In the early 1980s and 1990s, studies re-evaluated the biomechanical properties of keratoconus compared to normal corneas and noted that the stiffness in the anterior 200 μm of the cornea was significantly less (by a factor of approximately 1.5).7 Hans Oxlund et al. evaluated the load-strain and stress-strain values of corneas from eyes with keratoconus compared to normal eyes and found that the corneas from eyes with keratoconus had smaller values compared to normal corneas.4 They hypothesized that these eyes had fewer cross links compared to normal eyes. This was shown by multiple analyses looking at the percentage of various protein end groups and also by the amount of hydroxyproline (a moiety often cross linked in the collagen structure) that was able to be dissolved. They found that the corneas from eyes with keratoconus had a greater percentage (9%) that dissolved compared to normal corneas (4.7%), which suggested more cross links in the control eyes as well as a greater mechanical strength.4

Other important keratoconus-related studies included confocal laser-scanning microscopy and electron microscopy studies. The former found an irregular structure of keratocytes in the anterior stroma of eyes with keratoconus compared to controls,7 while the latter showed abnormal amounts of electron-dense material in the anterior stroma of the corneas with keratoconus. The abnormal electron dense material has suggested an “abnormal metabolism,” the nature of which has yet to be fully characterized.

Keratoconus continues to be one of the main indications for corneal transplantation in the United States and of the 41,652 corneal transplants performed in 2008, almost 15% (6,238) were indicated for keratoconus.8 It has long been known that keratoconus represents a spectrum of disease that ranges from mild and not very visually significant, to severe and visually disabling to the point of not being correctable by glasses or contacts. Early in its course, patients are typically asymptomatic and require little other than...
Corrective glasses or contacts. As the disease progresses, glasses are often no longer effective at correcting the irregular astigmatism, but rigid gas permeable contact lenses may still allow for adequate visual acuity. According to reports published in the 1980s, approximately 20% of patients with keratoconus will go on to require corneal transplantation.2,9 Given that topography has become increasingly available and more and more young adults have had screening topographies in the course of refractive laser surgery evaluations, it is likely that the reporting statistics for the frequency of keratoconus and the percentage of patients that require transplantation will change. Regardless, the disease continues to be one of the most common indications for corneal transplantation and because it occurs most often in young patients (typically under age 35), there has been much interest in understanding the disease and finding ways to treat it early in order to prevent irreversible vision loss and subsequent surgical intervention via corneal transplantation.

**CROSS LINKING—BACKGROUND**

The principle of cross linking has been used for decades in the polymer industry as the means to harden various plastics and other long chain molecules. Cross linking refers to the formation of covalent bonds between long polymer molecules and it therefore results in a chemical strengthening of material. In addition to the polymer industry, cross linking has been used in bioengineering to stiffen and maintain various tissues, as in the case of prosthetic heart valves.10

Cross linking involving corneal collagen was investigated initially in diabetics who had been noted to have a reduced incidence of keratoconus and it was therefore postulated that the collagen of diabetics was more cross linked.11 To study and show this, Maillard reactions, which refer to the addition of an aldehyde sugar to the amino group of protein chains, were investigated in the corneas of diabetics compared to controls. The products of Maillard reactions can undergo various rearrangements to form advanced glycation end products (AGEs).12 Diabetics have been found to accumulate more and more AGEs and collagen modification by AGEs results in covalent cross linking. Specifically, Nagaraj et al. showed that diabetics had increased AGEs and increased cross links from pentosidine, which is one of the AGEs formed.12 They also have been found to have more glucosyl-lysine cross links from the AGEs.12

In addition to AGEs related corneal cross linking, cross linking has been found to occur with age.11,12 The type of cross linking seen in aging has been shown to be due to the enzyme lysyl oxidase, which leads to aldehyde formation and further post-translational modifications, which leads to cross linking.12 Thus, cross linking can occur in a variety of settings and from different pathways, so if it could be purposefully driven and in a controlled setting, then it could be used as a treatment modality for keratoconus.

