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1 **Bulk changes in posterior scleral collagen microstructure in human high**
2 **myopia**

3

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28

29 **Abstract**

30

31 Purpose: We aimed to characterise any bulk changes in posterior scleral collagen fibril
32 bundle architecture in human eyes with high myopia.

33

34 Methods: Wide-angle X-ray scattering (WAXS) was employed to map collagen orientation at
35 0.5mm × 0.5mm spatial intervals across the posterior sclera of seven non-myopic human eyes
36 and three eyes with high myopia (>6D of refractive error). At each sampled point, WAXS
37 provided thickness-averaged measures of 1) the angular distribution of preferentially-aligned
38 collagen fibrils within the tissue plane and 2) the anisotropic proportion (ratio of
39 preferentially aligned to total collagen scatter).

40

41 Results: Non-myopic specimens featured well-conserved microstructural features, including
42 strong uniaxial collagen alignment along the extraocular muscle insertion sites of the mid-
43 posterior sclera and a highly anisotropic annulus of collagen circumscribing the nerve head in
44 the peripapillary sclera. All three myopic specimens exhibited notable alterations in the
45 peripapillary sclera, including a partial loss of circumferential collagen alignment and a
46 redistribution of the normally observed regional pattern of collagen anisotropic proportion.
47 Linear mixed model analysis indicated that mean fiber angle deviation from circumferential
48 in the peripapillary sclera of highly myopic eyes ($23.9^\circ \pm 18.2$) was significantly higher than
49 controls ($17.9^\circ \pm 12.0$) ($p < 0.05$).

50

51 Conclusions: Bulk alterations to the normal posterior scleral collagen microstructure can
52 occur in human eyes with high myopia. These changes could reflect remodelling of the
53 posterior sclera during axial lengthening, and/or a mechanical adaption to tissue stresses
54 induced by fluid pressure or eye movements that may be exacerbated in enlarged eyes.

55

56

57 **Introduction**

58

59 Myopia is the most common visual disorder, affecting 23% of the world's population, with
60 the number expected to reach 50% by 2050 [1]. Myopia is a type of refractive error defined
61 by the inability to see at greater distances and is caused, in major part, by an abnormal axial
62 lengthening of the globe - placing the eye's focal plane in front of the retina. Individuals with
63 myopia exceeding 6D are classified as having high myopia [2-4] and are at increased risk of
64 developing further complications that can lead to temporary or permanent loss of vision,
65 including glaucoma, cataract, macular degeneration and retinal detachment [5]. As its
66 prevalence continues to rise, gaining control of the escalating myopia problem is becoming a
67 growing global concern [6].

68

69 Myopic lengthening of the eye involves remodelling and biomechanical changes to its main
70 load-bearing tissue – the sclera, the white fibrous tissue that comprises about 85% of the
71 ocular tunic [7]. The sclera consists predominantly of densely woven fibrils of the complex
72 protein collagen that impart the tissue with mechanical rigidity and which, in turn, helps
73 maintain the eye's structural integrity and shape [8]. In the human sclera, about 90% of the
74 dry weight is due to collagen. After being secreted into the extracellular space, collagen
75 molecules assemble into fibrils, which have a wide range of diameters from 25 to 230nm [9]
76 and span many hundreds of microns in length in mature tissues [10]. The collagen fibril
77 bundles in the sclera are more complex and generally more disorganised than in the
78 neighbouring cornea and show a high degree of regional variability in their bulk orientation
79 between different areas of the tunic [11-13]. The collagen architecture of the posterior sclera
80 plays a major role in governing tissue deformation in response to changes in intraocular
81 pressure (IOP) and cerebrospinal fluid pressure (CSFP), and scleral stresses are readily
82 transmitted to the more compliant tissues of the optic nerve head (ONH) [12, 14]. The ONH
83 may be considered a “weak spot” in the scleral tunic, where the sieve-like lamina cribrosa
84 (LC) supports the exiting nerve axons, and where deformation forces are accumulated -
85 making it an area of particular mechanical interest [15, 16].

86

87 A number of alterations to both the scleral structure and neighbouring tissues have been noted
88 to occur with high myopia. With axial elongation of the eye globe the sclera, LC and choroid
89 have been noted to become thinner [17-19]. Sclera growth and remodelling in the myopic eye
90 is considered to be a dual process [20, 21]. The amount of collagen decreases by both a

91 down-regulation in the synthesis of the type I collagen and concomitant stimulation of
92 collagen degradation [22, 23]. The end result is a decline in existing collagen bundles and a
93 prevention of the formation of new ones. A decrease in collagen fibril diameter, particularly
94 near the posterior pole, has also been noted [24]. Studies in mammalian models further
95 confirm that the changes during myopia development are the result of active tissue
96 remodelling rather than just passive stretching of the sclera, contributing to a compromise in
97 the mechanical stability and integrity of the tissue [25, 26]. However, while there is
98 substantial evidence that collagen remodelling underlies the axial elongation of the myopic
99 sclera [20-26], it is not known to what extent this process manifests in terms of bulk changes
100 to the orientation of collagen in the tunic - a key determinant of its direction-dependent
101 biomechanical properties. Previously we have applied wide-angle X-ray scattering (WAXS)
102 to map the collagen fibrillar architecture in normal and glaucomatous posterior scleral shells
103 [12, 13]. The goal of the current study was to apply these methods to evaluate any bulk
104 changes to collagen orientation in the posterior sclera of highly myopic human eyes.

