SCLERAL CHANGES IN MYOPIA

A thesis submitted to The University of Manchester for the degree of Master of Philosophy in the Faculty of Biology, Medicine and Health Science.

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List of Abbreviations

AES: Aston Eye Study.

AFM: Atomic Force Microscope.

AL: Axial length.

CHASE: Child Heart and Health Study in England

CLEERE: Collaborative Longitudinal Evaluation of Ethnicity and Refractive Error

E^3: European Eye Epidemiology

ECM: Extracellular matrix

EOM: Extraocular muscle

FDM: Form deprivation myopia

GAG: Glucosaminoglycan

NT: Neurotransmitter

LIM: Len-induced myopia

MEPEDS: Multi-Ethnic Pediatric Eye Disease Study

MMP: Matrix metalloproteinases

NHNES: National Health and Nutrition Examination Survey

NICER: Northern Ireland Childhood Errors of Refraction

NIES: Norfolk Island Eye Study

PG: Proteoglycan

PAL: Progressive Addition Lenses

RPE: Retinal Pigment Epithelium

RT-PCR: Real-time Polymerase chain reaction

SER: Spherical Equivalent Refraction SS:

Statistically significant

TEM: Transmission Electron Microscope

MRSA: Methicillin-Resistant Staphylococcus Aureus

UVAF: Conjunctival UV-transmission filters

WAXS: Wide-angle X-ray scattering

HIV: Human Immunodeficiency Virus

Abstract

Background

Atomic Force Microscopy (AFM) imaging and biomechanics on bulk *ex vivo* human scleral tissue demonstrates the effect of chemical cross-linking on the nanomorphology of collagen fibrils, quantified using 2D-Fast-Fourier Transform. This study sets out to understand scleral role in myopia development and treatment by revealing the effect of cross-linking on scleral structure and mechanics.

Methodology

Four post-mortem longitudinally oriented scleral strips 7mmx3mm of healthy human scleras (2 male and 2 female donors; average age of 54.5 years) were used. They were randomly assigned into treatment groups (equatorial and posterior regions) each cross-linked with 0.5mM genipin and 0.1mM glutaraldehyde. Both cross-linkers mixed with Phosphate Buffered Saline Gibco™ Dulbeccos Phosphate-Buffered Saline (DPBS) with calcium, magnesium for specific number of hours at room temperature. The control groups (from same scleras and regions) were untreated. After dissection, samples were washed with sterile distilled water, dried and stored in the fridge (4^{o}C) overnight to stick onto a sterilized slide for AFM. The donors were free from diabetes mellitus/ HIV/MRSA. Scleras were obtained from the Manchester Eye biobank (NRES ethics 15/NW/0932). All the scleras had research permission from the donors' relatives where transplantation was not done. One-way ANOVA between and within the means of the treatment and control groups was done to determine the *F*-statistics and *P*-value. Histograms, bar graphs and box and whisker plots were used to show correlations in the different groups. 2-dimensional Fast-Fourier Transform was carried out on the images to calculate the D-periodicities of the tissue collagen fibrils.

Results

Although some AFM investigations were not completed, significant increase in biomechanical stiffness (higher reduced-YM) by up to 1.3-, 1.2- and 5.2-folds (F_1 = 27.64, p = 2.0323E-11; F_2 = 80.91, p = 1.6161E-28; F_3 = 213.26, p = 1.0364E-46) was observed with genipin crosslinking in three samples. Whereas a 2-fold increase in stiffness occurred in one of the glutaraldehyde cross-linked samples (F_3 = 213.26, p = 1.0364E-46). Also, a 2-fold increase in biomechanical stiffness was recorded after 12 hours of incubation with genipin ($F_{1,4}$ = 36.88, p = 0.00000001). No significant change was found in the D-periodicities ($F_{1,2}$ = 3.70, p = 0.19). However, the fibril 'packing' changed more significantly with genipin than with glutaraldehyde cross-linked samples.

Conclusion

Chemical cross-linking with genipin significantly alters collagen nanomorphology and biomechanics of the sclera, therefore presenting as a potential treatment intervention for human myopia. More samples should be studied, and the concentration/time of treatment considered in future work.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Chapter 1: Introduction

1.1. Brief description of early eye growth and myopia

The normal size of the eyes and its pre- and post-natal development is actively controlled by genes, transcription factors and visual experience (Smith et al., 2009a, Forrester et al., 2015). Genes and transcription factors direct eye growth from the inner parts of the neural folds/plate at around the 22nd day of human embryogenesis. The neural folds fuse into the neural tubes which then interact with the neighboring surface ectoderm to form some ocular tissues see figure 1a and b (Chai et al., 2017, Forrester et al., 2015). At around week 6 and 7, mesenchymal cells surrounding the growing eyes (optic cup) condense into the iris, ciliary body, choroid, cornea, and sclera. By week 12, fibroblasts are embedded into the scleral extracellular matrix (ECM) (Forrester et al., 2015).

At birth, the human eye is slightly hyperopic (far-sighted) or emmetropic ('normal' sight) at infancy with a normal distribution. Growth is rapid until age 3 it dwindles (Norton, 1999, Curtin, 1985). During post-natal growth, optical components (cornea and lens) combine with axial components (retina, choroid, and sclera) with inputs from visual environment, to bring about emmetropia (no refractive error or condition). This is the called emmetropization (Norton, 1999, Troilo, 1992, Rada et al., 2006).

