

1 **Determination of the relationship between mechanical properties, ultrastructural**
2 **changes and intrafibrillar bond formation in corneal UVA/riboflavin cross-linking**
3 **treatment for keratoconus**

4

5 Shao-Hsuan Chang¹, Ashkan Mohammadvali¹, Kai-Jung Chen¹, You-Ren Ji³, Tai-Horng
6 Young³, Tsung-Jen Wang^{4A,4B}, Colin E. Willoughby^{2A,2B}, Kevin J. Hamill^{2A*}, Ahmed
7 Elsheikh^{1,5}

8

9 ¹School of Engineering, University of Liverpool, United Kingdom

10 ^{2A}Department of Eye and Vision Science, University of Liverpool, United Kingdom

11 ^{2B}St Paul's Eye Unit, Royal Liverpool University Hospital, Liverpool, United Kingdom

12 ³Institute of Biomedical Engineering, College of Medicine and College of Engineering,

13 National Taiwan University, Taipei, Taiwan

14 ^{4A}Department of Ophthalmology, Taipei Medical University Hospital, Taipei, Taiwan.

15 ^{4B}Department of Ophthalmology, School of Medicine, College of Medicine, Taipei Medical

16 University, Taipei, Taiwan

17 ⁵NIHR Biomedical Research Centre for Ophthalmology, Moorfields Eye Hospital NHS

18 Foundation Trust and UCL Institute of Ophthalmology, United Kingdom

19

20 * Corresponding Author

21 Email:Kevin.Hamill@liverpool.ac.uk

22

23 **Key words:** Corneal Cross-linking, Dresden Protocol, Tensile Mechanical Property, Collagen

24 Fibril, Keratoconus

25

26

27

28 **Abstract**

29 **Purpose**

30 UVA/riboflavin induced corneal cross-linking is a common therapeutic approach used to
31 prevent progression in keratoconus. However, there is limited understanding of how changes
32 to the mechanical behavior in cross-linked corneas relate to changes in the corneal
33 ultrastructure. The aim of this study was to determine these relationships and, by so doing,
34 provide a reference against which future modifications to cross-linking approaches may be
35 compared.

36 **Methods**

37 Porcine corneas were treated following the “Dresden” protocol, the current gold standard for
38 clinical treatment, consisting of dropwise application of 0.1% riboflavin in 20% dextran
39 followed by 30 minutes of UVA irradiation. The effect of crosslinking was assessed using
40 uniaxial tensile testing, transmission electron microscopy, and Fourier Transform Infrared
41 Spectroscopy with results compared against corneas treated with each of the treatment
42 solution components individually

43 **Results**

44 Data revealed that UVA/riboflavin cross-linked corneas displayed $28\pm 17\%$ increase in the
45 material tangent modulus compared with dextran treatment alone, and altered collagen
46 architecture within the first 300 μm of stromal depth consisting of 5% increase in the
47 thickness of collagen fibrils, no significant changes to interfibrillar spacing, and an 8% to
48 12% decrease in number of fibrils per unit area. Fourier Transform Infrared Spectroscopy
49 confirmed formation of interfibrillar bonds ($p=0.012$) induced by UVA mediated
50 cross-linking.

51 **Conclusions**

52 Our data support a model wherein collagen fibril diameter and structural density are
53 fundamental parameters in defining tissue stiffening following UVA/riboflavin corneal
54 cross-linking, and provide benchmarks against which modifications to the Dresden
55 cross-linking protocol can be evaluated.

56

57 **Precis**

58 This paper provides the connection between ultrastructural and mechanical properties
59 changes induced by the current gold standard UVA/riboflavin cross-linking protocol. It

60 provides a benchmark against which future protocol modifications can be evaluated.

61 **Introduction**

62 Keratoconus is a corneal thinning disease which results in a focally reduced corneal radius of
63 curvature, abnormal wavefront aberrations, and a localised reduction in corneal thickness and
64 stiffness, these aberrations lead to a decline in visual function which ultimately may require
65 corneal transplantation¹. Corneal cross-linking with riboflavin and UVA is an established
66 treatment for progressive keratoconus and currently is the only therapeutic approach that is
67 capable of significantly altering disease progression² available for many years in Europe, this
68 treatment option has been recently approved by the US Food and Drugs Administration for
69 use in United States².. The so-called “Dresden” or “standard” protocol is the current standard
70 approach for corneal cross-linking for keratoconus; consisting of 30 minutes exposure to
71 3mW fluence (5,4 mJ/cm²) following 30 mins soaking in 0.1% riboflavin ³.

72

73 The Dresden protocol involves topical dosing of the cornea, following epithelial removal,
74 with 0.1% riboflavin in 20% dextran for 30 minutes, then exposure to ultraviolet light over a
75 30 minute period. This rationale behind this approach is that photo-polymerisation, in the
76 presence of the photosensitizer riboflavin, leads to creation of chemical bonds between

77 substrates within the corneal stroma, including between collagens and proteoglycans, and
78 other stromal proteins³⁻⁵. The effects of these induced cross-links have been variously
79 reported as increases in tissue stiffness, resistance to enzymatic digestion, changes to
80 ultrastructure and altered swelling behaviour⁶⁻⁹. However, these studies have generally
81 evaluated the overall effects of full cross-linking protocol without separating the contribution
82 of individual aspects of the treatment and there are some differences in outcome
83 reported^{5,6,10,11}.

