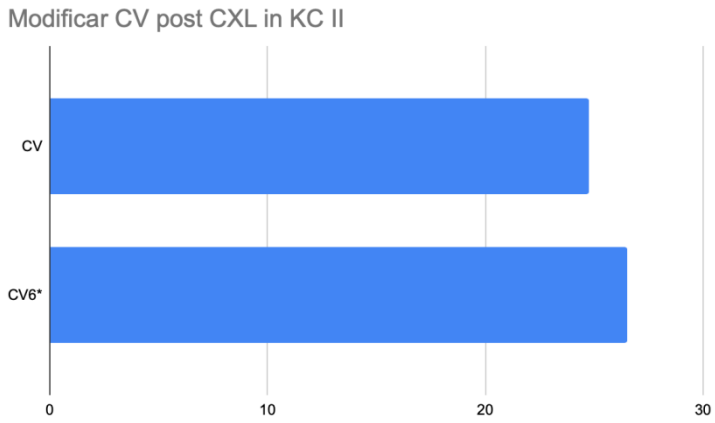


Figure 6. Increase in coefficient of variation at 6 months post CXL in KC II

The Stage III Keratoconus group was composed of 8 eyes. The paired T-test, comparing pre-CXL variables with post-CXL values at one and six months, reveals that CRF significantly improves after one month, and paracentral hexagonality is affected only after 6 months.

The Stage IV Keratoconus group was composed of 4 eyes. The paired T-test, comparing pre-CXL variables with post-CXL values at one and six months, reveals only a modified CRF average at one month after the procedure.

Correlations in the entire cohort

There were several statistically significant correlations between paracentral endothelial parameters and topographic variables in the initial cohort. There are significant, weak, positive correlations between paracentral cell variability and either average keratometry and minimum keratometry. There are also significant, moderate, negative correlations between the paracentral number of endothelial cells and either maximum, minimum or average keratometry.

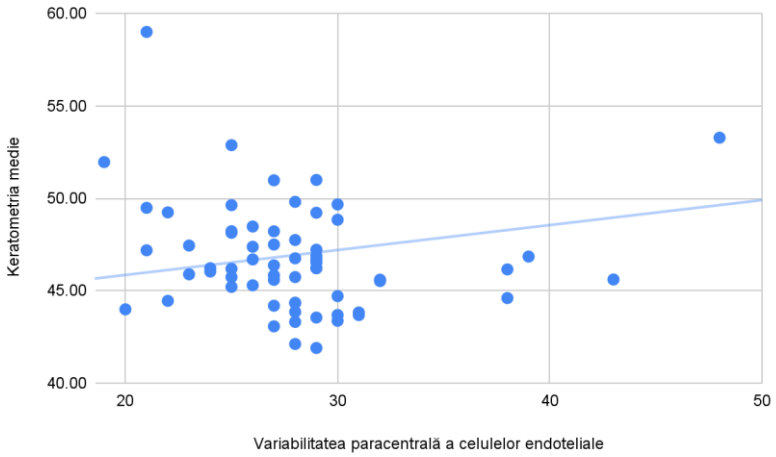


Figure 7. Correlations between mean keratometry and paracentral cellular variability, across the entire cohort studied before crosslinking.

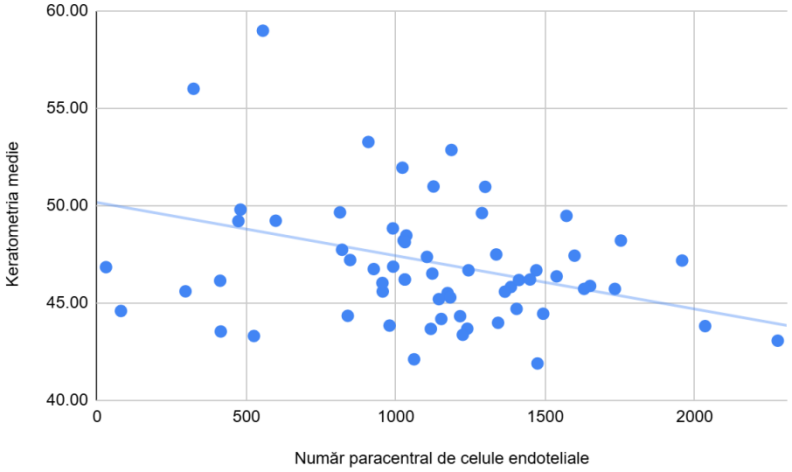


Figure 8. Correlations between mean keratometry and paracentral endothelial cell count, across the entire cohort studied before crosslinking.