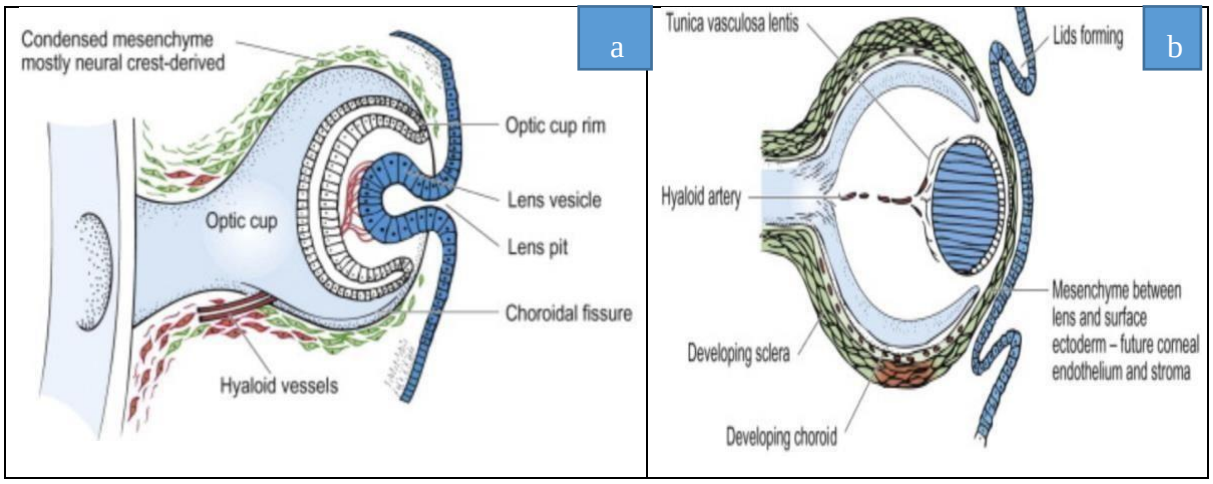


***Figure 1,a, ventrolateral view of developing eye (optic cup), 5th week of human embryogenesis, condensed mesenchyme (green) forms developing sclera in 1b. 1b,* 7th week of human embryogenesis, developing sclera (green) is more compacted. Adapted from Drzezo, (2019)**

Studies have established the presence of an emmetropization mechanism governed by genetic factors (Siegwart Jr and Norton, 2011), homeostatic nonvisual cues and retina

specific visually modulated control signals other than the brain or simple retinal blur (see figure 2 and 3) (Wallman and Winawer, 2004, Wildsoet et al., 2019, Schaeffel and Feldkaemper, 2015).

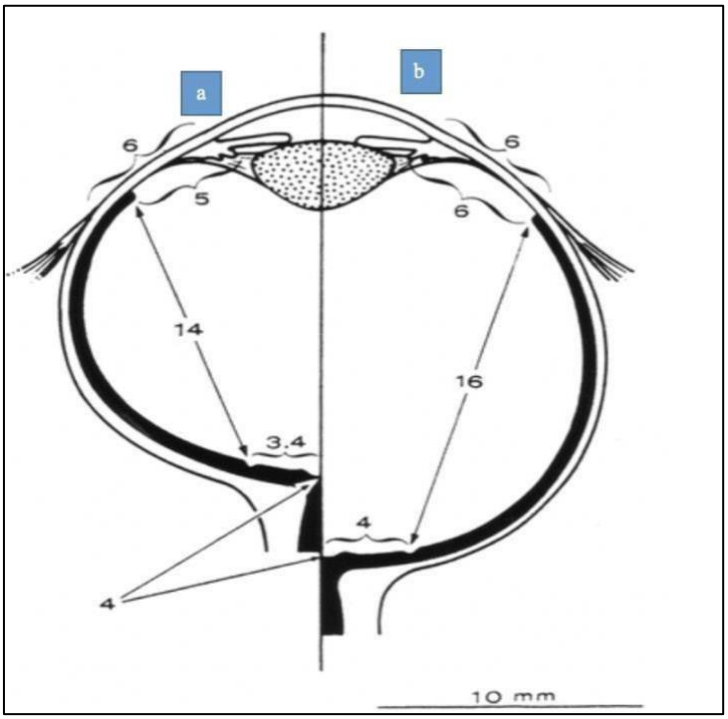


Figure 2 Cross-sectional view of normal vision (a) and form vision-deprived (b) eyes of growing monkeys (eyelids were sutured from 2 weeks to 18months). Measurements in mm. eye from form-deprived vision (a) is longer (16mm) than eye with normal vision(b) (14mm). Adapted from Wiesel and Raviola, (1977)

Also, it is stated that emmetropization exists in all vertebrates for images to be focused onto the retinal photoreceptors and for control of refractive error such as myopia (or nearsightedness), hyperopia and astigmatism (Schaeffel and Feldkaemper, 2015). The evidence for this is lens-induced myopia/ form-deprivation myopia (LIM/FDM) animal models (inducing myopia using minus lenses or translucent diffuser) (Smith III and Hung, 2000). Local visual deprivation of all types of form vision in chicks, not spatial frequency, may cause refractive error at specific retinal regions through lengthening of the vitreous chamber and scleral remodeling (Smith et al., 2009a). This results in a shift in the retinal focal plane (Wallman et al., 1987, Norton and Mcbrien, 1992, Smith et al., 2012, Schaeffel and Feldkaemper, 2015). Although recent studies show that man-made indoor and outdoor environments have spatial features that may drive myopia (Flitcroft et al., 2019a).

In addition, a retrospective study revealed high myopia prevalence in the 73 young humans compared to 12,000 normal subjects after pattern vision was binocularly deprived by cataract and other eye anomalies (see figure 3) (Rabin et al., 1981). A recovery (reduction in myopia) from the induced myopia has also been reported as part of the emmetropization phenomena and evidence of visually controlled eye growth (Rada et al., 2006).

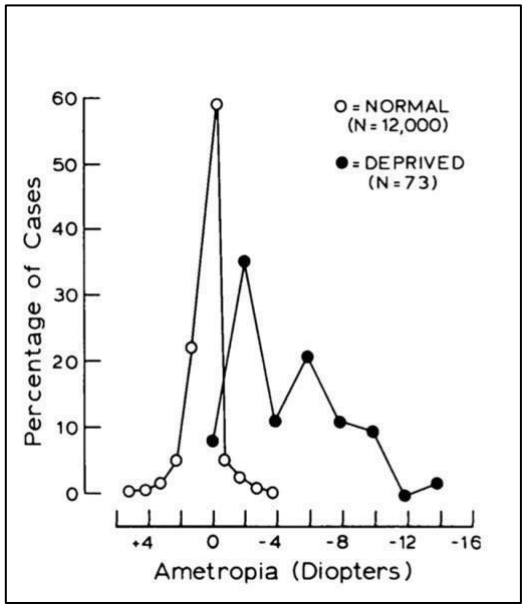


Figure 3 Graph showing the ametropia distribution in normal and binocular vision deprived eyes, deprived eyes (73) have more myopia, adapted from Rabin et al., (1981).