105

106 **Methods**

107

108 Tissue details and sample preparation

109

110 All experimental procedures were conducted in accordance with the Declaration of Helsinki.
111 Nine human ocular globes (seven non-myopic and two highly myopic) were obtained from
112 the Fondazione Banca degli Occhi del Veneto, Italy. In addition, one further highly myopic
113 eye was obtained from the Department of Ophthalmology, University of Hong Kong. All
114 specimens were acquired within a 13-18 hour window after death. Following removal of the
115 ocular contents, the intact scleral shells were stored in 4% paraformaldehyde at 277K. The
116 eyes were designated their myopic/normal status ($> 6D$ for highly myopic) via examination
117 by an ophthalmologist and none had a history of previous surgery involving the posterior
118 sclera. Furthermore, using the polar vector plot maps of collagen orientation from the
119 conducted WAXS experiments, we measured the distance between landmarks of the optic
120 nerve canal edge and the insertions of the inferior oblique muscle, as a measure of the degree
121 of scleral lengthening (Figure 1, Table 1). Scleral specimens were prepared based on
122 previously established protocols [12, 13]. The surrounding fat, muscle and episcleral tissues
123 were carefully removed before the optic nerve was excised with a razor blade flush to the
124 sclera [13]. The cleaned globes were dissected around the equator and the internal lens, retina

125 and choroid and subsequently removed. To prevent the formation of creases when flat
126 mounting the posterior cups, relaxing meridional incisions were made in the posterior sclera
127 from the equator to just outside the peripapillary region - defined as the 1.5mm-wide annular
128 scleral region immediately adjacent to the optic nerve canal opening. The specimens were
129 then returned to 4% paraformaldehyde until the time of the X-ray experiments. As shown in
130 our previous work, this mild fixation does not affect WAXS orientation measurements [27].
131 Details of the eyes used in this study are provided in Table 1. The mean donor age for the
132 control group of seven non-myopic eyes was 66.3 ± 7.1 years, while the mean donor age of the
133 three highly myopic specimens was 66.7 ± 8.3 years.

134

135 X-ray scattering data collection

136

137 Previously our group has developed a method for quantifying the bulk collagen fiber
138 orientation of the sclera using WAXS [12, 13]. When incident monochromatic X-rays pass
139 through the sclera, a portion of them are scattered at different angles and their direction will
140 reflect the sclera's intrinsic microstructure. A well-resolved single diffraction peak is formed
141 perpendicular to the fibril axis - referred to as the equatorial direction. This scatter pattern
142 arises from the regular ~ 1.6 nm lateral packing of the collagen molecules that make up the
143 fibrils [28]. The angular intensity distribution can be analysed to quantify the number of
144 fibrils in each direction within the tissue plane. A key advantage of this approach is that the
145 scleral tissue is not required to be sectioned, embedded, or stained for the experiments, thus
146 preventing artificial disruptions in the microstructure. Moreover, irrespective of the varying
147 diameter and packing of scleral collagen fibrils across the eye tunic, the diameter and packing
148 of the constituent collagen molecules from which the WAXS signal originates is highly
149 uniform, which gives rise to a sharp well-resolved signal that is relatively impervious to
150 variations in tissue hydration [12]. The technique provides quantification of the collagen
151 orientations as an average of the tissue thickness [29].

152

153 WAXS experiments were conducted at the Diamond Light Source (Harwell, UK), the UK's
154 national synchrotron facility. The specimens were measured using macromolecular
155 crystallography beamlines I02 and I03, which have identical capabilities. The beamlines were
156 operated in a custom-modified fiber-diffraction set-up to record WAXS patterns across each
157 scleral sample at 0.5mm (horizontal) \times 0.5mm (vertical) intervals using an integrated x-y
158 motor stage (Figure 2) [12, 30]. The whole of each posterior sclera cup was scanned for all

159 specimens, apart from highly myopic specimen HM3, where only a 16mm × 16mm square
160 region centered on the ONH was available to the study. To prevent tissue dehydration during
161 data collection, the specimens were wrapped in polyvinylidene chloride film and mounted
162 inside Perspex (Lucite Group Ltd, Southampton, UK) chambers with Mylar (DuPont-Teijin,
163 Middlesbrough, UK) windows. The incident X-ray beam was directed perpendicular to the
164 specimen surface, with an exposure time of 1s or 0.5s and recorded electronically on a
165 Pilatus-6MF silicon pixel detector (Dectris Ltd, Baden, Switzerland) placed 350mm behind
166 the specimen. The wavelength of the focused beam was 0.09795nm with a 150μm × 80μm
167 cross-sectional size.