**CROSS LINKING—CORNEA/ANIMAL STUDIES**

The application of cross linking to corneal tissue via riboflavin was first reported by Theo Seiler, Michael Huhle, and Eberhard Spoerl in 1998.11,13 As early as 1991, cross linking was shown to be induced using UV light with and without photosensitizers.11 Seiler et al. measured the stress-strain values for porcine corneas treated with the following: ultraviolet light alone (λ = 254 nm), 0.5% riboflavin plus ultraviolet light (λ = 365 nm), 0.5% riboflavin plus ultraviolet light (λ = 436 nm), 0.5% riboflavin with sunlight, 0.5% riboflavin alone, 0.1% glutaraldehye, 1% glutaraldehye, “Karnovsky solution 0.1%,” and no treatment. Glutaraldehye was a natural choice because it had been the standard for cross linking of biomaterials (either alone or when mixed with formaldehyde, when it becomes known as Karnovsky’s solution), but riboflavin had at the time only recently been tried as a photosensitizer.14 To test the stress-strain values, they first cut the corneas into very precisely sized strips (5 mm in length) and applied known strain forces and measured the stress values.11 They found that all groups were able to tolerate a higher level of stress compared to the control group except those treated with ultraviolet light alone or riboflavin alone.11 In addition, the riboflavin plus the UV light at 365 nm was found to strengthen the cornea the best compared to the 436 nm and 254 nm wavelengths. This study was a pivotal early study in showing the efficacy of 0.5% riboflavin-induced cross linking (using ultraviolet light at a wavelength of 365-370 nm), such that thereafter all studies were done in context of it, with the exception that the riboflavin concentration was changed from a 0.5% solution to 0.1% in all future studies.

The next important experiment was a much larger animal study using 0.1% riboflavin and UVA light (370 nm, 3 mW/cm2) for 30 minutes.7 The study treated 20 porcine eyes and compared them to 20 untreated porcine eyes. With each eye, two 200 μm thick flaps were cut with a microkeratome and then cut into strips 5 mm wide and 7 mm in length. In a similar manner, they treated 5 human donor corneas the same way. They found in both porcine and human corneas that the stiffening effect (as measured by testing the stress-strain behavior using a material...
Corneal Cross Linking for Keratoconus

To analyze the biochemical aspect of the cross linking, both enzymatic digestive studies and gel electrophoresis studies were done on riboflavin-induced cross linked porcine corneas compared to controls. The enzyme studies compared 60 porcine eyes treated with the riboflavin technique (de-epithelialization of the central corneas followed by application of riboflavin followed by 30 minutes exposure to 370 nm UVA light) compared to controls and looked at the length of time it took to be digested fully by pepsin, trypsin, and collagenase. They found that digestion by pepsin took 13 days in treated eyes compared to 6 days in the controls. Results were similar for the trypsin and collagenase treatments. This indicated a marked resistance to enzymatic digestion, suggesting high numbers of cross linked bonds requiring digestion. Next, Wollensak et al. took 20 porcine corneas and cross linked them using the riboflavin technique and afterwards extracted type I collagen from the treated area. They used 20 untreated porcine corneas as controls. After isolation of the type I collagen (selected because it comprises 90% of the collagen of the cornea), the extracts were run on sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis. They found the usual band pattern for type I collagen on the control corneas, but in the cross linked extracts they found another intense, much larger band (1000 kDa compared to the usual band sizes of 130 kDa, 200 kDa, and 300 kDa (32)). The 1000 kDa band that was found in the cross linked corneas was further tested and found to be resistant to heat denaturation, pepsin treatment, and mercaptoethanol treatment, showing its chemical stability. This large and stable band also strongly supports the previously held belief that the cross linking procedure actually induces multiple covalent bonds. However, the exact type of chemical cross linked bond that is induced is still not yet clear. Given that the band was resistant to digestion by mercaptoethanol, disulfide bonds would be unlikely.

One final important animal study that was completed prior to proceeding to in-vivo human studies was a hydration study and second analysis of the depth of treatment using optical coherence tomography (OCT). Wollensak et al. took 20 porcine eyes that were treated with the riboflavin UVA protocol. After treatment, the eyes were incubated for 24 hours in a moist chamber and 15 of the eyes were examined by biomicroscopy and OCT and 5 eyes were examined by light microscopy. They found that there was a characteristic swelling pattern that affected the cornea to a greater degree in the anterior than in the posterior portion and a cross linking pattern was also seen based on light microscopy. Specifically, they divided the cornea into 3 zones and noticed an anterior zone down to 242 µm, an intermediate zone encompassing the next 238 µm of tissue, and a posterior zone involving the remainder of the tissue (1355 µm for porcine corneas). The swelling was found to be increased by a factor of 2.2 in the intermediate zone and by a factor of 2.7 in the posterior zone. This study was useful in analyzing both the amount of swelling that occurs following treatment and the demarcation lines visible once the edema subsided that indicate treatment depth.
RIBOFLAVIN CROSS LINKING IN KERATOCONUS—HUMAN TRIALS