Also, as expected, there are significant correlations between biomechanical parameters and topographic parameters - CH has negative, moderate correlations with minimum, maximum and average keratometry, CRF has negative, moderate correlations with minimum, maximum and average keratometry and astigmatism, and CCT has negative, moderate-strong correlations with minimum, maximum and average keratometry.

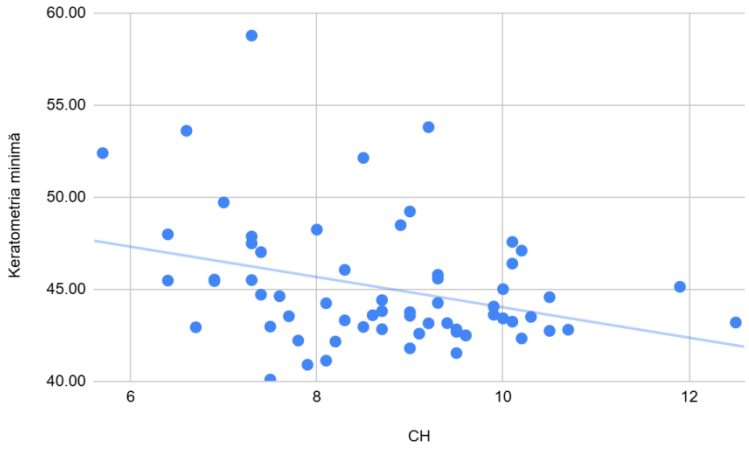


Figure 9. Correlations between minimal keratometry and corneal hysteresis, in the entire cohort studied before crosslinking.

Discussion

In this study we identified several differences between keratoconus eyes, depending on how advanced the corneal ectasia is, although this study was conducted on a small number of patients. According to the Amsler-Krumeich classification, the higher the stage, the higher the corneal astigmatism and average keratometry and the thinner the cornea, these significant differences being found in our cohort as well.

Sturbaum and Peiffer (1993) found that in the early stages of keratoconus, the endothelium appears normal. As the condition progresses and the corneal stroma thins, the endothelial cells flatten. This likely reflects the stretching of the endothelial cells as they attempt to maintain continuity across the progressively ectatic posterior surface [16]. Later in the course of the disease, endothelial changes include pleomorphism, polymegathism, endothelial cell degeneration, and exposure of fibrin, inflammatory cells, or both on the endothelial surface. Endothelial and Descemet membrane ruptures occur in 11% to 35% of keratoconic corneas, and healing of these defects can also lead to pleomorphism and polymegathism [16]. While advancing endothelial cell loss may decrease visual acuity through the loss of stromal transparency, unfortunately there is no definitive treatment to halt the loss of cells, except corneal transplantation in advanced cases [17]

There has been research in the literature hinting at an altered endothelial cell layer in keratoconus. A large study of over 700 eyes revealed that more advanced cases present a lower cell density and a higher variability [18]. This is probably due to the small number

of patients with advanced stages included in our study. These results are similar to ours, however only the variability was significant in our cohort. On the other hand, there are studies in the literature which found no differences in density, variability and hexagonality between Amsler-Krumeich KC stages [19].

A study applying deep learning technologies has found that an average number of cells in keratoconus is 175.00, comparable but slightly higher than our value of approximately 137 found in the present study [20]. More importantly, this average differed significantly to that of myopic eyes, serving as a control group [20]. Another recent study has included Egyptian keratoconus patients, with Stages 1-3 Amsler-Krumeich, and has found a significant decrease in cell density and an increase in variability [21]. Compared with our cohort, these parameters revealed a more significant alteration of the endothelial layer, suggesting that more research is needed to understand the endothelial behaviour of keratoconus in different populations and which factors may influence the endothelial health. Importantly, in our cohort, the average central endothelial cell density was sufficient to maintain corneal transparency (over 2900 cells/mm2), and the decrease in cell density was non-significant following CXL.

Keratoconus is a disease which involves the degradation of collagen fibers. Histologically, KC is characterised by a lower number of collagen fibers, in conjunction with a thinner stroma, abnormal keratocytes, affected Bowman's membrane and basement membrane with iron deposits [22]. Increased expression of the matrix metalloproteinase (MMP-1) leads to extracellular matrix degradation, affecting collagen, and a decreased expression of an enzyme responsible for natural collagen crosslinking contributes to a weaker corneal structure in keratoconus [23], with the addition of a higher lacrimal concentration of inflammatory markers in keratoconus patients [24]. As collagen is responsible for the elasticity and resistance of the cornea, it is known that CH and CRF are lowered in keratoconus. A large comparative study found an average of 7.5 mmHg and 6.2 mmHg for CH and CRF, respectively, significantly lower than the 10.8 and 11.0 averages in normal eyes [25]. These values are comparable with the averages obtained in our study. Further, as expected, keratoconus variables (average keratometry and corneal thickness) correlate well with CH and CRF, similar to other results in the literature [26].