168

169 X-ray scattering data processing

170

171 By analysing the angular distribution of intensity around the 1.6nm WAXS reflection (Figure
172 3A) a quantitative measure of the relative number of collagen fibrils orientated at a given
173 angle within the scleral plane can be acquired. We obtained from all specimens, at each
174 sampled point in the tissue: 1) the relative number of preferentially aligned fibrils at a given
175 angle over and above the underlying isotropic population, referred to as the *collagen*
176 *orientation distribution*, with the magnitude of the principal direction, referred to as the
177 *collagen anisotropy*. 2) the scatter due to preferentially aligned collagen divided by that from
178 the total fibrillar collagen content, referred to as the *anisotropic proportion*.

179

180 The quantification of scleral fiber collagen orientation from WAXS patterns is described in
181 detail elsewhere [12, 28]. The scatter profiles were analysed using a bespoke MATLAB
182 software script (MATLAB; The MathWorks, Natick, MA) that adapted a previously used
183 approach [12, 31]. 720 radial profiles (one every 0.5°) were extracted from each WAXS
184 pattern and a unique power-law background function was fitted and subtracted from each
185 (Figure 3B) [12, 13, 30]. The isolated scatter profiles along each direction were normalised
186 against X-ray beam current fluctuations and exposure time, radially integrated and the values
187 extracted to angular bins. The resulting angular intensity profiles were divided into two
188 components: isotropic and anisotropic scatter (Figure 3C) and the latter plotted in polar
189 vector coordinates. To take into account the fact that equatorial scatter occurs at right angles
190 to the collagen axis a 90° shift in the total collagen scatter distribution was performed. For
191 each sampled point in the scleral tissue the collagen orientation distribution could be
192 represented by a polar vector plot (Figure 3D). Individual plots were then assimilated into

193 montages and the anisotropy assigned color codes in MATLAB, representative of the highest
 194 degree of alignment (maximum vector length per plot). Contour maps of collagen anisotropic
 195 proportion were generated in MATLAB, by calculating the ratio of aligned against total
 196 integral collagen scatter, (Equation 1):

197

$$198 \quad \text{Anisotropic_proportion} = \frac{\int_0^{2\pi} I_a d\phi}{\int_0^{2\pi} (I_a + I_i) d\phi} \quad (1)$$

199 where I_a is the preferentially aligned collagen scatter (i.e above the isotropic threshold I_i) at
 200 angle ϕ (Figure 3C), and ϕ is the azimuthal fiber angle in the tissue plane (Figure 3A). To
 201 compare bulk collagen structural changes between myopic and non-myopic individuals we
 202 selected a fixed region of 64 sampling points within a 1.5mm radius of the optic nerve canal
 203 edge, representative of the peripapillary scleral region [12]. Sampling points outside of this
 204 region were considered to be part of the mid-posterior sclera. The peripapillary sclera was
 205 further divided into 4 quadrants based on their position: Superior-Nasal (SN), Superior-
 206 Temporal (ST), Inferior-Temporal (IT) and Inferior-Nasal (IN), and for all of the sub-regions
 207 an average for the collagen anisotropy was calculated. To quantify any distortion in the
 208 alignment direction of preferentially aligned collagen bundles in the peripapillary sclera, we
 209 compared the angular displacement of the main direction revealed by the polar vector plots
 210 (for individual myopic specimens and the averaged control) to an idealized angle distribution
 211 representative of the circumferential collagen fiber structure circumscribing the optic nerve
 212 that characterizes the normal human sclera [12, 13, 32]. The idealized distribution model
 213 (Supplementary Figure 1) was created in MATLAB and consists of one partial inner ring and
 214 three full outer rings of polar vector plots (total angular range: 0° to 180°). The distribution
 215 width of the idealized plot (dispersion around the main orientation) was computed from the
 216 average of the experimentally determined peripapillary scleral plots from the control
 217 specimens. Within each quadrant of the distribution there are $n+2$ polar plots per ring
 218 ($0^\circ/180^\circ$ and 90° are omitted from the partial inner ring) and a $90/(n+1)$ angular step, where n
 219 is the radial position of the ring with respect to the scleral canal edge (1 being the closest).
 220 This resulted in a total of 16 plots per quadrant, matching the spatial sampling of the
 221 peripapillary sclera in the WAXS experiments, arranged in as close to circumferential
 222 orientation as possible.

223

224 Statistical analysis

225

226 For statistical assessment of differences in collagen anisotropic proportion and main fiber
227 alignment direction between highly myopic and control eyes, we leveraged the 64 unique
228 spatial measurements recorded per eye from the peripapillary sclera and carried out a linear
229 mixed model analysis for repeated measures (considered as a nested variable) using SPSS
230 software ver. 24.0 (IBM Corp., Armonk, NY). Linear mixed model analysis allows for
231 marginal estimations through the increase in observations in the cluster variable - maximizing
232 the statistical power of the analysis. For statistical analysis, data from control specimens N1
233 and N2 (a pair from the same donor) were firstly averaged point-for-point. For the mixed
234 models, a compound symmetry variance/co-variance structure was selected according to a
235 smaller the better information basis, based on Hurvich and Tsai's criterion for small sample
236 sizes (other structures compared were: 1st order autoregressive and diagonal). A probability
237 threshold of $p < 0.05$ was considered significant for mean differences in anisotropic
238 proportion and fiber deviation angle (from circumferential) between control ($n=6$) and highly
239 myopic ($n=3$) groups.