However, the fact that FDM and LIM function through different mechanisms is overlooked in certain studies (Wolffsohn et al., 2019). For instance, optical defocus must reach a critical threshold/ stimulate whole visual field to cause significant myopia (Arumugam et al., 2016) and FDM depends on genetic and environmental factors (Troilo et al., 2019). Genetic influence is however not significant (Morgan and Rose, 2019).

Emmetropization may continue beyond adolescent age according to a recent Norwegian adolescent study (Hagen et al., 2019) and is interrupted when normal crystalline lens flattening, thinning, and stretching due to peripheral scleral growth ceases. According to Mutti et al, (2012). the posterior central sclera continues to grow independent of the crystalline lens (Mutti et al., 2012). Myopia susceptibility is highest at earlier ages (called the sensitivity period) which is around 5-8weeks in some animals and 7-15 years in humans (Siegwart Jr and Norton, 1998). In addition, myopia results from abnormal biochemical and cellular changes in retina, choroid, and sclera (Daw, 2014). This review focuses on the role of the sclera in myopia.

1.1.1. Scleral macro- and micro- structure

The opaque viscoelastic human sclera is the outermost coat of the eye and toughest of the three – retina, choroid, and sclera. It protects the inner tissues and maintains the size and shape of the eyeball by resisting internal and external forces, see figure 4. It allows for attachment/insertion of the eye's extraocular muscles. The ciliary muscles are supported by the sclera during lenticular accommodation (Metlapally and Wildsoet, 2015). The sclera is continuous with the cornea anteriorly and the brain's dura mater lining and the optic nerve posteriorly. Several nerves and blood vessels perforate the sclera through the posterior scleral foramen. The blood supply of the human sclera is from the episclera, choroid and vascular plexi of the tenon's capsule (Watson and Young, 2004). The mammalian sclera can check refractive error development due to its anatomy (Mcbrien et al., 2009).

Histologically, the fibrous tri-layered sclera is mostly composed of collagenous bundles, interspersed with elastic fibers, and interposed with fibroblasts which synthesize and maintain the extracellular matrix (ECM). The ECM and its fibrils determine scleral elasticity (Forrester et al., 2015). The irregular arrangement of the bundles/lamellae, higher water content, variable fibrillar spacing et cetera, account for scleral opacity, see table 1 (Meek, 2008).

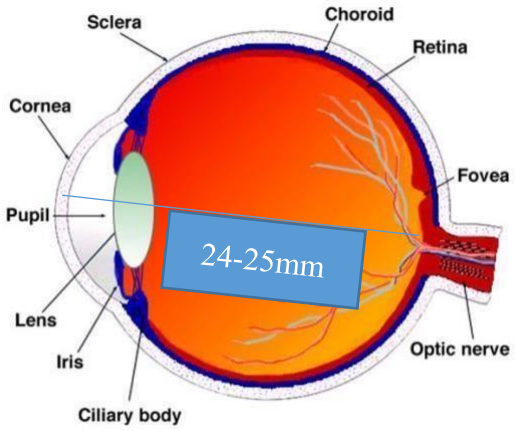


Figure 4 Model of emmetropic adult eye (vertical sagittal section), showing sclera and other ocular tissues. Normal adult eye axial length from cornea to retina is 24-25mm, adapted from Stovall, (2013)

The mechanical property of the sclera is modulated by collagen (Ricard-Blum, 2011). Collagen is the most abundant structural protein (between 25 -35%) in mammalian

ECM secreted by myofibroblast. Astbury was the first to study its primary structure in 1940 (Mayne and Burgeson, 1987, Forrester et al., 2015, Ricard-Blum, 2011). Although there is a wide variety of ECM molecules and collagen types (28-29 different types) (Ricard-Blum, 2011), extensive homology (such as recurring structural motifs and triple-helix of 3 polypeptide chains) exists. For instance, fibre-forming collagen (types I,II,III,V,XI) are homologous but different from non-fibrillar collagen (such IV,VIII,IX,XII, etc) (Forrester et al., 2015).

Collagen I is most abundant (>90%) and plays important role in corneal transparency and sclera opacity, Collagen II is main component in cartilage and Collagen 1V (Anchoring collagen) is common in basement membranes (Forrester et al., 2015). Also, the different collagen types aggregate to form supramolecules stabilized by intermolecular covalent bonds (Mayne and Burgeson, 1987, Forrester et al., 2015).

In addition to collagen's molecular similarity is the alignment of collagen molecules in a parallel staggered pattern within a fibril based on *Hodge-Petruska model* (1964). This results in repeating gaps or overlaps called D-spacing (periodicity), see figure 5 below (Bron et al., 1997, Meek, 2008, Boote et al., 2019). The D-spacing is of biological importance as it has a normal value of 67nm according to X-ray scattering data (Erickson et al., 2013). D-spacing variations occur due to tissue-dependent differences, methodology artifacts or disease states such as estrogen deprivation (Fang et al., 2012, Graham et al., 2010). The effect of refractive conditions such as myopia on scleral collagen D-banding has not been reported yet.