With the support of all of the in-vivo and ex-vivo animal and human corneal studies using the riboflavin UVA cross linking technique, beginning in 1998, Wollensak et al. began treating patients with keratoconus using this method.20 The method utilized for cross linking for this Dresden pilot study was the following: epithelial debridement of 7 mm using a blunt knife; application of 0.1% riboflavin for 5 minutes prior to UV irradiation and every 5 minutes during irradiation; UVA irradiation at 370 nm at irradiance of 3 mW/cm² at 1 cm distance for 30 minutes, followed by application of antibiotic ointment until re-epithelialization. 20 The results of the pilot study were published in 2003, showing that all treated eyes had a halting of the progression of the keratoconus and that 70% had regression and a reduction in the maximal keratometry readings by an average of 2 diopters. 20 The initial pilot study had follow-up ranging from 3 months to 4 years. 20

Since this pilot study showed such positive results, the idea spread to other centers and patients with keratoconus have been treated around the globe. The University of Florence in Italy was the next center to take to the riboflavin cross linking and to develop a UV machine marketed for such purpose. The treatment protocol has undergone mild variations, but has not deviated markedly from the initial pilot study. The other countries that have recently published results from prospective, randomized studies looking at riboflavin-induced cross linking for keratoconus include Turkey (at the Dunya Eye Hospital) and Australia (at the Royal Victorian Eye and Ear Hospital). While the number of patients varied and not all groups reported identical measurements, a summary of the results is listed in Table 1.

In the Dresden trial including 241 patients, they found that their results were statistically significant after the first year postoperatively and remained stable for the remainder of the follow-up, which extended up to 6 years. 21 Two patients had an acute exacerbation of neurodermatitis, after which there was progression of their keratoconus and they required repeat cross linking. 21 In regards to the observation that many of the patients had improvement in visual acuity, the explanation was felt to be due to a combination of a decrease in the amount of irregular astigmatism, a decrease in the corneal curvature, and a secondary improvement in the ability to fit contact lenses. 21 They found no statistically significant change in IOP at one year, although the smaller Turkish study found a slight increase in IOP averaging 2 mmHg in the first 5 to 12 months of follow-up. 21 Certainly the Dresden study involving ten times as many patients (241 compared to 19) and follow up for more than 12 months would seem to be more convincing, but further studies will help determine if there is a statistically significant IOP effect.

Small differences in the effect on the central corneal thickness (CCT) were seen among some of the studies. The Dresden study reported an increase in CCT of -2 +/- -12 μm after the first year and 21 +/- 31 μm after the second year. The study from Siena University found the mean CCT before treatment was 431.5 μm and after treatment was 463.3 μm, but this dropped to 450.6 μm at 3 months (so essentially an average of 20 μm increase in CCT). Thus, comparing the 2-year results of the Dresden study (increase in CCT on average of 21 μm compared to 20 μm at 3 months for the Siena group), the results were comparable. However, the Swiss study looking at 21 patients found a decrease in CCT by approximately 11 μm (11 +/- 22). 4 Given the standard deviations and the small number of patients in the later two studies, the effect on CCT appears to be similar, with a range of anywhere from a 30 μm increase in CCT to a 30 μm decrease.

Of note, the endothelial cell count was unchanged in all groups. In addition, no major adverse events were reported in any of these studies.

SAFETY OF RIBOFLAVIN-INDUCED CROSS LINKING

Leading up to these various trials, data has been published looking at the safety of riboflavin UVA cross linking. 24 Riboflavin, also known as vitamin B2, is a micronutrient and cofactor for a number of enzymes and plays a role in metabolism. There has been no evidence of riboflavin toxicity in human studies of oral intake up to 400 mg/day. It is yellow in color and is used as a food coloring and to fortify some foods, such as baby foods, breakfast cereals, and energy drinks. Although glutaraldehyde had been the original photosensitizer for cross linking, the initial animal studies on pig and rabbit corneas found riboflavin to be equally efficacious and much more preferable, given its nontoxic history. In addition, riboflavin is also soluble in water and can penetrate corneal stroma after debridement of epithelium. 11

Results regarding the safety of riboflavin UVA corneal cross linking were published in 2007 by Spoerl et al. 24 The two major potential damage mechanisms include direct damage from the UVA irradiation and indirect damage from any photochemically induced free radicals. In regards to the UVA irradiation, the energy used for the cross linking protocols has been 5.4 mJ/cm², which corresponds to an irradiant energy of 3 mW/cm². The aforementioned energy is below the known energy threshold for damage to corneal endothelium, lens, and
TABLE 1 Summary of the 5 major human studies involving riboflavin UVA cross linking