240

241 **Results**

242

243 In Figure 4 a polar plot map of collagen orientation is presented. The map is overlaid on top
244 of a photograph of the scanned posterior sclera of a non-myopic right human eye (specimen
245 N4). In accordance with previous WAXS studies, reproducible structural features
246 characteristic of the non-myopic sclera were found [12, 13]. These included the tendon
247 insertions of the inferior oblique muscle in the mid-posterior region, which were found to be
248 consistent in position from the landmark of the optic nerve canal (Table 1). Around the optic
249 nerve, the collagen bundles were preferentially aligned in a circumferential direction and this
250 feature exhibited noticeably higher collagen anisotropy. Another consistent feature was two
251 linear fiber bands that radiated tangentially from the peripapillary ring of aligned collagen
252 outwards into the mid-posterior scleral region [33]. All of these features were found to be
253 present in the other six non-myopic specimens from the control group (see supplementary
254 material).

255

256 In Figure 5 a comparison between a typical non-myopic scleral polar vector map and the
257 three highly myopic specimens is presented and reveals several marked differences in the

258 bulk collagen orientation. In non-myopics, there is consistently a disruption in the
259 circumferential collagen orientation in the SN quadrant of the peripapillary sclera, as found in
260 previous studies [12, 13] (Figure 5B). However, for myopic specimen HM1 two such regions
261 of disruption were observed instead, in the ST and IN regions (Figure 5D). HM2 exhibited
262 more widespread differences: the ONH appears wider and the surrounding annulus of
263 collagen, which had a noticeably larger interruption in its circumferential structure in the SN
264 quadrant, was spread over a larger radial distance - extending well into the mid-posterior
265 sclera (Figure 5F). HM3 also featured a larger SN interruption to the normal circumferential
266 structure (Figure 5G), and also a noticeable splitting of the fiber alignment into two sub-
267 populations over the majority of the peripapillary sclera. In particular, peripapillary fibers in
268 HM2 and HM3 showed a move away from circumferential alignment towards the radial
269 direction (Figure 5F, G).

270

271 For each sampled point of the posterior sclera, a value for the ratio of aligned to total collagen
272 (anisotropic proportion) was also extracted and plotted (Figure 6). The anisotropic proportion
273 values of the peripapillary sclera for the seven non-myopic posterior scleral specimens were
274 combined into an averaged control. This was justified based on the highly conserved collagen
275 structure of the posterior sclera in non-diseased eyes, as shown herein and previously [12].
276 Regional quantification of this data is shown in Figure 7. For all seven non-myopic
277 specimens the minimum collagen anisotropic proportion was consistently observed in the SN
278 quadrant and the maximum value observed in the IN quadrant (Supplementary Table 1 and
279 Figure 7). This pattern was not exhibited in the highly myopic specimens HM1 and HM2,
280 where the minimal value was in the ST and IT, and maximum in the IT and ST quadrants,
281 respectively - while HM3 displayed maximum anisotropic proportion in the IT quadrant
282 (Figure 7). The atypical results for the myopic sclera are highlighted in Figure 7, where the
283 myopic specimen values (apart from the SN quadrant of HM3) are clearly identifiable as
284 outliers to the box-plot data. The anisotropic proportion for the peripapillary sclera in
285 specimen HM2 generally demonstrated higher values than both the controls, HM1 and HM3
286 (Figure 6B, D, F, G). This appeared initially at odds with the vector plot maps, that indicated
287 overall lower collagen anisotropy for HM2 around the nerve head (Figure 5B, D, F, G).
288 However, the two observations may be reconciled if we consider that the collagen anisotropy
289 will scale directly with tissue thickness (and hence total collagen scatter), whereas the
290 anisotropic proportion will scale inversely with thickness. Hence, it is likely that excessive
291 tissue thinning around the posterior pole in myopic specimen HM2 would have manifested in

292 a lower total collagen scatter, and hence higher anisotropic proportion, while the absolute
293 number of fibrils along the preferred direction (defining the collagen anisotropy) was
294 relatively low. This would also be consistent with HM2 showing the largest indications of
295 scleral lengthening, as determined by the inferior oblique muscle to posterior pole distance
296 (Table 1). Statistical comparison of the difference in mean collagen anisotropic proportion in
297 the whole peripapillary sclera between control (n=6) and highly myopic (n=3) groups using
298 linear mixed model analysis fell below the $p < 0.05$ significance threshold (Figure 8A). This
299 is likely because of the cancelling effect of some tissue quadrants showing increases, and
300 some decreases, with myopia. Sample numbers were insufficient to do a quadrant-wise
301 statistical comparison.