Biomechanical evaluation of the cornea holds great value, as focal alterations may appear before the topographical alterations become evident, therefore holding great potential for early ectasia diagnosis [27]. CH, and even moreso, CRF, present good accuracy in differentiating normal and ectatic corneas [27]. Similarly, in our study only

CRF, not CH, has differed significantly between stages of keratoconus, supporting the role of diagnostic and staging in the variations of CRF.

Besides the variable relationship with the disease stage, in our cohort endothelial properties correlated with other parameters specific to KC - namely corneal astigmatism and keratometry readings (minimum, maximum and average). These findings have been previously suggested in the literature, with endothelial cell density being negatively correlated with the steepest and flattest corneal meridian, and positively correlated with the minimum corneal thickness [28].

Corneal crosslinking is a well established treatment in keratoconus, with known improvements in the visual acuity, refractive values and topographical aspect [29]. In our study, there have been no statistically significant changes in terms of corneal topography or biomechanics, as the follow-up reported here was only up to 6 months. Studies reporting from one year, up to 7 years of follow-up, describe significant improvements in keratometry and refractive errors, however less so in biomechanical parameters [30][31]. On the other hand, certain endothelial alterations have been observed in our cohort, such as increased cell variability and average cell area, and decreased paracentral number of cells. A study similar to ours, with a follow-up of one year, has not found any differences in terms of cell density, however has not investigated other endotelial parameters that we found different before and after CXL [32]. Another recent study has revealed a statistically significant decrease in cell density after 6 months, however the final density does not impede endothelial function and corneal clarity [33]. The significant variation of endothelial parameters in the paracentral area after crosslinking obtained in our study could also be explained by the fact that the paracentral area in the patients in the study is the most deformed area and with the lowest corneal resistance (it coincides with the area of corneal ectasia). While these modifications were statistically significant, it is unlikely that they are clinically significant. The usage of riboflavin prevents UVA damage to the endothelial cells, and more studies have reported no density decrease and far fewer reported decrease in endothelial cell density after CXL [34]. Most likely, significant changes occur in very thin keratoconic corneas, where the endothelium experiences a higher dose of UVA [35]. The UVA reaction from the crosslinking process damages subbasal nerves [36][37], which are essential in the endothelial pump. In this way, changes occur in the endothelial cells. Over time, endothelial cells that have not been affected tend to replace the damaged cells [36].

In our study, a significant increase in hexagonality resulted. In Stage IV nerve fibers are more evident and probably through the irradiation process the function of the endothelium is affected and subsequently the regeneration process leads to an increase in hexagonality. Stromal edema begins to reduce after the first month after CXL, when nerve fiber regeneration and an increase in stromal density also occur [37]. However, the low number of subjects in the AK stage IV represents a limitation of our study, therefore more research is needed to determine if this post-CXL increase in hexagonality is reproduced in other cohorts.

Study II: Comparing standard and accelerated crosslinking in keratoconus

Materials and methods

This retrospective study evaluates the evolution of keratoconus cases one year after accelerated or standard CXL were performed using epi-off method. All patients who presented at the Oftaclinic Clinic in Bucharest between 2021 and 2022 and were screened for inclusion criteria. Patients and their legal representatives (in the case of minors), offered informed consent. The study was approved by the Oftaclinic Ethics Committee.

All patients underwent a complete ocular consultation, which also included records of refraction (using auto-refractometer records), maximum and minimum corneal dioptres measured with Aladdin corneal topography (Topcon, Tokyo, Japan), demarcation line depth, measured with Optical Coherence Tomography (OCT), central corneal thickness (CCT), corneal biomechanical properties: corneal hysteresis (CH) and corneal resistance factor (CRF), measured with Ocular Response Analyzer (ORA) (Reichert Ophthalmic Instruments Inc, Depew, NY, USA).

Patients were included if they met criteria for progressive keratoconus: increase of corneal diopter on the steepest meridian (Kmax) more than 1 D, increase of average of corneal diopter more than 0.75 D, increase of spherical equivalent (SE) more than 0.50 D, thinning of corneal thickness more than 5% or more than 20 microns, history of decreased visual acuity.

Exclusion criteria were represented by corneal thickness less than 400 microns, history of keratitis or corneal surgery, pregnancy and corneal scars.