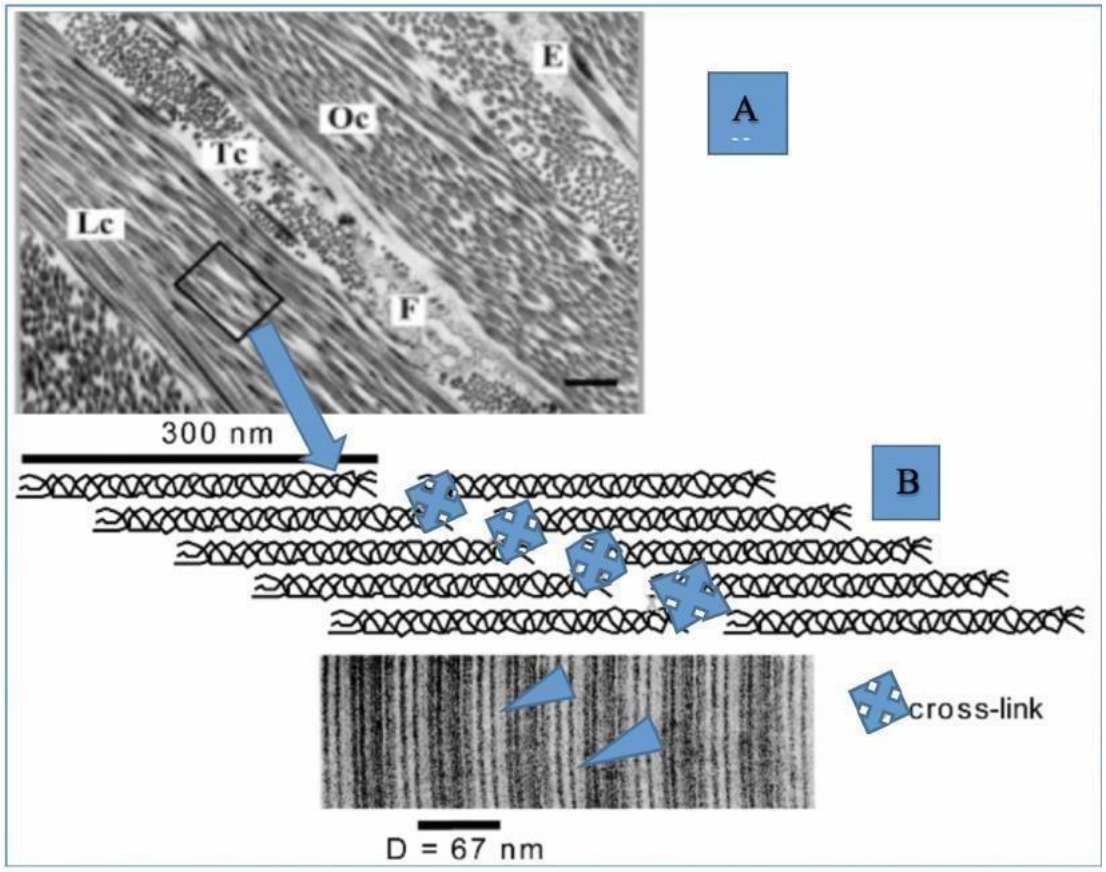


Figure 5. Outline of scleral morphometry and collagen structure. A = TEM image of the outer scleral stroma, revealing lamellar structure formed by collagen fibril bundles in longitudinal (Lc), transverse (Tc) and oblique (Oc) portion. A fibrocyte (F) and elastin fibre (E) are also shown. Bar: 1.5 µm. B = Illustrates N- and C- terminal organization resulting in spontaneous arrangement of collagen fibrils stabilized by covalent cross-links (x). TEM image of stroma from a different specimen at higher magnification, showing D-periodic banding (D=67nm) of individual fibrils in longitudinal section. PG are present as fine filaments (blue arrowheads) linked with the collagen fibrils. Bar: 250 nm, adapted from Meek, (2008).

Collagenous fibrils also determine scleral thickness. Scleral thickness varies from 1mm at the posterior pole (the most extensible region due to its slack broad-angle fibrillar weave) to 0.3mm at the equator and 0.8mm around limbus (Boote et al., 2019). Anterior fibril diameter is larger than posterior (De La Maza et al., 1994). The fibrils in the outer sclera are aligned in bundles with whorl and arc-like patterns while bundles in deeper areas have more rhombic arrangement (Forrester et al., 2015). Light and electron microscope-based studies showed that around week 6 of human embryonic development, the neural crest and mesoderm differentiates into scleral fibrils. By the 24th week, the

fibrils attain the mature size three times their diameter at the outset ultra-structurally (Sellheyer and Spitznas, 1988, Rada et al., 2006). Sclera grows from the corneoscleral limbus progressing posteriorly, hence variations in features with scleral region (Rada et al., 2006, Forrester et al., 2015, Meek, 2008). Major studies have not concentrated on the role of variations in human scleral regions and layers in refractive error development (Wolffsohn et al., 2019).

Table 1; Macroscopic, microscopic and nanoscopic features of the human cornea and sclera, Meek, (2008).

FEATURE	TISSUE	TISSUE
MACROSCOPIC	**CORNEA** (15% outer coat)	**SCLERA** (85% outer coat)
	Centre; 0.5mm thick, periphery; 0.7mm thick	Limbus (anterior); 0.53mm, equator; 0.39mm, peripapillary; 0.9mm. Less extensible in anterior and equatorial regions and more extensible in the posterior.
MICROSCOPIC	Comprise 5 layers; collagen fibrils I,III,IV,V especially in bowman's membrane, fibrils regularly packed in lamella, more in stroma. Lamella in outer and middle layers bifurcate and are interwoven. In inner layers, they are stacked. Stroma contains keratocytes responsible for slow turnover of collagen and other components. Half-life of collagen type I is unknown.	Comprise 3 layers; collagen I,III,V,VI,VII;99% Collagen is type I; proteoglycan; PG (decorin, biglycan, aggrecan(aggrecan is more in posterior sclera)) which regulates fibril assembly, interaction, diameter and hydration, glucosaminoglycan (GAG); hyaluronan), 2% elastin and fibroblasts. Fibrils are packed irregularly in bundles. Fibril orientation varies with region and is governed by intraocular tension and EOM pull. Fibrils in outer sclera are thicker than those in inner, this is called trans-scleral diameter gradient; its more marked in posterior sclera and increases with age. Collagen turnover is unknown.