84

85 Mechanical properties of biological tissues are largely dependent upon the intertwining of
86 collagen fibrils, linked lamina layers and interfibrillar spacing¹²⁻¹⁴. Therefore, the efficacy of
87 the Dresden corneal cross-linking protocol is believed to be dependent on changes to the
88 mechanical properties of the tissue induced through modifying the characteristics of collagen
89 fibrils within the cornea, and the induction of intrafibrillar bonds, with the overall level of
90 effect being dependent on treatment depth^{15,16}. Previous studies have attempted to evaluate
91 mechanical properties and the effective cross-linking penetration depth by specifically
92 examining anterior and posterior corneal layers^{9,17,18}. These studies demonstrated that the

93 collagen cross-linking effect is predominantly located within anterior stroma but did not
94 specify the parameters that may be involved in determining and defining the amount of the
95 tissue stiffening induced. Indeed, the relationship between the mechanical behaviour of
96 cross-linked cornea and its ultrastructure is poorly explored, and the specific contribution of
97 the dextran within the riboflavin solution has not been reported^{18,19}.

98

99 numerous modifications to the Dresden protocol are being trialed in clinical settings therefore
100 defined knowledge of the effect of the current treatment regime is needed to provide a
101 benchmark against which these modifications can be compared and moreover for rationale
102 design of alternative approaches. The present study aimed to improve this understanding by
103 systematically investigating the role of dextran and the effect of riboflavin and UVA
104 cross-linking in inducing mechanical and ultrastructural changes in the porcine cornea.

105

106 **Materials and methods**

107 **Cross-linking Procedure and Experimental Design**

108 Fresh porcine eyes were collected from an abattoir, washed with phosphate-buffered saline
109 (PBS) (Sigma, Dorset, United Kingdom), the central corneas excised and the corneal
110 epithelium removed. To control for inter-animal variation, each cornea was cut into two
111 segments in a superior-inferior fashion with one half being used as the test sample and the
112 second for the control treatment (Fig. 1).

113

114 The treatment groups were assigned into groups as follows: Group 1 (PBS vs. PBS): 6
115 corneas; each half cornea was topically treated with PBS in 3 minute intervals for 1 hour.
116 used to examine the intrinsic differences between the two segments of each porcine cornea.

117 Group 2 (PBS vs. Riboflavin + PBS): 6 corneas; each corneal segment was topically treated
118 in 3-minute intervals for 1 hour with either PBS or 0.1% riboflavin (Sigma) prepared in PBS.

119 Group 3 (PBS vs. Riboflavin + Dextran): As for Group 2, except that the riboflavin was
120 prepared in 20% dextran (Sigma). Group 4 (Dextran vs. Riboflavin + Dextran + UVA): 10

121 corneas; one half treated following the conventional Dresden protocol³, the anterior surface

122 of the corneas were treated with 5ml of 0.1% riboflavin in 20% dextran every 3-minutes
123 intervals for 30 minutes, followed by UVA (370nm) illumination at 3 mW/cm² (Opto XLink,
124 Mehra Eyetech Pvt Ltd, Delhi, India) for a further 30 minutes. Topical dosing of riboflavin
125 with dextran drops was continued every 3 minutes manner during the UVA irradiation (for
126 full cross-linking details following the standard convention, see table 1)²⁰. Cross-linked
127 corneas were compared to fellow segments treated with 20% dextran only.

128

129 Following treatment, the corneal tissues were dissected for mechanical testing and for
130 ultrastructural analysis (Fig 1).

131

132 **Tensile Testing**

133 Corneal stiffness was examined using a uniaxial tensile tester, Instron 3366 machine (Instron
134 Engineering Corp., MA, USA), equipped with a 10N load cell. Two strips (3mm width x
135 6mm length) cut in the superior-inferior direction at the central cornea were inserted
136 vertically into custom designed clamps and the protocol set to apply a maximum loading
137 stress of 0.125 MPa, slightly above the stress expected under an intraocular pressure of 80

138 mmHg²¹. A constant extension of 1 mm/min was applied and the corresponding stress
139 (applied force divided by cross-sectional area) and strain (extension over original length)
140 measured continuously. Five conditioning cycles²¹, with four-minute recovery periods
141 between each two cycles, were performed. The tangent modulus (Et), the gradient of a
142 tangent of the stress-strain behavior pattern, was calculated at different stress levels to derive
143 the overall stiffness of the tissue^{22,23}.

144

145 **Transmission electron microscopy**

146 Transmission electron microscopy (TEM) was performed as described previously⁶. Briefly,
147 the specimens were isolated from the central regions of cornea (Fig 1) and fixed overnight
148 with 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd, Reading, UK) in 0.1% tannic
149 acid. Thereafter, specimens were dissected into 1mm x 2mm blocks and incubated with 4%
150 osmium (TAAB), followed by serial dehydration through an acetone gradient (30%, 50%,
151 70%, 90%, 100%)(Sigma). Specimens were then infiltrated and embedded in medium resin
152 (TAAB) and ultrathin 70 nm-thickness sections cut using a diamond knife microtome and
153 collected onto 200 mesh copper grids (Ted Pella, Inc., CA, USA). Sections were examined

154 using a Tecnai G2 spirit BioTWIN Transmission electron microscope (FEI Company, OR,
155 USA) operated at 120kV and 60k-fold magnification with a CCD camera. Tissues were
156 sampled at five depth intervals from top of the anterior stroma 0-50µm, 80-150µm,
157 200-250µm, 300-350µm, 400-450µm.