302

303 In order to further quantify the structural differences between the non-myopic control group
304 and the two highly myopic eyes, we compared the angular displacement of the collagen
305 vector plots from an idealized circumferential distribution (Supplementary Figure 1). The
306 right eye was chosen as default and for left eyes a mirror image of the polar vector maps was
307 taken. Figure 9 shows maps of the angle difference between the idealized circumferential
308 distribution and (A) averaged control, (B) myopic specimen HM1, (C) myopic specimen
309 HM2 and (D) myopic specimen HM3. The results indicate how closely the non-myopic
310 structure follows the idealized circumferential orientation around the ONH (Figure 9A). HM1
311 followed the pattern to a lesser degree and diverged markedly from the idealized distribution
312 in the ST quadrant with a maximum deviation of 74° (Figure 9B). For HM2 the differences
313 were most pronounced on the outer parts of the peripapillary region in the SN quadrant, with a
314 maximum deviation of 83° (Figure 9C). For HM3, SN disturbances were again most
315 pronounced, with a maximum deviation of 79° (Figure 9D). These differences are further
316 highlighted in the box-plot data in Figure 10, where the majority of the HM data is again
317 visible as outliers to the control data. Mean fiber deviation (from circumferential) in the
318 whole peripapillary sclera was statistically different between control ($17.9^\circ \pm 12.0$, n=6) and
319 highly myopic ($23.9^\circ \pm 18.2$, n=3) groups ($p < 0.05$) using linear mixed model analysis
320 (Figure 8B).

321

322 **Discussion**

323

324 This paper presents the first application of WAXS mapping to determine bulk collagen
325 orientation changes in human eyes with high myopia. The results verify that in non-myopic

326 human posterior sclera the collagen orientation distribution is highly conserved between
327 individuals, while in specimens with high myopia a marked loss of the normal
328 microstructural organisation was observed. Previous research has provided evidence on
329 remodelling of the scleral extracellular matrix with myopia progression [21, 34]. However,
330 until now it has remained unknown how bulk scleral collagen fibril orientation is affected in
331 myopia. The presented results provide evidence that highly myopic posterior sclera do not
332 follow the normal fibrillar organisation, with all three myopic specimens exhibiting notable
333 changes in the peripapillary sclera. Specifically, the high myopia group showed a statistically
334 significant increase in fiber angle deviation away from the normal circumferential
335 arrangement with more radially oriented fibers present in the peripapillary sclera overall.
336 Notable regional variations between the three myopic specimens studied likely reflect
337 different stages of myopic lengthening, rather than natural variations between individual
338 patients, since we have established herein and previously [12] that the collagen
339 microstructure of the peripapillary sclera is well-conserved between individuals not affected
340 by posterior scleral disease. Unfortunately, the limited number of highly myopic specimens
341 available to the study precluded us from being able to statistically compare individual HM
342 specimens either with each other or to the control group, or to carry out a quadrant-wise
343 analysis within the peripapillary sclera.

344

345 The existence of a distinct ring of peripapillary collagen fibers around the optic nerve was
346 reported for the first time less than a decade ago and since then has been documented to exist
347 in humans as well as a number of animals [27, 31, 35-38]. The circumferentially orientated
348 fibril bundles provide mechanical stability to the ONH as they limit the IOP-related
349 expansion of the scleral canal and reduce the in-plane tensile strains within the LC [14, 16,
350 29, 39, 40]. As such, changes to the peripapillary collagen architecture in myopia may be
351 linked to an increased susceptibility to ONH damage in glaucoma [12, 41, 42]. All highly
352 myopic specimens in this study displayed noticeable disruption in the preferential orientation
353 of the collagen fibrils around the ONH. It is possible that remodelling of the extracellular
354 matrix has occurred as a result of myopia and that, given the mechanical role of the
355 peripapillary sclera, that this may, in turn, affect the mechanical environment of the ONH and
356 its physical response to IOP and CSFP fluctuations [14, 41, 43, 44].

357

358 While the structural changes reported herein could be a consequence of scleral remodelling
359 during axial lengthening, a further conceivable possibility is that they may represent a

360 mechanical adaptation to increased tissue stresses in an enlarged eye. In respect of this, it is
361 worthwhile to note that a change in collagen fiber orientation in myopic eyes could
362 potentially be due to loads other than IOP and CSFP. During horizontal eye movements, the
363 optic nerve can exert a traction force on the eye globe to shear and deform the ONH tissues
364 [45, 46]. In high myopia, the optic nerve traction force could be significantly increased
365 because of an elongated eyeball, thus yielding a higher amount optic nerve straightening for
366 the ONH to travel the same distance as that in a healthy eye. In highly myopic eyes with
367 staphylomas, it has also been shown that the optic nerve traction force can be so large to even
368 retract the eyeball within its orbit [47]. If such a traction force were to increase in high
369 myopia, then its contribution on collagen remodeling might become more important than that
370 of IOP or CSFP, and collagen fibers may try to orient along the direction of higher stress. In
371 adduction (left eye movement for a right eye), the dura will transmit higher stress in the
372 temporal side of the peripapillary sclera, which could plausibly result in a radial alignment of
373 collagen fibers in that area. Interestingly, in this study we found disrupted collagen fiber
374 orientations in both nasal and temporal regions. However, in high myopia several
375 morphological changes such as the presence of a tilted disc or peripapillary atrophy (delta and
376 gamma zones [48]) may also affect stress distributions within the peripapillary sclera and
377 other remodeling scenarios may be plausible. Stress levels can also be high in the nasal
378 quadrant of the sclera in abduction [46]. To better understand this phenomenon, we are
379 currently building growth and remodeling computational models to help us tease out the most
380 relevant forces responsible for a change in collagen fiber orientation.