The Amsler Krumeich classification was used to stage the keratoconus cases, a system which accounts for the degree of myopia and astigmatism, mean keratometry and minimal central corneal thickness [38]

To identify significant differences between time points of the same patient (before and after CXL), the Levene's Test, followed by the dependent t Test, was performed. In order to identify significant differences between either the accelerated and the standard procedure, the Levene's Test, followed by the independent t Test, was performed. The degree of correlation between variables was calculated using Pearson's correlation coefficient ("Pearson's r"), and correlations were deemed weak (Pearson's r between 0.3 and −0.3), moderate (Pearson's r 0.3 - 0.5 or −0.3 - −0.5) and strong (Pearson's r over 0.5, under −0.5). The p value of 0.05 was considered the threshold for statistical significance. Statistical analysis was performed using the Statistical Package IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA).

Results

The **accelerated** CXL cohort was composed of 20 eyes, 15.00% female and 85.00% male, with an average age 24.55 ± 6.27 years old. According to the Amsler Krumeich criteria, two eyes were diagnosed with stage I keratoconus, ten eyes were stage II, two eyes were stage III and six eyes were in stage IV.

The **standard** CXL cohort was composed of 21 eyes, 33.33% female and 66.67% male, with an average age 19.80 ± 5.99 years old. According to the Amsler – Krumeich criteria three eyes were diagnosed with stage I of keratoconus, nine eyes with stage II, two eyes with stage III and seven eyes with stage IV.

The two cohorts had similar baseline characteristics. However, the age was significantly lower (p value 0.018), and the refractive cylindric value was significantly higher in the standard procedure group (p value 0.026).

The accelerated crosslinking protocol led to significant improvements. After CXL, the cylindrical refractive value, spherical equivalent and Kmax were significantly lowered, and CCT and CRF were significantly higher.

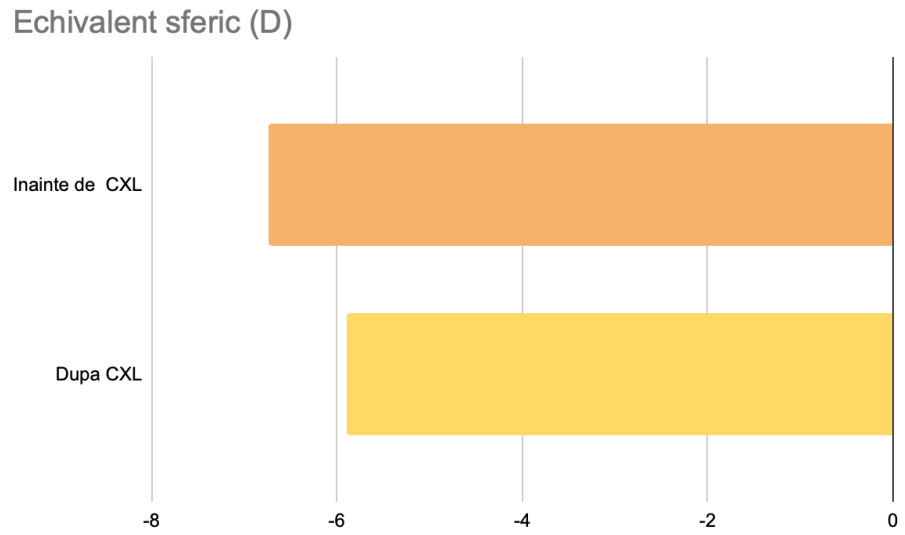


Figure 10 Spherical equivalent in patients who underwent accelerated CXL after and before the intervention

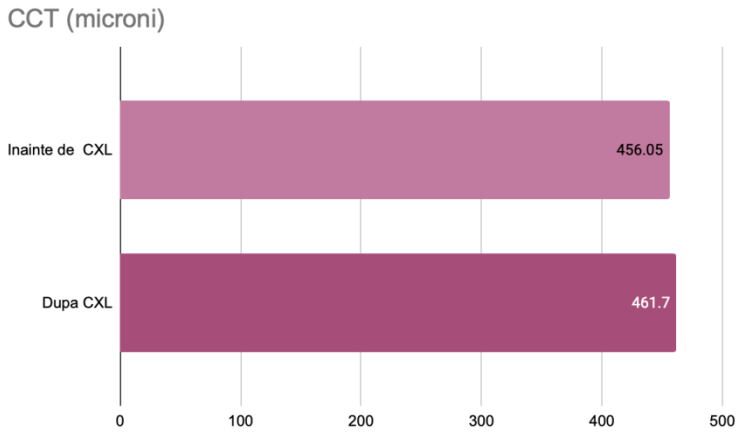


Figure 11 Central corneal thickness in patients who underwent accelerated CXL before and after the intervention

Similar results were found in the standard crosslinking protocol group. After CXL, the cylindrical refractive value, spherical equivalent and Kmax were significantly lowered, and CCT and CRF were significantly higher.