158

159 Collagen fibrils in longitudinal, frontal and oblique profiles were observed in TEM images;
160 only those in frontal profiles were used for quantitative analysis with analyses performed
161 using Fiji software (National Institutes of Health, Bethesda, MA, USA). Ultrastructural
162 parameters evaluated were; i) mean diameter of collagen fibrils ii) interfibrillar spacing and
163 iii) number of fibrils per unit area. For i) circular spots in frontal profiles were isolated,
164 exported and diameters measured. ii) The calculation of interfibrillar spacing was generated
165 from equation (1) while assuming that the collagen fibrils were evenly distributed.

166
$$R + D = \sqrt{A/N} \quad (1)$$

167 Here R was defined as interfibrillar spacing (nm), D as mean diameter of collagen fibrils
168 (nm), A as the area of selected zone of measurement (nm²), N as the number of fibrils
169 (circular spots) within the selected zone. iii) fibrils per unit area was determined by randomly

170 localising a window of fixed unit size (300 x 300 dpi) and the number of circular spots
171 counted.

172 The distribution curve of collagen fibrils was assessed with a bespoke code using MATLAB
173 2016b (The MathWorks Inc., Natick, MA, USA). Original images were converted to binary
174 using adaptive thresholding and a custom designed collagen fibril detection system, using the
175 Circular Hough Transform (CHT) based algorithm^{24,25}, was established to analyse the radius
176 of circular objects in frontal profiles of TEM images. Distribution curves were generated by
177 plotting the frequency in 2nm increments against fibril radius.

178

179 **Fourier Transform Infrared (FTIR) Absorption Spectroscopy**

180 Fresh porcine corneas were treated with either PBS, 0.1% riboflavin in 20% dextran or the
181 full Dresden protocol (6 per group), lyophilized for 3 days, then FTIR spectra measured using
182 a Nicolet 6700 FTIR spectrometer (Thermo Scientific, MA, USA) with a attenuated total
183 reflection (ATR) module. 64 accumulative scans were taken with a resolution of 4 cm⁻¹
184 between 800 cm⁻¹ to 4000 cm⁻¹. Data was collected using OMNIC software (Thermo
185 Scientific, MA, USA). Analyses of FTIR spectra of each condition were obtained with a

186 combination of four spectral intervals [$\nu(\text{C}=\text{O})$ absorption of amide I (1,680–1,630 cm^{-1}),
187 $\delta(\text{NH}_2)$ absorptions of amide II (1,570–1,515 cm^{-1}), $\nu(\text{C}-\text{N})$ absorptions of amide III (1,350–
188 1,200 cm^{-1}), and $\nu(\text{C}-\text{O})$ absorptions of carbohydrate moieties (1,150–1,000 cm^{-1}]. Area
189 under the curve at each interval was analyzed using KnowItAll® ID Expert™ (Bio-Rad, CA,
190 USA).

191

192 **Statistical Analysis**

193 Results for mechanical, ultrastructural and FTIR spectra analyses are presented as means \pm
194 standard deviation (SD) and statistical significance calculated using one-way analysis of
195 variance (ANOVA) with Turkey's HSD post-hoc tests. The Mann-Whitney U test (two-tailed)
196 was used for analyzing the statistical difference of the distribution curves, and significance
197 differences accepted where $p < 0.05$.

198

199 **Results**

200 **UVA/riboflavin treatment and dextran mediated dehydration both contribute to**
201 **mechanical property changes in corneal cross-linking.**

202 In order to isolate the effect of the individual components of UVA/riboflavin crosslinking
203 procedure, uniaxial tensile experiments and transmission electron microscopy were
204 conducted on a set of porcine split into paired comparison groups. To account for
205 inter-animal variability, each cornea was cut in two and one half treated with the treatment
206 and compared against the control (Fig 1), tensile measurements and ultrastructural analyses
207 were performed on the same cornea). Comparison groups were: PBS vs. PBS, riboflavin in
208 PBS vs. PBS, riboflavin in 20% dextran vs. PBS and riboflavin in 20% dextran+UVA vs.
209 20% dextran (Fig 1).