381

382 A number of studies have linked a significant increase in the prevalence of glaucoma with
383 high myopia [49-51]. Studies conducted by Jonas et al. (1988), Saw et al. (2005) and Kimura
384 et al. (2014) indicate that highly myopic patients have larger optic discs [5, 52, 53]. Jonas et
385 al. (1988) described them as “secondary acquired macrodisks”, which are accompanied by
386 larger peripapillary atrophic regions [52]. Saw et al. (2005) added to the list of abnormalities
387 a tilt to the optic disc as well as a thinner LC [5]. Bellezza et al. (2000) concluded that a
388 larger optic disc is more susceptible to IOP-related damage, which could link to the
389 pathological changes to the scleral architecture presented here [54]. Specifically, in specimen
390 HM2 the scleral canal was noticeably enlarged, with the width of the aligned collagen ring
391 spanning a larger radius than in the control specimens. This could be a direct result of
392 elongation of the eye. Based on the polar vector plot map for HM1 the optic nerve canal
393 appears to be stretched in the ST-IN direction, in which there also a smaller amount of

394 preferentially aligned collagen. This is reminiscent of the findings of Pijanka et al. (2012) for
395 glaucomatous specimens, which showed a significantly lower degree of peripapillary
396 collagen alignment in glaucomatous eyes [12]. Furthermore, the Beijing eye study found that,
397 while there was no significant difference in IOP between highly-myopic and non-myopic
398 eyes, the former group exhibit a significantly higher onset of glaucoma [49]. This could
399 further suggest that a greater risk of developing glaucomatous damage might be linked with
400 structural changes occurring with high myopia, such as those in the peripapillary sclera noted
401 herein.

402

403 Several experimental limitations and factors must be taken into account when drawing
404 conclusions from the present study. Firstly, the number of highly myopic specimens used in
405 the study was small due to the universally limited availability of suitable posterior scleral
406 tissue from donors of known myopic status. **While** the structure of **all three** myopic eyes did
407 noticeably deviate from the non-myopic eyes, whose structural features were, in contrast,
408 highly reproducible between specimens, **caution should be used when applying the results of**
409 **the current preliminary work to human high myopia in general without validation in a larger**
410 **sample population.** Secondly, the axial length of the specimens was not determined. This was
411 compensated by calculating the distance from the edge of the optic nerve canal to the
412 insertion of the inferior oblique muscle for each posterior shell, as a measure of the scleral
413 tissue elongation. Notably, the results were highly consistent between controls (Table 1), with
414 a marked increase for myopic specimens HM2 and HM3. This calculation was not possible to
415 do accurately for HM1 because the wide-spread nature of the structural deformations present
416 in this specimen precluded the use of the inferior oblique muscle insertion as a reliable
417 landmark. However, the specimen was confirmed to be highly myopic in the clinic, with >6D
418 of refractive error. Nonetheless, further studies are required to correlate axial length with
419 scleral microstructural changes in order to shed further light on the role of collagen fiber
420 remodelling in myopia progression. Also related to this point, it is unknown if any of the
421 donors from the current study had myopia since early childhood (early onset myopia), or else
422 developed myopia later in life; or in the former case how the original disease associated with
423 high myopia. How these factors might relate to the microstructural alterations described in
424 the current study warrants further investigation. Thirdly, information about the sex and
425 ethnicity of the donors was not available. While the potential effect (if any) of sex on scleral
426 microstructure is not known, there is some limited documented evidence that collagen fiber
427 arrangement [55] and structural stiffness [56] of the posterior sclera may vary between ethnic

428 groups. Fourthly, there are inherent limitations to the WAXS method itself. As mentioned,
429 WAXS yields thickness-averaged results and cannot provide clarity on structural composition
430 throughout the tissue depth. Pijanka et al. (2015) showed that the circumferentially aligned
431 collagen fibers do not persist through the entire tissue depth but rather the outer two-thirds of
432 the stroma [30]. Thus it remains unknown if the observed changes in myopic specimens are
433 present through the entire depth of the scleral tissue. Flattening of the scleral coat may have
434 released some of the residual stress that is present in the intact tissue, potentially causing
435 changes in the typical collagen fibril orientation. It has been shown, however, that this effect
436 is more profound at a macro (organ) level and less prominent at the collagen microstructure
437 level [57]. Moreover, the relaxing incisions used to flatten the tissue did not penetrate the
438 peripapillary tissue where the quantitative analysis in this paper was concentrated. In
439 addition, original fixation of the eye tunic in its natural curvature should have further limited
440 the extent of any fibrillar reorganization upon subsequent dissection. Nevertheless,
441 considering the limited number of specimens available to the study, it was decided not to
442 include the mid-posterior tissue in the quantitative analysis, as minor changes in fiber
443 alignment near the cuts cannot be ruled out.