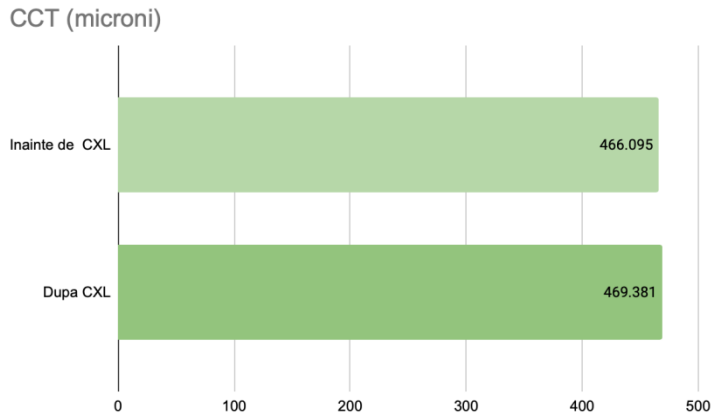


Figure 12 Central corneal thickness in patients who underwent standard CXL before and after the intervention

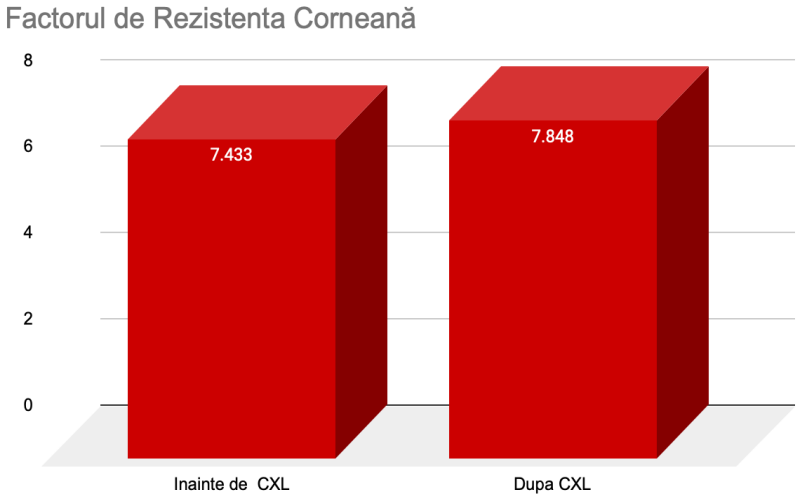
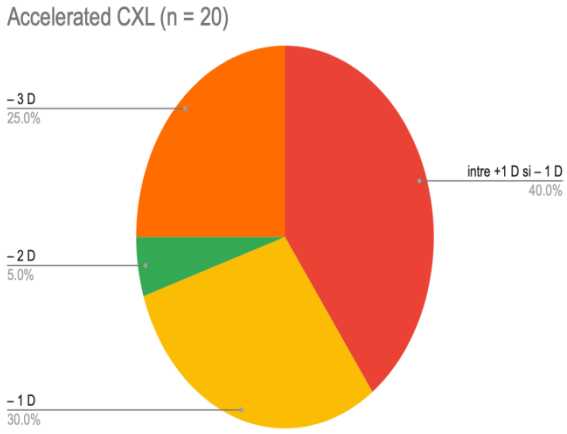


Figure 13. Corneal resistance factor in patients who underwent standard CXL before and after the intervention

When comparing the two protocols, both were proven to be similarly efficacious. There were no statistically significant differences in the magnitude of improvement in SE, CCT, maximum and minimum keratometry, CH, CRF or the depth of the demarcation line.

Importantly, no patients had an increase of Kmax over one diopter following the intervention - see Table IV. A similar proportion of patients had a stable Kmax (changes + 1 D to – 1 D) in the two groups (40.0% for Accelerated-CXL and 38.09% for Standard - CXL).



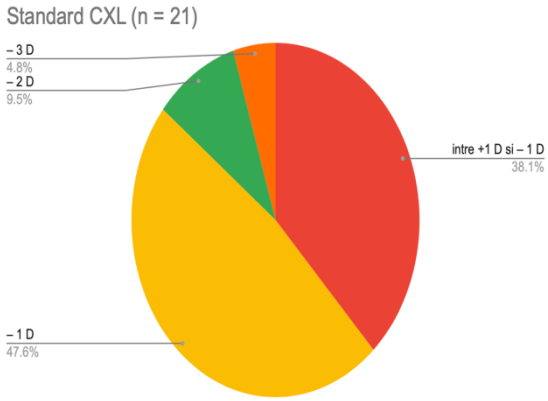


Figure 14 Distribution of patients who underwent accelerated CXL according to the degree of Kmax flattening and **Figure 15** Distribution of patients who underwent standard CXL according to the degree of Kmax flattening

