Table 2
Fibril Waviness.

Authors	Methods			Conclusion
	Tissue	Imaging	Measurement	
Bradford et al. [70]	Rabbit: in vivo	SHG: cross section	Traced fibrils	1% decreased crimp after UVA CXL
Tan et al. [32]	Porcine: ex vivo	SHG: en face	2D FFT	Decreased waviness after UVA CXL
Germann et al. [72]	Porcine: ex vivo	SHG: en face	Order coefficient	Increased order and straighter fibrils after UVA CXL
Bueno et al. [73]	Avian/ Rabbit: in vivo	SHG: en face	Degree of isotropy, preferential orientation, and structural dispersion	The more ordered the original collagen structure, the less increase in order seen after UVA CXL

of a 45 μm^2 region of interest. In contrast, the previous study measured multiple fibers across the entire width of a 427 μm region of interest, which lowers the possibility for sampling error. Moreover, where the previous study measured waves moving in the x-z direction (anterior to posterior), any crimp measured by Tan et al. would be within the same x-y plane, since any fibers moving with depth into the stroma would not produce a strong SHG signal. Furthermore, these measurements were taken immediately after treatment, when the dehydrating effects of the riboflavin dextran solution are still relevant. This is especially important since SHG imaging has also been used to show increased fiber packing in the anterior corneal stroma immediately after dextran exposure [99]. Taking into consideration all the listed differences of the two studies, it is still possible that the described results are not entirely contradictory, since the imaging was performed from different angles. A fiber that appears wavier when viewed from the top, may appear straighter and shortened when viewed from the side because the observed waves are secluded to the cross sectional plane. It is also possible that crimping may occur on a molecular scale, within fibrils, to induce wavier, shortened fibers.

Disagreement in this area is prevalent in the literature [100]. For this reason, another study has attempted to explain these discrepancies. Bueno et al. claims results of these studies differ not only due to their many different methodologies of tissue preparation, imaging, and imaging analysis, but because of the different species used [101]. Bueno's study compared SHG imaging analysis after in vivo UVA CXL in both avian and rabbit corneas, Figs. 2 and 3. The rabbit corneas, which are naturally less organized than avian corneas, were more structurally affected by the procedure. The authors qualitatively described the treated rabbit cornea as having collagen bundles which "appeared more delineated and less interwoven," particularly in the posterior region. Similarly the avian cornea, Fig. 2, showed similar interweaving in the anterior and mid stroma in both treated and untreated. In the posterior, however, where the untreated stroma no longer showed such a high degree of initial order, the treated cornea showed a more drastic change, with long collagen fibers running parallel to each other after treatment. When the authors analyzed the images to calculate the degree of isotropy (DOI) of the samples, they discovered that treated rabbit corneas experienced an increase in DOI at every depth, while avian corneas, with much higher initial DOIs, did not experience any significant increase in DOI at any depth. The authors concluded that the more ordered the original collagen structure, the less increase in order seen due to the procedure. Additionally, to our knowledge, all SHG studies have performed imaging within tissue, and no study has been performed in a collagen gel. It could be useful to observe the effects of UVA CXL within a simplified structure. A comparison of the methods and main results of these studies can be seen in Table 2, as well as a diagram to summarize these results in Fig. 4.

3. Cellular effects

3.1. Epithelium

Epithelial debridement is a necessary step in the traditional Dresden protocol for UVA CXL. Without it, riboflavin penetration does not occur in concentrations required for effective crosslinking. Epithelial debridement is painful for the patient, delays visual recovery, and increases the risk of bacterial keratitis and corneal ulceration [102–104] and requires 2 days to heal in rabbits or as long as 3 weeks in humans [43,105].