210

211 The tangent modulus (E_t) versus stress (σ) for each corneal strip was determined and the
212 overall stiffening effect indicated by the ratio of the tangent modulus ($E_{t_{\text{experimental}}}/E_{t_{\text{control}}}$).
213 Comparisons concentrated on tangent modulus ratios at a stress of 0.03MPa, which is
214 equivalent to a physiological intraocular pressure (IOP) of around 25mmHg²¹. As expected,

215 no significant differences were observed where both corneal segments were treated
216 identically with PBS, confirming the validity of our intra-eye control system ($E_{t \text{ experimental PBS}}$
217 vs. $E_{t \text{ control PBS}}$: 1.36 ± 0.32 vs. 1.43 ± 0.30 at 0.03MPa, respectively, $p = 0.075$, Figs 2A and 2B,
218 S1 Table). Riboflavin in PBS treatment also caused no stiffening (riboflavin in PBS
219 1.45 ± 0.15 vs. PBS 1.38 ± 0.17 , $p = 0.448$, Figs 2C, S1 Table). However, the riboflavin in 20%
220 dextran group displayed a $13 \pm 9\%$ tangent modulus increase compared to their internal PBS
221 control (riboflavin in dextran: 1.52 ± 0.17 vs. PBS 1.34 ± 0.18 , $p = 0.011$, Figs 2D, S1 Table).
222 Comparing the effect of the full Dresden crosslinking protocol to the effect of dextran
223 treatment alone, we observed a $28 \pm 17\%$ increase in tangent modulus in the Dresden treated
224 corneal segments (riboflavin in dextran + UVA 2.09 ± 0.17 vs. dextran 1.62 ± 0.18 $p =$
225 $5.67E-05$, Figs 2E, S1 Table).

226

227 The differences observed with dextran alone were somewhat surprising however, upon
228 comparing the tissue thicknesses following treatments, we observed statistically significant
229 reduced thicknesses in the dextran treated samples compared with their PBS controls,
230 indicative of dehydration (mean thickness riboflavin in dextran 0.79 ± 0.04 mm, PBS

231 1.05±0.39 mm, $p = 1.39 \times 10^{-6}$)(Fig 2F and S1 Table). Correcting the tangent modulus
232 readings with these thickness measurements removed the apparent dextran effects, whereas
233 residual stiffening effect was still observed in the Dresden protocol treated samples after
234 correction (Fig 2G). Together these data demonstrate that dextran treatment alone causes
235 dehydration and therefore an apparently increased stiffening, whereas UVA exposed
236 riboflavin treated corneas exhibit further increased stiffening beyond that caused by the
237 dextran treatment alone.

238

239 **UVA/riboflavin treatment causes depth dependent changes to collagen fibril**
240 **ultrastructure.**

241 Next we measured changes to the collagen ultrastructure of specimens, measured at five
242 different depth intervals using TEM (S1 Fig, Fig. 2A; representative images at 80-150mm)
243 and the obtained images used to determine collagen fibril diameter (Fig 2B), interfibrillar
244 spacing and fibril number per unit area (density)(S2 Table). The overall morphologies of the
245 collagen ultrastructure did not change following PBS or riboflavin in PBS treatment in the
246 absence of UV (S1 Fig, S2 Table). However, the dextran treatment led to significantly thinner

247 collagen fibrils (Fig 3A), as well as reduced interfibrillar spacing and denser packing at all
248 depth intervals compared to its PBS control (S1 Fig). As dextran also causes loss of tissue
249 hydration we used a mathematical method to correct for dehydration effects, in order to
250 identify the true structural changes²⁶. The relationship between thickness (T) and hydration
251 (H) was modelled using the equation $T = 0.2 * e^{(0.33 * H)}$ which has been shown to be
252 effective for these types of calculation²⁶. The reduction in thickness followed an exponential
253 decrease in tissue hydration, which we used to calculate the hydration state of the tissues and
254 therefore calculate swelling factors at each depth interval (S3 Table). Analysis of fibril
255 diameter at each depth interval revealed that although, in the uncorrected data, dextran
256 treatment caused a reduction in the frequency of large collagen fibrils compared to PBS
257 treatment (Figs 3B; 80-150mm, S2 Table and S3 Table), when correction for swelling factors
258 was included, no significant differences were detected (Fig 3B and S2 Fig), indicating that
259 dextran treatment alone has no effect on collagen fibril diameter.

260

261 In analyzing the effect of the Dresden UVA/riboflavin crosslinking protocol on corneal
262 ultrastructure, both segments within our intra-eye comparisons were treated with dextran

263 therefore any residual difference between the internal controlled comparison groups reflected
264 a true effect of the crosslinking procedure. Analysis revealed a small but statistically
265 significant increase in collagen fibril diameter in the cross-linked group at 80-150 μ m ($5\pm 2\%$,
266 $p<0.01$) and 200-250 μ m depths ($6\pm 3\%$, $p<0.01$) with no differences observed at any other
267 depth intervals (Figs 4C and 4D, S2 Fig). No statistical differences in interfibrillar spacing
268 between treatments were observed at any depths (Fig 4E), however the Dresden protocol
269 caused a decrease in the number of fibrils per unit area in the anterior 250 μ m of the tissue
270 (0-50 μ m $6\pm 5\%$, $p<0.05$; 80-150 μ m $11\pm 7\%$, $p<0.01$; 200-250 μ m $11\pm 5\%$, $p<0.01$) (F5 and S2
271 Table). Together these data indicate that the UVA crosslinking procedure creates relatively
272 small, depth-localized changes to the collagen ultrastructure.