The demarcation line was identified using Anterior Segment OCT and the depth reported to the total corneal thickness was calculated. The average absolute depth was at 278.90 micrometers in the accelerated group and at 280.43 micrometers in the standard group. The average depth relative to the corneal thickness was 61.21% in the accelerated group and at 60.04% in the standard group. The two groups did not differ significantly in terms of the depth of the demarcation line (p value 0.904) or in terms of the relative depth (p value 0.574)

There were several significant correlations between the difference before and after the procedure and the baseline characteristics of the patients. The degree of refractive improvement (i.e. the decrease in SE) was higher the more myopic and astigmatic the patient was (negative correlation r with baseline sphere -0.332, p 0.034 and with baseline cylinder r -0.319, p 0.042), and the steeper the initial corneal shape was (positive correlations with Kmax r 0.529, p value <0.001, with Kmin r 0.465, p value 0.002).

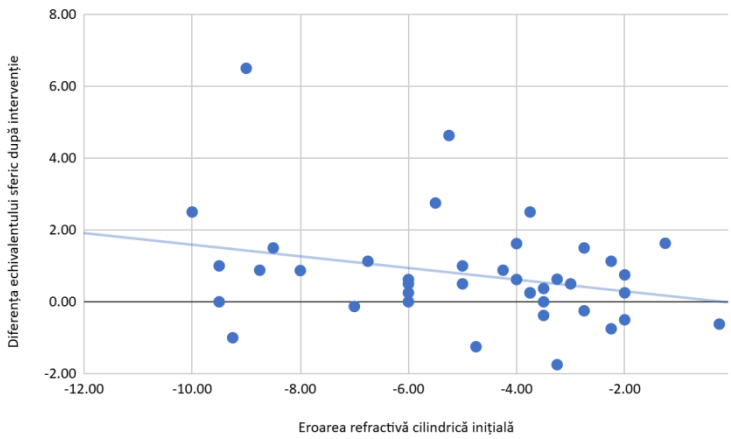


Figure 16. Correlation between initial relative cylindrical error in the entire cohort and SE improvement after the CXL procedure.

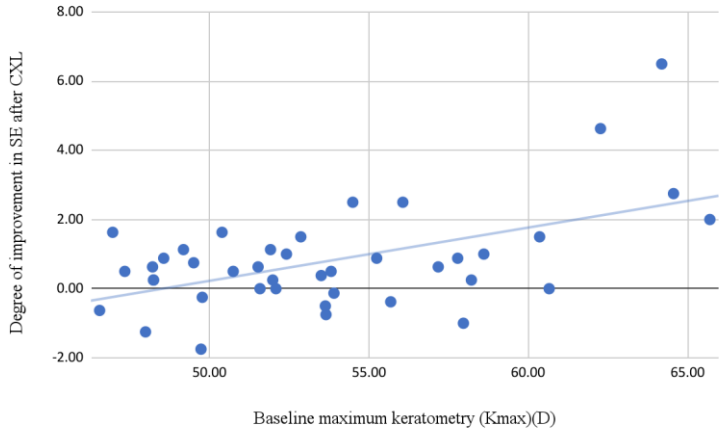


Figure 17. Correlation between the baseline Kmax in the entire cohort and the improvement in SE after the CXL procedure.

The biomechanical improvement of CRF increase was correlated positively with the cylindrical values (r 0.326, p value 0.038) (i.e. the lower the cylindrical error, the better the CRF improvement). The topographical improvement of Kmax was also positively correlated with the baseline cylindrical value (r 0.327, p value 0.037), SE (r 0.417, p value 0.007) and CRF (r 0.378, p value 0.015), and negatively correlated with baseline Kmin (r -0.515, p value 0.001) and Kmax (r -0.532, p value <0.001) - i.e. the steeper the cornea was and the higher the astigmatism before the procedure, the more the Kmax has flattened after the procedure.

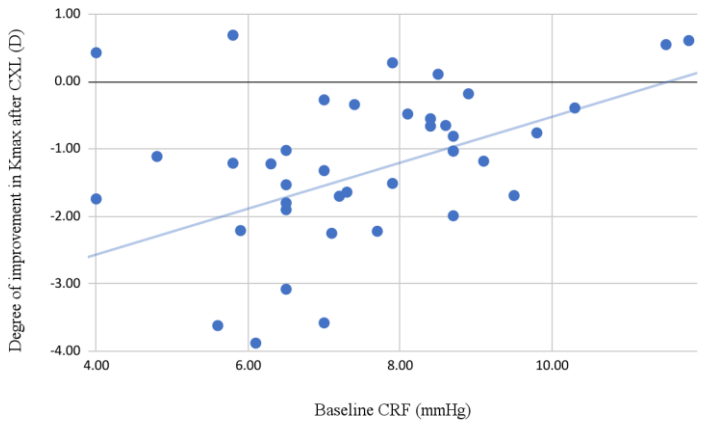


Figure 18. Correlation between the baseline CRF in the entire cohort and the improvement in Kmax after the CXL procedure.