<table>
<thead>
<tr>
<th>Authors</th>
<th>Center</th>
<th>Type of Study</th>
<th># Eyes Treated</th>
<th>#/type of control</th>
<th>Machine</th>
<th>Flattening of Kmax</th>
<th>Spherical Equivalent change</th>
<th>Range of Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Raiskup-Wolf et al.</td>
<td>Carus University Hospital, Dresden, Germany</td>
<td>Long-term retrospective study</td>
<td>241</td>
<td>None</td>
<td>UV-X Peschke</td>
<td>1.46 D (at 1 year), but Kapex reduced 2.68 D</td>
<td>BCVA improved at least 1 line in 53%</td>
<td>12 months to 6 years</td>
</tr>
<tr>
<td>2 Caporossi et al.</td>
<td>Siena University, Italy</td>
<td>Second-phase, prospective, nonrandomized open study</td>
<td>10</td>
<td>Fellow eye</td>
<td>Exerion-Sas</td>
<td>Mean K reduction of 2.1 +/- 0.13 D in central 3.0 mm</td>
<td>-2.495 D +/- 3.06 at 3 months</td>
<td>3 months</td>
</tr>
<tr>
<td>3 Coskunseven et al.</td>
<td>Dunya Eye Hospital, Turkey</td>
<td>Prospective comparative study</td>
<td>19 eyes</td>
<td>19 (fellow eye)</td>
<td>UV-X PESCHKE</td>
<td>Kmax decreased 1.57 +/- 1.14 D</td>
<td>-1.03 +/- 2.22 D</td>
<td>5-12 months</td>
</tr>
<tr>
<td>4 Koller et al.</td>
<td>Institute for Refractive and Ophthalmology, Switzerland</td>
<td>Prospective, randomized study</td>
<td>21 eyes</td>
<td>21 (fellow eye)</td>
<td>UV-X PESCHKE</td>
<td>Kmax decreased 0.66 D +/- 0.9</td>
<td>Not available</td>
<td>12 months</td>
</tr>
<tr>
<td>5 Wittig-Silva et al.</td>
<td>Royal Victorian Eye and Ear Hospital, Australia</td>
<td>Prospective, randomized, controlled</td>
<td>33 eyes</td>
<td>33</td>
<td>UV-X IROC, Zurich</td>
<td>Kmax decreased 1.45 D +/- 1.00 at 12 months</td>
<td>Increase in BCVA of 0.12 log mar</td>
<td>3 to 12 months</td>
</tr>
</tbody>
</table>
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Table 1 shows the published trial data from around the globe. Study efforts have begun in the United States in an attempt to receive Food and Drug Administration approval to begin riboflavin cross linking in the US. One multicenter study sponsored by Peschke Meditrade using the Peschke UV light source has completed enrollment and is currently undergoing data analysis. The second FDA trial is currently unfunded and based out of Buffalo, NY, at the SUNY Buffalo School of Medicine using the Caporossi-Baiocchi-Mazzotta (CBM) X-linker. Massachusetts Eye and Ear Infirmary has recently received IRB approval to join this study and plans to begin treatment early in 2010. Please see clinicaltrials.gov for more details.

### ADDITIONAL APPLICATIONS OF RIBOFLAVIN CROSS LINKING

In addition to keratoconus, cross linking is being looked at to treat various other corneal conditions, including post-LASIK ectasia, and to treat corneal melting conditions or infectious keratitis. In the case of the latter two conditions, it is felt that the cross linking can strengthen the collagen of the melting cornea and in the case of infectious keratitis, that the UV radiation kills the infectious agent. Seiler et al. recently reported the efficacy of cross linking for infectious keratitis associated with corneal melting. They reported a halting in the progression of the melt and avoidance of emergent penetrating keratoplasty in all cases. Four of the five patients had a history of laser-assisted in situ keratomileusis (LASIK) with an early post-operative infiltrate at the interface. In all cases, the infiltrates progressed with thinning of corneal stroma, despite intensive topical antibiotic use. While elective keratoplasty was performed subsequently for two of the patients, one of the key points is that emergent keratoplasty was avoided. The microorganism cultured included *Mycobacterium* species in two patients, *Fusarium* in one and unknown in the final two.

### CONCLUDING REMARKS

Riboflavin-induced UVA cross linking for keratoconus has established itself as a safe and what so far appears to be an efficacious and lasting treatment when detected in the early stages of the disease. The widespread availability of corneal topography and the now common detection during laser vision correction screening evaluations makes detection of keratoconus in its early stages much easier. Although improvement in vision has been noted in a large percentage of patients after cross linking, it is not a definite expectation. Patients should realize that the goal is to halt the progression of the disease before vision decreases.
to a point requiring surgical intervention. The cut-off values at which point cross linking would not be indicated is not entirely clear, but any patient with a corneal steepness above 60 diopters or a visual acuity not correctable to 20/40, could be cut-offs for which it would be unlikely to be beneficial. What is clear is that the corneal thickness must be a minimum of 400 μm in order to avoid damage to corneal endothelium.

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