273

274 **Individual ultrastructural measurements are insufficient to predict mechanical changes.**

275 As we had performed the tangent modulus and ultrastructural measurements upon the same
276 eye (Fig 1), we were able to directly compare the values obtained (Fig 5). Note comparisons
277 were made between mechanical outcomes measured at 0.03MPa stress and structural
278 parameters were determined from tissues in relaxed states. Collagen fibril diameter at

279 80-150 μ m, intrafibril spacing increases, and collagen fibril density decreases each displayed
280 correlation with tangent modulus increases across the test population (diameter; $r^2=0.23$, Fig.
281 5A, spacing $r^2= 0.39$, Fig. 5B, density $r^2=0.52$, Fig. 5C). When percentage change on an
282 individual eye basis was plotted, the none of the individual ultrastructural parameters were
283 independently indicative of the overall tissue stiffness (Fig. 5D). However, when considered
284 in combination, the ultrastructural measurements performed better, with a positive correlation
285 of 0.177 (collagen fibril diameter + fibril density - interfibrillar spacing, Fig. 5E).

286

287 **Fourier Transform Infrared Spectroscopy Reveal the Formation of New Amide Bonds** 288 **in Cross-linked Corneas**

289 To assess the detailed chemical reactions and the conversion of chemical bonds within the
290 cornea tissue after UVA/riboflavin cross-linking, Fourier Transform Infrared Spectroscopy
291 measurements were performed on corneas treated with either PBS, riboflavin+dextran or
292 riboflavin+dextran+UVA (Fig 6A and B). The relevant characteristic bands were; amide I
293 C=O stretching vibration (1,680–1,630 cm^{-1}), amide II NH_2 bending vibration (1570-1515
294 cm^{-1}), amide III C-N stretching vibration (1,350–1,200 cm^{-1}), and C-O bond stretching

295 vibration ($1,150\text{--}1,000\text{ cm}^{-1}$). The area under each band was calculated, and the deformation
296 vibrations of CH_2 ($1,485\text{--}1,360\text{ cm}^{-1}$) used as an internal standard to determine the intensity
297 ratios (Fig 6C, S4 Table). These analyses revealed significant increases in the C-O stretch
298 peak, decreased C-N stretch and increased NH_2 deformation following crosslinking (Fig
299 6C). Plotting the ratio of C-N stretch to NH_2 deformation suggests that the decrease
300 intensities of amide II infrared absorption bands are likely to be accompanied by an increase
301 in the formation of amide III bonds (riboflavin+dextran+UVA 2.5 ± 0.5 compared to PBS
302 1.7 ± 0.3 , and riboflavin+dextran 1.8 ± 0.2 Fig 6D, S4 Table).

303

304 **Discussion**

305 In this study, we have characterized how corneal mechanical properties relate to
306 ultrastructural changes following Dresden protocol treatment, and identified the contribution
307 of the different components of the protocol to observed effects. Specifically, our data
308 demonstrate that the increases in corneal stiffness measured following Dresden protocol
309 treatment arise from combination of dextran-mediated dehydration along with
310 UVA/riboflavin induced new bond formation and depth-dependent increases in collagen fibril
311 diameter.

312 Tissue stiffness is considered as a combination of the internal geometry of the tissue and the
313 properties of the material itself. Therefore, although our data indicate that almost half of the
314 measured stiffening effect of the Dresden protocol comes from the dextran component of the
315 protocol, these effects can be explained by dehydration effects. However, we did observe an
316 increase in C-O stretching force in the riboflavin in dextran treated corneas compared with
317 controls indicating that dextran does itself induce collagen changes. A potential explanation
318 for these new bonds is that the dextran induced dehydration increases the swelling pressure of
319 tissue and therefore the resistance pressure of proteoglycan matrix^{26,27}, giving rise to

320 intermolecular forces. These dextran and dehydration effects could help explain some of the
321 apparently contradictory reports in the literature^{5,6,10,11}. Interestingly tissue hydration states
322 have been reported to affect the efficacy of cross-linking treatment²⁷, therefore, hydration
323 states and the osmolarity of the riboflavin solutions could be important factors to consider in
324 protocol modifications.

325

326 Our FTIR data revealed significant changes in C-O stretching force and conversion rate of
327 amide bonds following the full Dresden protocol. Lysine-based cross-links following
328 UVA/riboflavin cross-linking have previously been postulated but not been found
329 chemically^{3,28} but it has been proposed that cross-links form through endogenous carbonyl
330 groups including imidazole formation²⁹. Our data support a model where the increased
331 swelling pressure and the involvement of endogenous carbonyls (allysine) leads to the new
332 bond formation²². This leads to a broader mechanism where UVA/riboflavin induced
333 intra-fibril bonds, presenting as thickened collagen fibrils and less dense overall structure and
334 drive the increased tissue mechanical strength and resistance to dehydration (Fig. 7).

335

336 It should be noted, however, that the ultrastructural changes we measured do not fully
337 account for the mechanical effects suggesting that collagen fibril diameter and spacing are
338 not the only aspects of stromal biology affected by UVA/riboflavin crosslinking. Effects
339 upon other stromal proteins and particularly the interactions of collagen with proteoglycans,
340 could be contributing to the stiffening. Proteoglycans within the corneal stroma have been
341 proposed to play a pivotal role in regulating the fibril-fibril spacing and
342 hydration-dehydration properties³⁰, however, the dehydration induced decrease in interfibril
343 spacing has prevented us from being able to determine the sole effect of cross-linking on
344 proteoglycans within our experimental system.