NANOSCOPIC	Fibrils have same diameter; lie parallel to lamella; stabilized by covalent cross-links. Interfibrillar space (1.8nm lateral spacing) is filled with (PG). Centre-to-centre interfibrillar spacing is ~57nm. Increases towards limbus. Type I is predominant while type IV is slightly more in stroma. Water content is 76**%. D-periodicity is**	Fibrils have non-uniform diameter (btw 25&230nm) and thickness (btw 0.5-0.6μm). Water content of 68% Modulus of elasticity of anterior sclera is $2.9X10^6Nm^{-2}$and $1.8X10^6$ Nm^{-2} for stress levels from $2X10^5$ to $2.6X10^6$N. **D-periodicity is 67nm**
	65nm due to fibrillar tilting, Reduced Young's modulus is +/-0.207MPa	

Scleral fibril number decreases with age, but its concentration factor is unchanged in ageing monkeys (Wollensak and Spoerl, 2004, Girard et al., 2009). However, the experimental temperature is not the physiological body temperature (22^oc instead of 37^oc) (Girard et al., 2009). Myopia has also been reported to alter fibril number in animal sclera (Mcbrien et al., 2001). Chick's sclera has an outer fibrous layer and an inner cartilaginous scleral layer common in most vertebrates (Wallman et al., 1995). The growth of the cartilaginous sclera is regulated at certain life stages. However, the vertebrate fibrous sclera has similar collagen type and dynamic ECM constituents remodeled by retinal signals and visual environment (Rada et al., 2006).

Studies propose scleral ECM and fibril changes occurring in human myopia and high myopia result in biomechanically weaker and thinner sclera mainly peripapillary. This transformation is different from mere enhanced scleral tissue growth. It is called scleral remodeling and will be discussed later in this review (Mcbrien et al., 2001, Boote et al., 2019). The lamina cribrosa biomechanics is affected due to fluctuating IOP force. Glaucoma and myopic retinopathy risk increases (Harper and Summers, 2015). However, the eyes used in most of these studies had glaucoma not clearly myopia. (Jonas et al., 2011, Metlapally and Wildsoet, 2015, Norman et al., 2011). Unusual star-shaped fibrillar structures were discovered in human pathological myopia (Curtin et al., 1979) proposed to be a result of abnormal scleral catabolism (Rada et al., 2006), and abnormal fibrillar alignment in high myopia (Markov et al., 2018). This recent study by Markov et al. (2018) using WAXS (Wide-angle X-ray Scattering) to show bulk biomechanical and structural alterations in the high myopic peripapillary sclera used three specimens, neither analyzed the entire scleral tissue depth (Markov et al., 2018). In my opinion, the specific posterior scleral region should be considered, and the entire scleral depth studied (Yan et al., 2011, Girkin et al., 2017).

1.1.2. **Scleral biomechanics**

The mammalian sclera may have higher modulus of elasticity (3 - 3.5 times more) than the cornea based on findings using porcine eyes (Asejczyk-Widlicka and Pierscionek, 2008). Modulus of elasticity or Young's modulus is the measure of the ability of a material to withstand changes in length when subjected to compression, stress or strain (Girard et al., 2009). Whereas the reduced Young's modulus is the elastic deformation that takes place on the indentation probe and sample (Ma et al., 2004). The human sclera is biomechanically stiffer than rabbit or porcine sclera (Wollensak and Spoerl, 2004). The peripapillary (near optic nerve head) and posterior sclera of young monkeys is thicker and less stiff compared that of old (Girard et al., 2009, Girkin et al., 2017).

The biomechanics or stress-strain behavior of the fiber-reinforced sclera has been studied by various researchers since late 1960's using experimental and computer models and has been characterized by ex vivo and in vivo procedures (Woo et al., 1972, Curtin, 1969, Jia et al., 2016, Boote et al., 2019, Girard et al., 2009, Girkin et al., 2017). The three key biomechanical properties are stress-strain response heterogeneity, anisotropy (scleral stiffness ratio at different fibril orientation) and nonlinearity (ability of the fibrils to uncrimp with stretching). These properties are interrelated, vary with anatomical region and help protect the sclera from large deformations when exposed to mechanical forces such as IOP. However, the posterior sclera is the most deformed region compared to the rest (Woo et al., 1972, Girard et al., 2009, Girkin et al., 2017). The scleral fibrils also display hysteresis and tensile strength which vary with scleral region or depth (Forrester et al., 2015, Boote et al., 2019). Scleral biomechanics is affected by ageing and diseases such as glaucoma and myopia (Girard et al., 2009, Girkin et al., 2017, Dikici et al., 2016) and improved by cross-linking (Wollensak and Spoerl, 2004, Levy et al., 2018). Although they have been studied in animals such as sheep using polarized microscopy (Jan et al., 2017) or pigs using optical elastography probe called Brillouin microscope (Shao et al., 2016), biomechanics in animal and human sclera can be better studied with atomic force microscope (AFM) for a classic link between scleral micro-structure and biomechanics (Boote et al., 2019).

Ex vivo characterization has been done successfully with uniaxial tensile testing (use of scleral strips). It gives inaccurate results due to its loading mode (Wollensak and Spoerl, 2004, Jia et al., 2016). Biaxial testing (use of square tissue patches) is uncommon but gives more information in terms of human scleral physiology *in vivo* (Eilaghi et al., 2010, Perez et al., 2014, Boote et al., 2019). Perez et al. performed biaxial testing on porcine scleras, but the

microstructural analysis was lacking (Perez et al., 2014). Other *ex vivo* methods include inflation testing (which the IOP-associated deformation/displacement patterns of the sclera is delineated/tracked) with electronic speckle pattern interferometry (ESPI) (in monkey sclera) (Girard et al., 2009), ultrasound speckle tracking (regional displacements in human scleras) (Ma et al., 2019) and graphite powder markers tracked with charge-coupled device (CCD) cameras and digital image correlation (DIC) (Coudrillier et al., 2012, Boote et al., 2019). Also, compression and indentation procedures have been performed to determine scleral pressuredeformation response. In 2014, Leung et al., using a camera-mounted stereomicroscope and indentation technique showed that when a porcine sclera is stressed, the tangent modulus increases (Leung et al., 2014). These studies have analysed 3D scleral deformations even with high resolution but have measured mostly surface displacements and the experimental conditions have not well mimicked *in vivo* environment. Also, it may be difficult to compare techniques due to the use of different scales of stiffness measurement (Boote et al., 2019).

In vivo characterization has been explored by Girard in 2013 in which the IOP-induced deformations on peripapillary scleral were delineated using an algorithm based on digital optic nerve head volume correlation with the optical coherence tomography (OCT)(Girard et al., 2013). Others have recently used shear wave elastography (SWE) to reveal increase in scleral stiffness due to glaucoma in humans *in vivo* (Dikici et al., 2016). However more research is ongoing such as the use of OCT-based technologies to characterize scleral biomechanics in health and disease conditions (Boote et al., 2019).