444

445 In conclusion, using WAXS we have mapped the bulk posterior scleral collagen structure of
446 three human eyes with high myopia. In comparison to non-myopic eyes, all three highly
447 myopic specimens showed disruptions in the characteristic circumferential collagen fibril
448 organisation in the peripapillary sclera, as well as changes in the normally well-conserved
449 regional pattern of anisotropic proportion. The results support the idea that pathological
450 structural remodelling takes place with high myopia that accompanies axial lengthening and
451 mechanical alteration of the scleral tissue. Further research is required to ascertain whether
452 these structural changes are a direct result of remodelling of the posterior sclera during axial
453 lengthening, or else could be a mechanical adaptation to tissue stresses induced by fluid
454 pressure or eye movements that may be exacerbated in enlarged eyes. Structural changes in
455 the peripapillary region may link to the increased susceptibility of myopic eyes to glaucoma
456 development. The present data will enhance future modelling studies of ocular biomechanical
457 changes in myopia and glaucoma.

458

459

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461

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465

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617

618 **Figure Legends**

619

620 **Figure 1:** Calculating the distance between the edge of optic nerve canal and the tendon
621 insertions of the inferior oblique muscle. WAXS polar vector plots (plot interval: 0.5mm)
622 reveal circumferential collagen annulus around the canal and oblique uniaxial alignment of
623 muscle insertion region. Canal edge is denoted by curved line. Three individual
624 measurements (line lengths) were performed and a mean taken as the representative value.
625 (A) Non-myopic posterior sclera N6. (B) Highly myopic specimen HM2. Note marked
626 increase in line length for myopic specimen, indicative of axial lengthening of globe.

627

628 **Figure 2:** Beamline I03 at the Diamond Light Source operating in a custom fiber-diffraction
629 set-up. The goniometer (A) provides directional translation of the sample holder (B) between
630 X-ray exposures. A flat-mounted posterior sclera is shown mounted between Mylar sheets.
631 After the specimen is positioned a further Mylar sheet (C) in which a lead beam stop (D) is
632 attached, preventing undiffracted X-rays from reaching and damaging the detector positioned
633 out of shot.

634

635 **Figure 3:** X-ray scattering data analysis. (A) WAXS pattern from peripapillary human sclera
636 N4. The area bounded by the two concentric circles corresponds to the collagen scatter. The
637 X-ray scatter intensity spread as a function of the azimuth angle ϕ around the collagen peak
638 can be analysed, which provides the distribution of fibril orientations. The presented two-
639 lobed WAXS pattern is indicative of the uniaxial fiber alignment at that point in the tissue.
640 (B) Power-law background function (green line) fitted to a radial intensity profile (red line)
641 through the pattern shown in (A). The blue open circle marks the peak in collagen intensity,
642 while the blue crosses show the fitting points of the background function. For each WAXS

643 pattern, a background function was independently fitted along the 720 equally spaced radial
644 directions, which allows extraction of the collagen signal in two dimensions. (C) Angular X-
645 ray scatter intensity profile for the pattern presented in (A). The collagen scatter intensity
646 may be represented as two components – scatter from the isotropically aligned collagen
647 fibrils (*I_i*) and anisotropic scatter (*I_a*) arising from preferentially aligned collagen. (D)
648 Corresponding polar vector plot of the collagen alignment. The anisotropic collagen scatter is
649 displayed in polar coordinates, where the length of vector **r** is proportional to the relative
650 number of collagen fibrils orientated along the preferred direction.

651

652 **Figure 4:** WAXS polar vector map showing preferential collagen orientation across non-
653 myopic flat-mounted posterior sclera N4, overlaid over a photograph of the tissue before
654 scanning. The superior direction of the specimen is indicated with an arrow. Polar vectors are
655 color coded according to bar, with warmer colours indicative of higher degrees of collagen
656 anisotropy. Note highly aligned collagen annulus circumscribing the nerve head (black line
657 bounded region), two tangential fiber bands (black arrows) and uniaxial alignment of the
658 ocular muscle insertion regions, with the inferior oblique highlighted (red arrow).

659

660 **Figure 5:** WAXS polar plot vector maps comparing one non-myopic (A-B) and two highly
661 myopic (C-F) posterior scleras. (A) Full map of non-myopic specimen N4; (B) 30 × 30 vector
662 plot zoom of N4; (C) Full map of highly myopic specimen HM1; (D) 30 × 30 vector plot
663 zoom of HM1; (E) Full map of highly myopic specimen HM2; (F) 30 × 30 vector plot zoom
664 of HM2. (G) Map of myopic specimen HM3. The zoomed regions are denoted by a red
665 square on the full maps. Peripapillary scleral region is shown bounded by black lines, in
666 which largely circumferential collagen alignment is observed. Arrows: interruption of the
667 circumferential collagen orientation (normally limited to the SN quadrant in non-myopic
668 eyes) is more extensive in highly myopic specimens. S, N, I and T denote superior, nasal,
669 inferior and temporal directions, respectively.