345

346 Understanding the stabilization mechanism of UVA/riboflavin cross-linking is clinically
347 relevant when evaluating modifications to the Dresden protocol and developing optimal
348 protocols or new keratoconus treatments. This study establishes the standards in terms of
349 mechanical, chemical and structure/biological changes induced by the Dresden protocol and
350 therefore provides the baseline against which modifications can be judged.

351

352 **Acknowledgments**

353 The authors thank Alison Beckett at Biomedical Electronic Microscopy Unit for teaching
354 techniques and preparing materials, and Brendan Geraghty at Biomechanical Engineering
355 Group in the University of Liverpool for help with mechanical settings. SHC performed
356 experiment, analyzed data, developed methodology, prepared figures and wrote manuscript
357 text. AM was involved in data curation and manuscript writing and editing. YRJ collected
358 FTIR data, THY and TJW supervised data acquisition. CEW, KJH and AE provided resources
359 and supervision, were responsible for overall conceptualization, manuscript writing and
360 editing.

361

362

363 **References**

- 364 **1.** Andreassen TT, Simonsen AH, Oxlund H. Biomechanical properties of
365 keratoconus and normal corneas. *Exp. Eye Res.* Oct 1980;31(4):435-441.
- 366 **2.** Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr.*
367 *Opin. Ophthalmol.* Aug 2006;17(4):356-360.
- 368 **3.** Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen
369 crosslinking for the treatment of keratoconus. *Am. J. Ophthalmol.* May
370 2003;135(5):620-627.
- 371 **4.** Brummer G, Littlechild S, McCall S, Zhang Y, Conrad GW. The role of
372 nonenzymatic glycation and carbonyls in collagen cross-linking for the treatment
373 of keratoconus. *Invest. Ophthalmol. Vis. Sci.* Aug 11 2011;52(9):6363-6369.
- 374 **5.** Hayes S, Kamma-Lorger CS, Boote C, et al. The effect of riboflavin/UVA collagen
375 cross-linking therapy on the structure and hydrodynamic behaviour of the
376 ungulate and rabbit corneal stroma. *PloS one.* 2013;8(1):e52860.
- 377 **6.** Akhtar S, Almubrad T, Paladini I, Mencucci R. Keratoconus corneal architecture
378 after riboflavin/ultraviolet A cross-linking: ultrastructural studies. *Mol. Vis.*
379 2013;19:1526-1537.
- 380 **7.** Spoerl E, Wollensak G, Dittert DD, Seiler T. Thermomechanical behavior of
381 collagen-cross-linked porcine cornea. *Ophthalmologica.* Mar-Apr
382 2004;218(2):136-140.
- 383 **8.** Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea
384 against enzymatic digestion. *Curr. Eye Res.* Jul 2004;29(1):35-40.
- 385 **9.** Wollensak G, Wilsch M, Spoerl E, Seiler T. Collagen fiber diameter in the rabbit
386 cornea after collagen crosslinking by riboflavin/UVA. *Cornea.* Jul
387 2004;23(5):503-507.
- 388 **10.** Wang X, Huang Y, Jastaneiah S, et al. Protective Effects of Soluble Collagen during
389 Ultraviolet-A Crosslinking on Enzyme-Mediated Corneal Ectatic Models. *PloS one.*
390 2015;10(9):e0136999.
- 391 **11.** Wollensak G, Aurich H, Pham DT, Wirbelauer C. Hydration behavior of porcine
392 cornea crosslinked with riboflavin and ultraviolet A. *J. Cataract Refract. Surg.* Mar
393 2007;33(3):516-521.
- 394 **12.** Boote C, Dennis S, Newton RH, Puri H, Meek KM. Collagen fibrils appear more

- 395 closely packed in the prepupillary cornea: optical and biomechanical
396 implications. *Invest. Ophthalmol. Vis. Sci.* Jul 2003;44(7):2941-2948.
- 397 **13.** Depalle B, Qin Z, Shefelbine SJ, Buehler MJ. Influence of cross-link structure,
398 density and mechanical properties in the mesoscale deformation mechanisms of
399 collagen fibrils. *Journal of the mechanical behavior of biomedical materials.* Dec
400 2015;52:1-13.
- 401 **14.** Whitford C, Studer H, Boote C, Meek KM, Elsheikh A. Biomechanical model of the
402 human cornea: considering shear stiffness and regional variation of collagen
403 anisotropy and density. *Journal of the mechanical behavior of biomedical
404 materials.* Feb 2015;42:76-87.
- 405 **15.** Raiskup F, Spoerl E. Corneal crosslinking with riboflavin and ultraviolet A. I.
406 Principles. *The ocular surface.* Apr 2013;11(2):65-74.
- 407 **16.** Schumacher S, Mrochen M, Wernli J, Bueeler M, Seiler T. Optimization model for
408 UV-riboflavin corneal cross-linking. *Invest. Ophthalmol. Vis. Sci.* Feb 16
409 2012;53(2):762-769.
- 410 **17.** Kohlhaas M, Spoerl E, Schilde T, Unger G, Wittig C, Pillunat LE. Biomechanical
411 evidence of the distribution of cross-links in corneastreated with riboflavin and
412 ultraviolet A light. *J. Cataract Refract. Surg.* 2006;32(2):279-283.
- 413 **18.** Seifert J, Hammer CM, Rheinlaender J, et al. Distribution of Young's modulus in
414 porcine corneas after riboflavin/UVA-induced collagen cross-linking as
415 measured by atomic force microscopy. *PloS one.* 2014;9(1):e88186.
- 416 **19.** Choi S, Lee S-C, Lee H-J, et al. Structural response of human corneal and scleral
417 tissues to collagen cross-linking treatment with riboflavin and ultraviolet A light.
418 *Lasers in medical science.* 2013;28(5):1289-1296.
- 419 **20.** Randleman JB, Santhiago MR, Kymionis GD, Hafezi F. Corneal Cross-Linking
420 (CXL): Standardizing Terminology and Protocol Nomenclature. *J. Refract. Surg.*
421 Nov 1 2017;33(11):727-729.
- 422 **21.** Elsheikh A, Wang D, Brown M, Rama P, Campanelli M, Pye D. Assessment of
423 corneal biomechanical properties and their variation with age. *Curr. Eye Res.*
424 2007;32(1):11-19.
- 425 **22.** Boyce B, Jones R, Nguyen T, Grazier J. Stress-controlled viscoelastic tensile
426 response of bovine cornea. *J. Biomech.* 2007;40(11):2367-2376.
- 427 **23.** Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and