1.1.3. Scleral biomechanics (in myopia)

Ultramicroscopic investigation of human scleras in the late 1900s and recent anatomical studies reveal that structural, biomechanical, and molecular changes in myopic and pathological myopic scleras are similar (Curtin, 1969, Curtin et al., 1979, Mcbrien, 2013). Although most studies focused on high axial myopia. The time-determined viscoelastic features vary in myopic scleras making them susceptible to IOP stress and resulting in elongation with time (Mcbrien et al., 2001, Elsheikh and Phillips, 2013).

Scleral stress-strain response is influenced significantly by glaucoma and myopia (Coudrillier et al., 2012, Mcbrien, 2013). Studies have discovered individual posterior scleral fibrils and bundles decline in size resulting in a thin and weak sclera, but the main cause is not conclusive (Harper and Summers, 2015, Jonas and Xu, 2014). The myopic posterior sclera was however found to be 31% thinner and biomechanically weaker than that of healthy eyes. Over 200% increase in sclera extension over time when a constant load is applied (called creep rate)

to the weak myopic sclera, see figure 6 (Curtin, 1969, Metlapally and Wildsoet, 2015, Curtin et al., 1979).

Siegwart and Norton concluded in a study using tree shrew sclera that the timedependent creep rate, not modulus of elasticity is significantly upregulated by FDM and LIM. Also, although, FDM and LIM function through different mechanisms, they affect the sclera similarly. Increased creep rate and biomechanical remodeling of the scleral ECM causes axial elongation by exposing the 'failed' sclera to the effect of IOP-associated strain and stress (Siegwart Jr and Norton, 1999, Phillips et al., 2000, Jia et al., 2016, Tao et al., 2013). Moreover, light and electron microscopy of tree shrew scleras after monocular deprivation of pattern vision at short and long-term periods revealed remarkable collagen fibril diameter changes comparable with that in human high myopia structurally and ultra-structurally (Mcbrien et al., 2001).

In 2015, a similar study revealed temporary biomechanical alterations of tree shrew scleras during LIM and recovery. Scleral collagen fibril crimp (strain level at which the fibrils straighten) angle time-dependent modification with axial elongation rate suggested that scleral remodelling mechanism was the cause rather than scleral growth (Grytz and Siegwart, 2015). More recent studies with porcine and shrew scleras discovered that *ex vivo* scleral crosslinking (SXL) with genipin (22.1mM and 0.25mM respectively) hampers eye elongation linked remodeling by controlling IOP-associated cyclic inelastic scleral micro-deformations (Wang and Corpuz, 2015, Levy et al., 2018) . They concluded that this is a potential myopia control, yet the process is not fully understood. My view is that although genipin (chemical SXL) has low cytotoxicity and gives safer SXL than UV-based SXL, more *ex vivo* and *in vivo* studies ought to be tried with many specimens, from animals and humans to understand the remodelling process and myopia control better (Boote et al., 2019).

Scleral remodelling involves perpetual turnover of ECM components and significant time-dependent alteration of the scleral biomechanics at both posterior and equatorial regions, and post-natal change in vitreous depth during visually guided active emmetropization or refractive error development (Siegwart Jr and Norton, 1998, Phillips et al., 2000, Rada et al., 2006). It also involves scleral collagen metabolism and mechanical properties (Ouyang et al., 2019). Using swept-source OCT, to carry-out *in vivo* evaluation of the sclera of human eyes with high myopia, it was observed that the scleral tissue was deformed (Ohno-Matsui et al., 2012). In summary, some methodologies used in the studies reviewed above are invasive and lack accuracy to show the difference *in vivo* measurements between an emmetropic and a myopic sclera. More studies are currently been carried out (Wolffsohn et al., 2019).

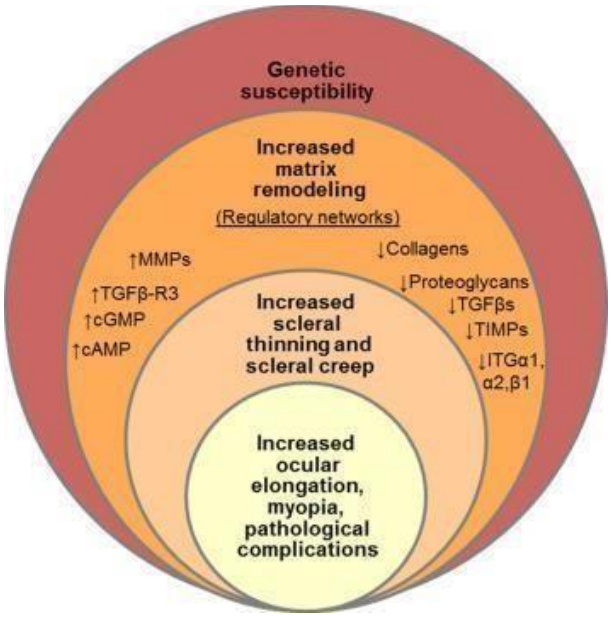


Figure 6 Schematic model of scleral remodelling mechanisms and implications in myopia. Genetic susceptibility (red circle) combines with increased matrix remodelling (orange) and increased scleral thinning and creep (peach) to cause myopoia development and its complications (yellow). Adapted from Metlapally and Wildsoet, (2015).

1.1.4. Scleral biochemical and molecular components in myopia

Ageing, stress and eye disease affect the production, regulation and functioning of ECM components including its lipid and protein content and concentration (Broekhuyse and Kuhlmann, 1972, Frost and Norton, 2012). Myopia-related stress on the sclera affects the production, regulation and functioning of ECM biochemical components which are important for scleral rigidity, strength, and elasticity (Mcbrien et al., 2009, Wollensak and Spoerl, 2004). Most research works reviewed here focus on tree shrew sclera as they are like human sclera (single fibrous layer) (Metlapally and Wildsoet, 2015). The major ECM constituents include GAG, PG, metallinoproteinases or matrixins (MMP) and their tissue inhibitors (TIMP), collagen proportion and fibrils assembly (Rada and Brenza, 1995).

Collagen make up 80% of scleral dry weight. Age, glaucoma and myopia affect scleral col percentage (Mayne and Burgeson, 1987, Forrester et al., 2015, Bailey, 1987).