The absolute demarcation line depth had a negative correlation both with Kmax (r -0.416, p value 0.007) and with Kmin (r -0.314, p value 0.045)(i.e. the DL was deeper the flatter the cornea was).

Discussion

In this study, we showcase the efficacy of corneal collagen crosslinking in halting, and even reversing, the progression of keratoconus. By applying either the accelerated or the standard protocol, statistically significant improvements are obtained in several essential parameters.

The efficacy and safety of the standard CXL protocol has been proven by numerous studies, however to diminish the discomfort of the prolonged intervention, the epi-off accelerated CXL protocol was introduced in clinical practice [39]. This requires a change in the riboflavin solution composition, to enhance the stromal penetrance. It is well known that the photooxidative crosslinking and its safety depends on the amount of riboflavin in the corneal stroma. In the present study, we used a riboflavin solution containing hypromellose, which facilitates the diffusion of the active substance into the stromal layer. The selection of patients for either the standard or the accelerated procedure is based on their age. It is known that the disease progression is more likely in young patients and that studies show the efficacy of the standard method [40].

Some authors recommend accelerated CXL for children, as the procedure is quicker, while others suggest that longer follow-up periods may reveal diverging evolutions of the two groups [39]. As such, the efficacy of the two epi-off procedures were compared, and a study from 2020 found that after 2 years of follow-up, children who underwent the accelerated procedure had a worse evolution than those who underwent the standard procedure, in terms of refraction, topography and visual acuity [41]. A recent meta-analysis compared the two protocols used in paediatric keratoconus and found no difference in terms of Kmax, SE and CCT but with a better evolution of standard CXL in terms of visual acuity after two years [42]. This is why, in the present study, the two protocol groups differed significantly: the patients in the standard group were significantly younger and had significantly higher astigmatism, as the standard procedure was preferred in young, paediatric patients.

Importantly, in the present cohort there were no differences in the depth of the demarcation line (as percentage of the entire corneal thickness) - which has been suggested

as an efficacy surrogate indicator [39]. Therefore, our results may suggest that, in this cohort, the efficacy is comparable between standard and accelerated corneal crosslinking.

In the present study, both protocols were useful in halting the progression of the corneal ectasia and even reversing refractive, pachymetry, topographic and biomechanical parameters, after one year. When studying the two groups individually, similar results were found. The spherical, cylindrical refractive values and maximum keratometry were decreased, and CH and CRF were increased.

Evaluation of patients revealed no keratoconus progression following the procedure. 40% of the cases in the accelerated protocol group and 38.09% of the cases in the standard protocol group have maintained the parameters constant, and approximately 60% of the cases have presented improvements. An Australian registry revealed that both CXL protocols are safe and effective, however the standard procedure leads to an improved visual acuity, a more significant flattening of the steepest meridian, along with a higher chance of an effect greater than one diopter power[43].

Similar results to ours were obtained in the literature, by comparing standard and accelerated epi-off CXL procedures. In a two-year follow-up of a Romanian cohort, visual acuity, refraction, corneal thickness and the steepest corneal meridian improved similarly, small differences being that BCVA improved more quickly in the accelerated group and in the standard group a deeper demarcation line was obtained [29].

In our study, a decrease in cylindrical refractive error, and of spherical equivalent was observed, in relationship with the decrease in maximum keratometry. This change was larger in the standard group (Kmax decreased on average by 1.462 D, compared to 1.276 D in the accelerated group), which may be explained by the younger patients in the standard group, still undergoing growth and hormonal fluctuations [44].

As the CXL procedure strengthens the links between collagen fibrils in the corneal stroma, it has been suggested in the literature that the biomechanical properties of the cornea should improve after the procedure [45]. Preclinical studies involving ex vivo human corneas prove that the rigidity is more than triple [46], and the diameter of collagen fibers increases following crosslinking in animal models [47].[45][46]

Given the small number of cases for each study group, in order to perform a significant statistical analysis, correlations were evaluated for the entire cohort of cases. Several correlations were observed between the degree of improvement in corneal parameters and the initial characteristics of the patients. The decrease in spherical equivalent was more pronounced in cases with higher initial values regarding the

cylindrical components of refraction and corneal dioptric values. The CRF difference was correlated with the initial cylindrical value, and the Kmax difference was correlated with the CRF, SE and baseline Kmax and Kmin. [27]. According to the statistical analysis, the improvement of the corneal resistance factor was positively correlated with the improvement of the corneal dioptric value on the most refractive meridian as a result of the histopathological changes induced by crosslinking, namely the increase in the diameter of the collagen fibers and the formation of new connections between them. A multivariate analysis identified high baseline SE and Kmax as predictors of significant Kmax flattening [28].