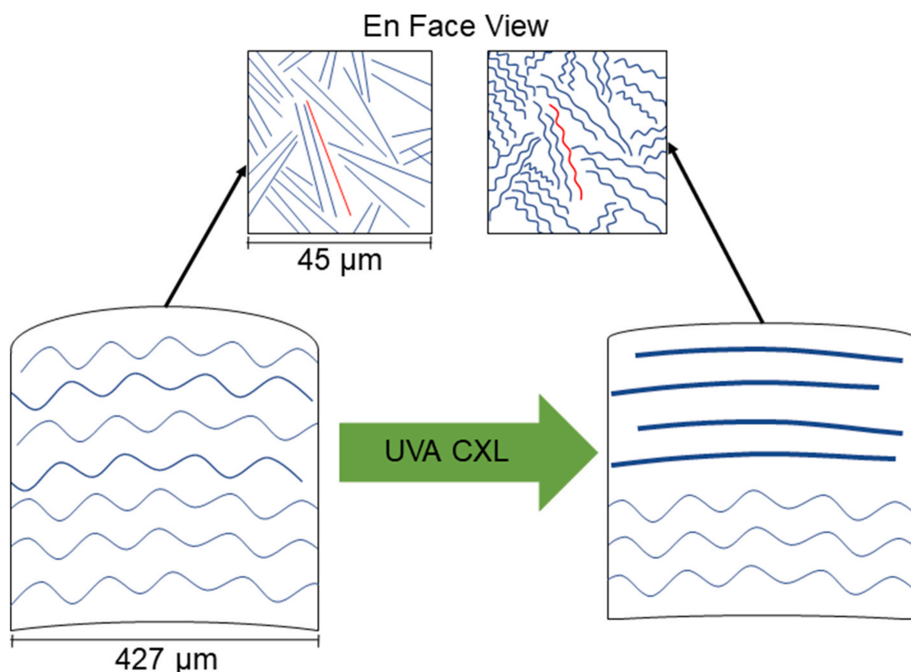

Fig. 4. Suggestive SHG diagram. Literature reviewed in this article has shown collagen fibers to be straighter after UVA CXL when viewed in a cross section (bottom) [98], and wavier when viewed in an en face orientation (top) [37]. Literature also suggests that observed corneal flattening could be due to the shortening of collagen fibers as they straighten in one orientation, and crimp in another, (Right). The red fibers before and after crosslinking were highlighted to illustrate the transition from a long straight fiber to a shortened wavier fiber after treatment.

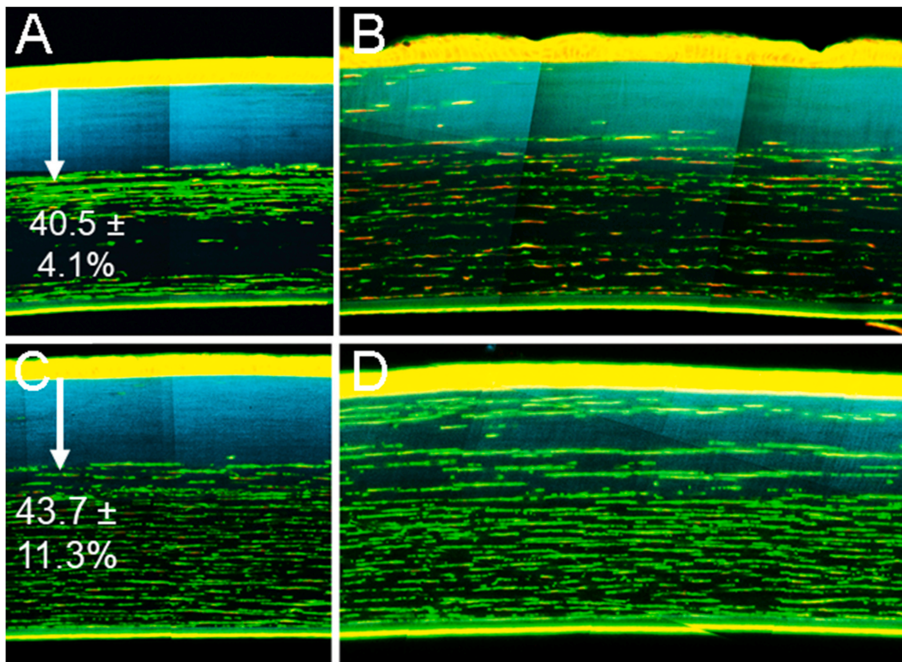

Fig. 5. Cell Staining
The top row of images represents samples from the central CXL region and the edge of the CXL region bordering the periphery from one month samples (A and B respectively). The bottom row represents corresponding images from three month samples (C and D). Staining with Phalloidin (green; 1:100) and Propidium Iodide (red; 0.01 mg/ml) showed little cellular repopulation into the central CXL region, shown with blue CAF, at either time point. Images from the periphery show migrating cells into the CXL region. The depth of the acellular zone in the central cornea, indicated by arrows, was measured to be 40.5 ± 4.1%, and 43.7 ± 11.3% of the stromal thickness on average for one and three month samples. This corresponds to the measured depth of CAF. Also, in two of the four one month samples, a second deeper acellular region was noted, pictured in A [98].

To avoid epithelial debridement research has focused on the development of a transepithelial UVA CXL protocol. Various methods have been used to enhance transepithelial riboflavin penetration without debridement prior to UVA exposure. Though clinical studies have reported fewer severe complications from experimental transepithelial UVA CXL techniques on average, there has also been minimal success, with reported stabilization of KMax (maximal corneal curvature) of only 43% compared to 93% reported after traditional UVA CXL [20,57]. Additionally, the line of demarcation in the stroma, the presumed zone of transition between treated and untreated tissue often used as a measure of the depth of treatment, was observed to be shallower when the epithelium remained intact [20]. This is likely due to the epithelium acting as a barrier to UVA light as well as riboflavin penetration. Riboflavin within the epithelium could absorb much of the UVA light and reduce the UVA intensity deeper within the stroma. Research has also shown extensive epithelial damage after transepithelial UVA CXL, in response to both the UVA exposure and the methods used to facilitate riboflavin penetration [62,77,78,106–108]. Benzalkonium Chloride (BAK), commonly used as an excipient, is especially toxic to cells and has been shown to damage the epithelial layer even without subsequent UVA exposure [106,109]. Taneri et al. reported that epithelial defects and perceived pain were common following various transepithelial UVA CXL techniques, though these instances were less common using methods that produced less stromal riboflavin penetration [107]. This indicates an inverse relationship between riboflavin penetration and epithelial disruption. Additionally, Chow et al. reported more extreme epithelial damage following accelerated transepithelial UVA CXL, in which higher intensity UVA light is used to shorten the procedure time [62]. In this study 64% of eyes experienced complications including large epithelial defects and diffuse punctate epithelial erosions, with 100% of patients reporting significant postoperative pain. This indicates that epithelial damage is not only a result of varying riboflavin penetration techniques, but also due to UVA exposure itself. The free oxygen radicals which induce crosslinking in the stroma also damage epithelial cells.