Using various biological techniques such as RT-PCR and immunohistochemical analyses, researchers have reported the expression of collagen types XIII and XII in fetal and adult scleral tissues at mRNA and protein levels (Sandberg-Lall et al., 2000, Wessel et al., 1997). The expression of other collagen subtypes including collagen types III, IV, V, VI, VII, VIII except type II, and certain ECM genes have been also confirmed to be present

in the sclera (Young et al., 2003, Rada et al., 2006). Certain collagen types, GAG synthesis and TGF- decrease significantly in myopia governed by TGF- , see figure 7 and 8 (Mcbrien, 2013, Rada et al., 2006, Akhtar et al., 2008).

The spacing of the fibrils within the lamellae is maintained by long unbranched mucopolysaccharide molecules called GAG. GAGs make up approximately 0.2% of the scleral dry weight (a property of the ECM) and may be involved in regulating the scleral creep rate in tree shrews' sclera and collagen fibril strength and assembly (Scott, 1990, Moring et al., 2007, Rada et al., 2006, Trier et al., 1990). Moring et al. (2007) used few tree shrews with age-matched controls, but AL measurement was carried out with inaccurate ultrasound measurements. LIM was only considered, and few GAGs were concentrated on in this study. In 2016, Murienne et al. used human posterior scleral shells from 11 nonmyopic donors to show the role of GAG in sclera biomechanics but donor ethnicity was not mentioned (Murienne et al., 2016). Repeating the experiments thrice and averaging the results and studying all GAGs (including most abundant – dermatan sulfate (DS) and chondroitin sulfate (ChS)) would be beneficial in my view (Rada et al., 2000).

Using cellulose acetate electrophoresis and optical scanning, 5 different GAGs were identified in human sclera and their proportion at various scleral locations was given. They include ChS, hyaluronic acid (HA), DS, heparan sulfate (HS), keratan sulfate (KS) (Moring et al., 2007). HA staining was highest at the equator. ChS and uronic acid in nmol/ng were found to be highly concentrated in the posterior scleral regions while DS was highest around the papilla region of the eyeball compared to sclera from the equator or limbus (Rada et al., 1997, Trier et al., 1990). The relative proportions of sulphated (HA) and unsulfated GAG's (such as ChS, DS) from results of capillary electrophoresis of tree shrew's sclera has been shown to reduce remarkably under myopiagenic visual conditions and at several definite time conditions (Moring et al., 2007, Trier et al., 1990). Although a recent study revealed the role of sulphated GAGs (s-GAG) on human scleral biomechanics and thickness, a single eye was studied for s-GAG content (Murienne et al., 2016).

Comparing different scleral regions and more specimens would give better information.

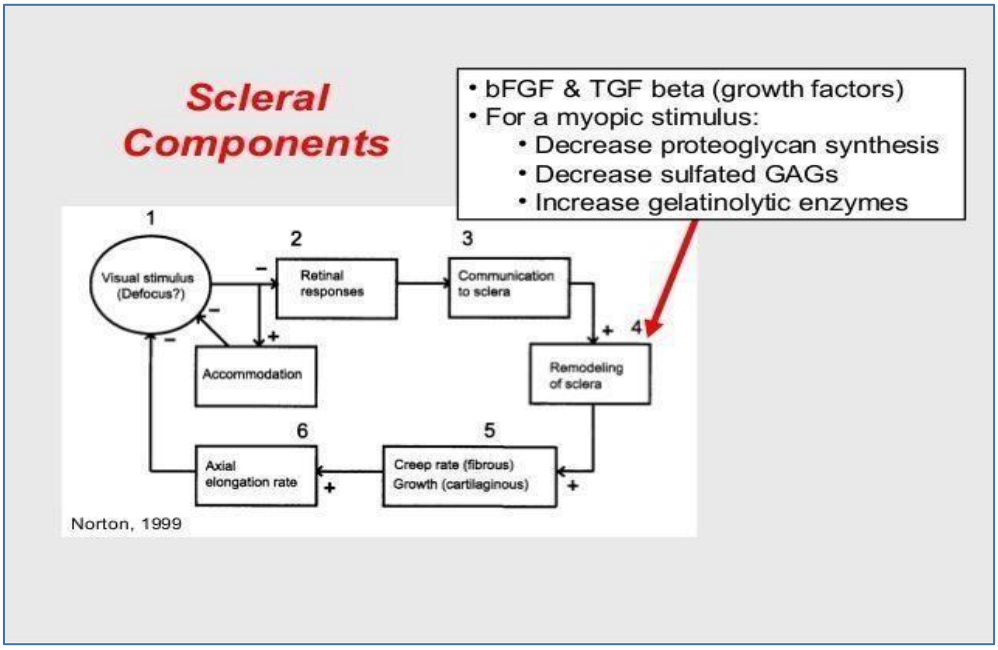


Figure 7 showing sclera components associated with eye elongation and emmetropization. Visual stimulus (1) causes the retina (2) to send signals to the sclera (3). In the sclera, the growth factors and other regulatory networks combine to increase (+) axial length elongation rate (6), adapted from Norton, (1999)

Furthermore, using immunochemistry and laser scanning confocal microscopy, melatonin and melatonin receptors were localized in cornea and sclera of Xenopus laevis and may be involved in mechanism for refractive anomalies development and ECM turnover (Wiechmann and Rada, 2003). The relationship between melatonin and eye growth has been shown in several animal and human studies (Rada and Wiechmann, 2006, Kearney et al., 2017, Iuvone et al., 1991). Using (3H) thymidine and inducing myopia in tree shrews with translucent occluders and negative lenses, Gentle and McBrien (1999) reported that alteration of scleral fibroblast proliferation affected vitreous chamber depth during myopia development (Gentle and Mcbrien, 1999).