- 428 porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J. Cataract*
429 *Refract. Surg.* 2003;29(9):1780-1785.
- 430 **24.** Atherton TJ, Kerbyson DJ. Size invariant circle detection. *Image and Vision*
431 *computing.* 1999;17(11):795-803.
- 432 **25.** Yuen H, Princen J, Illingworth J, Kittler J. Comparative study of Hough transform
433 methods for circle finding. *Image and vision computing.* 1990;8(1):71-77.
- 434 **26.** Hatami-Marbini H, Etebu E, Rahimi A. Swelling pressure and hydration behavior
435 of porcine corneal stroma. *Curr. Eye Res.* 2013;38(11):1124-1132.
- 436 **27.** Hodson SA. Corneal stromal swelling. *Prog. Retin. Eye Res.* 1997;16(1):99-116.
- 437 **28.** Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp. Eye Res.*
438 1998;66(1):97-103.
- 439 **29.** McCall AS, Kraft S, Edelhauser HF, et al. Mechanisms of corneal tissue
440 cross-linking in response to treatment with topical riboflavin and
441 long-wavelength ultraviolet radiation (UVA). *Invest. Ophthalmol. Vis. Sci.*
442 2010;51(1):129-138.
- 443 **30.** Hahn RA, Birk DE. beta-D xyloside alters dermatan sulfate proteoglycan
444 synthesis and the organization of the developing avian corneal stroma.
445 *Development.* 1992;115(2):383-393.

446

447

448

449 **Figure legends**

450 **Fig 1. Experimental scheme.** Each cornea was cut into two segments in a superior-inferior
451 fashion. The right-hand segment was used as an experimental segment, while the left-hand
452 segment was used as its corresponding control. Four experimental groups were designed,
453 Group 1: PBS vs. PBS; Group 2: PBS vs. riboflavin+PBS; Group 3: PBS vs.
454 riboflavin+dextran; Group 4: dextran vs. riboflavin+dextran+UVA. After each treatment,
455 specimens were dissected and processed for tensile testing (green hollow rectangle) and TEM
456 (red hollow rectangle). S: superior; I: inferior.

457

458 **Fig 2. The tangent modulus (E_t) versus stress (σ) behaviour and ratio of tangent**
459 **modulus of paired samples.** (A) The tangent modulus versus stress behavior of right-hand
460 corneal flaps versus left-hand flaps from 6 are plotted. Both flaps were identically treated by
461 soaking in PBS. The ratio of tangent modulus between (B) PBS versus PBS (n=6), (C)
462 riboflavin in PBS versus PBS (n=6), (D) riboflavin in 20% dextran versus PBS (n=6), (E)
463 riboflavin in 20% dextran+UVA exposure versus 20% dextran only (n=10). Values from
464 each individual cornea pair tested are indicated by grey lines. Average stiffening ratio \pm

465 standard deviation are indicated by the bold line and error bars. The red dash line represented
466 the value of 1; i.e. no difference between paired samples. Gray shaded region in A-E plots
467 represents 0.03MPa. (F) average thickness measurement of each comparison group after
468 treatment. (G) the stiffening ratio at 0.03MPa with and without correction for tissue thickness
469 changes. Values in F and G denote mean \pm standard deviation (SD). * p value of < 0.05 , **
470 $p < 0.01$.

471

472 **Fig 3. Transmission electron microscopy (TEM) images of porcine corneas imaged at**
473 **depth of 80-150 μ m (A)** Representation TEM images of PBS, riboflavin in dextran, and
474 dextran only and riboflavin/dextran/UVA cross-linked corneas. Bar 500nm. An area of 300 x
475 300 dpi (yellow dash-square) is shown at higher magnification in (B) with example of the
476 measurement of the area and density of collagen fibrils.