A new technique to imbibe the corneal stroma with riboflavin has shown promise in minimizing epithelial damage prior to crosslinking therapy. By focusing a high pulse energy infrared femtosecond beam into small, widely spaced spots, a pattern of small channels can be drilled into the surface epithelium. At 2 μm in diameter and only 25 μm long, each channel causes minimal cellular disruption while allowing free passage of riboflavin through the epithelium. Microchannels combined with more concentrated riboflavin drops were shown to facilitate similar levels of stromal riboflavin concentration as the standard epithelial debridement method [106]. Additionally, epithelial damage due to the microchannels was undetectable after 24 h of organ culture, as evidenced by phalloidin and propidium iodide cellular staining (Fig. 5).

3.2. *Stromal keratocytes*

Stromal Keratocyte death occurs due to UVA exposure of around 0.5 mW/cm^2 [42,110], resulting in an acellular zone within the anterior stroma to a depth of 200–300 μm [41,43,84,98,111]. Post treatment activation and migration of keratocytes into corneal fibroblasts from adjacent regions back into the acellular zone and the expression of disordered extracellular matrix produced by those fibroblasts is responsible for the development of haze, as these cells scatter light more than their non-activated counterparts [112]. Wollensak et al. detailed severe haze in rabbit corneas lasting one week, while other studies showed haze peaking at one month [43,84,98]. Clinically, haze has been shown to persist for six to eight months, and over a year in 10% of cases, leading to permanent corneal scarring in 2.9% of cases [44,71,113]. It has previously been observed by Wollensak and Kozobolis via light microscopy that keratocyte repopulation begins around one month post crosslinking in rabbits, with full cellular repopulation reported by six weeks [43,44]. Both reported a continuing presence of acellular areas and apoptotic changes such as apoptotic bodies, shrunken cell nuclei, and chromatin condensation at 4 weeks, especially around the periphery of irradiation. Kruger also observed cellular repopulation by 6 weeks using a combination of confocal laser scanning microscopy and two photon excited fluorescence, albeit with a lower cellular density than seen in controls [30]. By contrast, we reported persistent acellular regions at three months post UVA CXL, as well as an additional acellular region below the region of crosslinking, using a combination of in vivo CMTF and cellular fluorescence staining, as seen in Fig. 5 [84,98]. Additionally, studies of corneal scrape and freeze injuries have shown stromal cellular repopulation 3–14 days after injury, suggesting the

delayed repopulation after UVA CXL may be due to either the biomechanical changes causing fibril stiffening and thinning, or to the UVA exposure itself [114].

4. Conclusion

In the last two decades since corneal UVA CXL has been developed research has focused on discovering the mechanisms that govern the process and the effects it has on corneal tissue. The main objective of the procedure is to enhance stromal mechanical stiffness via alteration of the corneal collagen structure, and for that reason research has been focused on structural changes. There is a general consensus that the procedure mechanically strengthens the corneal stroma and leads to a low degree of corneal flattening, collagen fibril thickening, and halted progression of corneal ectatic disease. The mechanism of these changes are not often agreed upon, however. For example, some authors claim increased fibril waviness plays a role in corneal flattening while others claim the opposite [37,98]. Though collagen structure and mechanical changes are important factors underlying UVA CXL, the procedure can be damaging to certain layers of the cornea. For this reason, safety studies focus on cellular responses to UVA CXL. Studies on the timeline of keratocyte repopulation are plagued with the same inconsistency in results as structural studies, with repopulation being reported anywhere from six weeks to three months or longer [30,43,44,98].

In either case, the truth is likely more complicated than any one study suggests. Taken as a whole it is difficult to compare most studies to each other, due to their varying protocols, measurement techniques, and even species of test subjects used. There is also a lack of consistency in definitions. For example, some studies which describe full cellular repopulation also describe continuing acellular regions or lower than normal cellular density [30]. It is important to take all of these factors into account when comparing various studies to determine whether differing results between individual studies could simply be a case of one question being viewed from many different perspectives.

Finally, as more researchers begin to turn their focus to alterations of UVA CXL it has become necessary to predict how various changes to the standard protocol would ultimately affect the results of the procedure. This has proved difficult, however, since the effects of the original procedure are not wholly understood. Building up a larger body of research focused on the effects addressed in this review would enable researchers to better predict the outcomes of protocol adjustments, allowing for more customized procedures to treat each individual patient's needs.

Disclosures

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