670

671 **Figure 6:** WAXS contour maps of collagen anisotropic proportion for one non-myopic (A-B)
672 and two highly myopic (C-F) posterior scleras. (A) Full map of non-myopic specimen N4;
673 (B) 30 × 30 point zoom of N4; (C) Full map of highly myopic specimen HM1; (D) 30 × 30
674 point zoom of HM1; (E) Full map of highly myopic specimen HM2; (F) 30 × 30 point zoom
675 of HM2. (G) Map of myopic specimen HM3. The zoom regions are denoted by a red square

676 on the full maps. Peripapillary scleral region is shown bounded by black lines. S, N, I and T
677 denote superior, nasal, inferior and temporal directions, respectively.

678

679 **Figure 7:** Box plots of mean collagen anisotropic proportion in the peripapillary sclera by
680 quadrant for the non-myopic control group (SN: Superior-Nasal, ST: Superior-Temporal, IT:
681 Inferior-Temporal, IN: Inferior-Nasal). Specimen-specific corresponding values for highly
682 myopic specimens HM1, HM2 and HM3 are shown for comparison and denoted by circles,
683 asterisks and triangles, respectively. Note that the majority of the myopic data lie outside the
684 non-myopic range.

685

686 **Figure 8:** Group-wise statistical comparison of mean (A) collagen anisotropic proportion and
687 (B) fiber angle deviation from circumferential in the whole peripapillary sclera using linear
688 mixed model analysis. Asterisk denotes significance at the $p < 0.05$ level.

689

690 **Figure 9:** Variation from idealized circumferential angle distribution (with respect to the
691 nerve canal edge) of the polar vector plots from the peripapillary sclera. Averaged control (A)
692 is shown alongside the three highly myopic specimens HM1 (B), HM2 (C) and HM3 (D)
693 following the orientation of a right eye viewed from the back: Top – Superior, Left – Nasal,
694 Bottom – Inferior, Right – Temporal. Marked deviations from circumferential alignment
695 show up as hot-spots in the myopic maps.

696

697 **Figure 10:** Box plots of mean collagen fiber deviation from circumferential orientation in the
698 peripapillary sclera by quadrant for the non-myopic control group (SN: Superior-Nasal, ST:
699 Superior-Temporal, IT: Inferior-Temporal, IN: Inferior-Nasal). Specimen-specific
700 corresponding values for highly myopic specimens HM1, HM2 and HM3 are shown for
701 comparison and denoted by circles, asterisks and triangles, respectively. Note that the
702 majority of the myopic data lie outside the non-myopic range.

703

704 **Table 1:** Details of the eye specimens used in the current study. Optic nerve head (ONH)
705 canal edge to inferior oblique (IO) muscle insertion distance is included as a measure of
706 relative axial globe elongation for all specimens, apart from HM1 which was not measurable
707 (as denoted N.A.). Note the consistent ONH-IO distance for normal (non-myopic) specimens,
708 which was markedly increased for highly myopic specimens HM2 and HM3.

709

710 **Supplementary Figure 1:** Idealized mathematical polar vector distribution for perfect
711 circumferential alignment, used to compare control and myopic collagen orientation in the
712 largely circumferential peripapillary region. Numerical values from 0 to 180 degrees denote
713 the main orientation angle.

714

715 **Supplementary Figure 2:** WAXS polar plot vector maps of three non-myopic (A-F)
716 posterior scleras. (A) Full map of non-myopic specimen N1; (B) 30×30 vector plot zoom of
717 N1; (C) Full map of non-myopic specimen N2; (D) 30×30 vector plot zoom of N2; (E) Full
718 map of non-myopic specimen N3; (F) 30×30 vector plot zoom of N3. The zoomed regions
719 are denoted by a red square on the full maps. Peripapillary scleral region is shown bounded
720 by black lines. Discontinuations of the circumferential collagen orientation in the SN
721 quadrant are indicated by arrows. S, N, I and T denote superior, nasal, inferior and temporal
722 directions, respectively.

723

724 **Supplementary Figure 3:** WAXS polar plot vector maps of three non-myopic (A-F)
725 posterior scleras. (A) Full map of non-myopic specimen N5; (B) 30×30 vector plot zoom of
726 N5; (C) Full map of non-myopic specimen N6; (D) 30×30 vector plot zoom of N6; (E) Full
727 map of non-myopic specimen N7; (F) 30×30 vector plot zoom of N7. The zoomed regions
728 are denoted by a red square on the full maps. Peripapillary scleral region is shown bounded
729 by black lines. Discontinuations of the circumferential collagen orientation in the SN
730 quadrant are indicated by arrows. S, N, I and T denote superior, nasal, inferior and temporal
731 directions, respectively.

732

733 **Supplementary Table 1:** Comparison of mean collagen anisotropic proportion by quadrant
734 for non-myopic control group specimens. Minimum and maximum values for each specimen
735 (denoted by – and + symbols) are consistently observed in the SN and IN quadrants,
736 respectively.

737