477

478 **Fig 4. Measurement of ultrastructural parameters and collagen fibril distribution at**
479 **different depth intervals.** (A) Mean collagen fibril diameters of PBS and riboflavin in
480 dextran at each depth interval. (B) Collagen fibril diameter distributions plotted as either

481 uncorrected values from of riboflavin in dextran group (top panel, black filled squares), or
482 corrected for dehydration (top panel, gray squares) or its corresponding PBS control (middle
483 panel, blue filled squares). Traces are shown overlaid in bottom panels. Grey filled
484 background added to aid visualization. (C) Collagen fibril diameter distribution curve of
485 cross-linking group (green boxes) and its dextran control (black boxes) at each depth interval.
486 (D) Relative collagen fibril diameter, (E) relative interfibrillar spacing, (F) relative collagen
487 density of cross-linking group relative to its dextran group at each depth interval. Values are
488 plotted as mean±SD from n=6 (A and B) or n=10 (C, D, E and F). Asterisks denote
489 significant differences from control groups with * p < 0.05, ** p<0.01.

490

491 **Fig 5. Correlation between ultrastructural parameters and tangent modulus.** Tangent
492 modulus at 0.03MPa versus (A) collagen fibril diameter, (B) interfibrillar spacing and (C)
493 collagen fibril density. Black boxes; dextran treated eyes, green boxes;
494 Riboflavin+UVA+Dextran treated eyes, (D) percentage change in tangent modulus versus
495 percentage change in each ultrastructural parameter. Each box represents the measurements
496 from one eye for either fibril diameter (red), interfirbil distance (orange) or fibril density

497 (yellow). (E) Percentage change in tangent modulus plotted against the cumulative effects of
498 percentage change in fibril diameter+interfibril spacing-fibril density. Each box represents
499 one eye. Green line; linear line of best fit. Red dotted line; 100% correlation.

500

501 **Fig 6. Absorption FTIR spectra of porcine corneas following cross-linking treatments.**

502 (A) Representative FTIR spectra of corneas treated with PBS (bottom, gray),
503 riboflavin+dextran (middle, black) or riboflavin+dextran+UVA (top, green). (B) Regions
504 where the characteristic bands are located shown at higher magnification and regions of
505 interest indicated: Amide I ($1,680-1,630\text{ cm}^{-1}$), Amide II ($1570-1515\text{ cm}^{-1}$), Amide III
506 ($1,350-1,200\text{ cm}^{-1}$), and CO absorption band ($1,150-1,000\text{ cm}^{-1}$). (C) Relative intensity ratio
507 of each characteristic band and (D) conversion rate of amide II to amide III.

508

509 **Fig 7. Proposed model of UVA/riboflavin cross-linking with the presence of dextran.** The

510 black solid circle, blue solid line, green solid line and red solid line represent the collagen
511 molecules, proteoglycans, original cross-links formed between collagen molecules and
512 additional cross-links induced by UVA/riboflavin cross-linking treatment, respectively.

513

514 **Supplementary Information Legends.**

515 S1 Fig. Transmission electron microscopy (TEM) images of porcine corneas imaged at
516 different depth intervals following PBS, dextran and/or UVA/riboflavin treatment.

517 Representative TEM images of PBS (A), Riboflavin+PBS (B), Riboflavin+Dextran (C), or
518 Riboflavin+Dextran+UVA (D) and their corresponding controls at five depth intervals of
519 0-50 μ m, 80-150 μ m, 200-250 μ m, 300-350 μ m, 400-450 μ m.

520

521 S2 Fig. Collagen radius distribution of each treatment group at each depth intervals. The
522 distribution of control segments (blue); (A) PBS, (D) PBS, (G) PBS, and (K) Dextran, and
523 the experimental segments (orange) (B) PBS, (E) Riboflavin+PBS, (H) Riboflavin+Dextran,
524 and (L) Riboflavin+Dextran+UVA. Dashed lines represent trend curves. The overlapped
525 curves (C, F, I, J, M) were used for detecting the shifting of distribution in radius between
526 control and experimental segments. The data were obtained at five depth intervals of 0-50 μ m,
527 80-150 μ m, 200-250 μ m, 300-350 μ m, 400-450 μ m. *p* value of < 0.05 is indicated by an
528 asterisk compared with its internal control.

529

530 S1 Table. Summary of the average thickness and stiffening ratio at 0.03MPa of each group
531 before and after thickness correction. Values denote mean \pm standard deviation (SD). *p* value
532 of < 0.05 is indicated by an asterisk compared with its control. Red asterisks indicate increase
533 compared with control, green represents decrease.

534

535 S2 Table. Mean diameter, interfibrillar spacing, and density of collagen fibrils of corneal
536 segments at different depth intervals following PBS, dextran and/or UVA/riboflavin
537 treatment. Values denote mean \pm standard deviation (SD). *p* value of < 0.05 is indicated by an
538 asterisk compared with its control. Red asterisks indicate increase compared with control,
539 green represents decrease.

540

541 S3 Table. Summary of area ratio corresponding to indicated characteristic bands in FTIR
542 spectra. Values denote mean \pm standard deviation (SD). *p* value of < 0.05 is indicated by an
543 asterisk compared with its control. Red asterisks indicate increase compared with control,
544 green represents decrease.

545

546 S4 Table. Parameters (decreased percentage, hydration loss percentage, remaining hydration

547 percentage and swelling factor) used in correction of the collagen fibril radius distribution

548 curves.

549