In terms of procedural safety, the corneal thickness of all patients in our study was over 400 micrometres, a value that avoids the UVA toxicity to the corneal endothelium [48]. Anterior segment OCT is essential in investigating corneal thickness and detecting the depth of the demarcation line, visualised as a hyperreflective band in the mid-stroma [49]. In the current study, the demarcation line was identified at an average depth of 278.90 micrometers in the accelerated group and at 280.43 micrometers in the standard group. The demarcation line was detected at a greater depth in cases with lower corneal dioptric power values.

Conclusions and Personal Contributions

1. In more advanced keratoconus, there is an increase in variability, which would seem to be correlated with the advancement of corneal ectasia. Increased polymegetism is the first sign of endothelial damage. Although the increase is not clinically significant, there is a possibility that it could be justified by the existence of corneal protrusion causing stress in the deeper layers of the cornea.
2. In more advanced keratoconus, hexagonality, although without statistical significance, was more increased, which may attract attention when the cornea becomes steeper, corneal pleomorphism increases, trying to compensate for the degree of corneal tension

that occurs. None of the patients in the present study had a value of hexagonal cells within the limits, which reinforces the idea that the structure of the endothelial bed manages to mention its structure and function even in the case of advanced keratoconus.

3. The results determined that once keratoconus progresses, the values of the central corneal thickness decrease, the mean values of the corneal resistance factor decrease, and the mean values of the mean keratometry and astigmatism increase.
4. Our study, as in the case of stage I of keratoconus, after performing corneal crosslinking, there is a significant decrease in the means of some endothelial parameters 6 months after the procedure, respectively the number of cells, hexagonality and cell densities for the central compared to the baseline group, and the cellular variability increased significantly at 6 months.
5. Regarding stage II, the endothelial changes that occurred strictly at 6 months consisted of a significant decrease in the mean values of the cell density and a significant increase in the mean value of cellular variability.
6. Stages III and IV of keratoconus in our study reveal minimal changes in the endothelial parameters, there is only an increase in the mean value of the central hexagonal cells in stage III.
7. Correlation between paracentral corneal endothelial parameters and initial topographic variables, across the entire study cohort, showed that there is a significant, weak and positive correlation between paracentral cellular variability and mean and minimum keratometry and significant, moderate, negative correlations between maximum and minimum paracentral endothelial cells.
8. Regarding study II, as expected, both procedures determined a similar efficiency of topographic indices and corneal biomechanics, thus both procedures bring a significant reduction in corneal astigmatism, spherical equivalent and maximum keratometry and an increase in corneal resistance factor and corneal thickness.
9. From a statistical point of view, between the two groups of patients there are no statistically significant differences between the magnitude of corneal topographic and biomechanical parameters.
10. The two groups did not differ significantly in terms of the depth of the demarcation line or in terms of relative depth, the treatment being effective in both types of procedures.
11. In our study, the mean absolute depth and the mean depth of the demarcation line relative to corneal thickness were similar in both groups of patients, a depth that did not cause corneal endothelial toxicity.

Personal Contributions

The only treatment method to stop the progression of keratoconus in its early stages is corneal crosslinking. The pathology of this condition remains poorly understood today, with studies demonstrating damage to the corneal stroma and epithelium, Bowman and Descemet membranes, but with regard to changes in the endothelial layer, research remains contradictory. The personal contributions of Study I consisted in identifying the differences and correlations between corneal topographic, biomechanical and endothelial parameters, between different stages of progression of this pathology, as well as before and after the crosslinking intervention using the “epi-off” technique. Study II consisted in correlating the evolutionary parameters of the disease, topographic and biomechanical, and monitoring the effect of the two epi-off crosslinking procedures, respectively standard and accelerated, through the demarcation line. The two procedures were applied in study II, ensuring statistically similar efficacy and do not produce different effects on the corneal endothelium, both having a similar depth of the demarcation line.

Considering that keratonus is a condition that mainly affects the young population, in the future it is important to expand the study group, especially in advanced and undiagnosed cases, but also to follow up the current cohort on a longer term.

Given the development of new technologies, which seek to study this pathology in more detail, providing more precise indices and early detection of the disease, we want this pathology to be as well understood as possible.

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