Four years later, Gentle and colleagues (2003) revealed a reduction in collagen type1 expression in tree shrew scleras with FDM using RT-PCR. Collagen types III and V were not affected (Gentle et al., 2003). That same year, Luebke and Rada reported that insulinlike growth factor-1 (IGF-1) enhanced the rate of PG synthesis remarkably in human scleral fibroblasts isolated by explant culture procedures and porcine scleral equatorial region processed by organ culturing. The human scleras with significant effects were from young adults of varying ages (between 24 and 33 years) (Luebke and Rada, 2003). Recently, a link between IGF-1 gene polymorphisms and myopia using polymerase chain reaction–

restriction fragment length polymorphism (PCR-RFLP) analyses was reported (Zidan et al., 2016).

Mutations in ECM proteins cause disease (Zanotti et al., 2005). Using TEM it was reported that the elastin fibres and their adjacent ECM in murine sclera and choroid is similar in appearance to that in the human eye. Also, the elastin fibres have low turnover rate throughout life and were more prevalent around the papilla of the murine sclera (Gelman et al., 2010).

Researchers have used immunochemical techniques to identify the presence of glycoprotein (PG) fibrillin in the microfibrils in human choroid and sclera stroma involved in controlling the strength and elasticity and disease states of these ocular connective tissues (Wheatley et al., 1995, Ashworth et al., 2000). The 6 cell-adhesion glycoproteins found by immunostaining in the lamina cribrosa, pial septa and sclera of sections from elderly donors reveal similarities and slight differences in these structures. They include laminin, fibronectin, vitronectin, tenascin (not identified in the sclera), thrombospondin (not identified in the pial septa, enactin/nidgen (Fukuchi et al., 2001, De La Maza et al., 1994). Non-collagenous cell-adhesion laminin, fibronectin and vitronectin proteins which bind ECM to myofibroblast have been revealed in foetal life. Their concentrations decline with age according to the findings (Kim et al., 1999, Fukuchi et al., 2001, De La Maza et al., 1994). However, laminin had a linear-like distribution mostly in scleral blood vessel basement membranes and sclemm's canal while fibronectin and vitronectin were highly concentrated in the sclera, lamina cribrosa and pial septa (Chapman et al., 1998, Fukuchi et al., 2001, De La Maza et al., 1994). More studies are needed in other age ranges to show the turnover rate and link to ECM remodelling and refractive error.

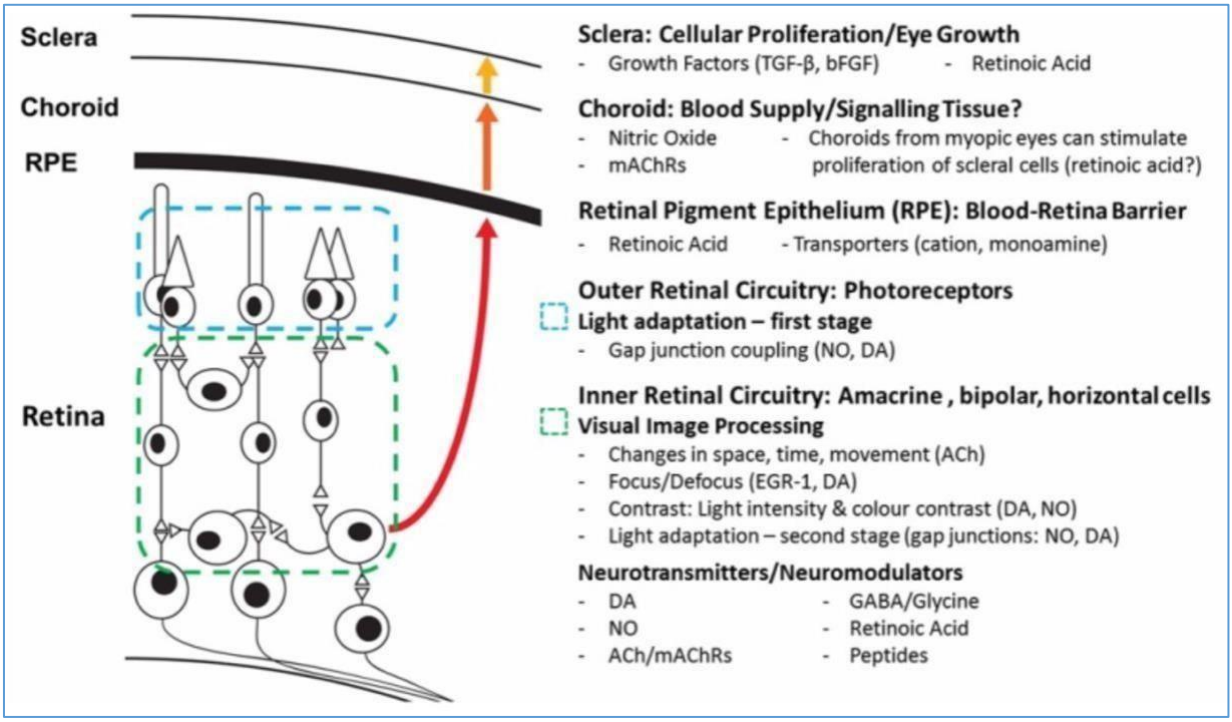


Figure 8 Illustrating the signalling cascade from the retina and choroid to the sclera during myopia and high myopia related to eye elongation. Visual stimulus such as defocus stimulate retinal cells such as amacrine cells to release dopamine (DA) which passes through the choroid (arrows) to the sclera, where other factors are released to increase eye growth, adapted from Carr and Stell, (2017)

Molecular studies using murine eyes and examining the sclera by TEM showed the importance of leucine-rich lumican and fibromodulin in maintaining scleral biomechanics by ordering collagen microfibrils (Young et al., 2003). Also, genes of some ECM proteins such as fubulion-1, transgelin and synedecan, thrombospondins, dystroglycans et cetera, were reported to be expressed in human scleras of nonmyopic donor eyes using cDNA library clones and microarray analyses. Their role in myopia is yet to be ascertained (Young et al., 2003, Young et al., 2002). In 2013, using quantitative PCR and other biotechniques and at certain time points, Guo et al, reported the existence of a similar gene expression signatures or patterns in juvenile tree shrew's scleras exposed to various visual conditions that cause axial elongation or myopia (Guo et al., 2013).

In summary, more quantitative study of human scleral microstructure, to determine the regional specializations and produce high resolution 3-dimensional entire-depth physiologically accurate images and data would be beneficial to understanding myopia mechanisms and discovering therapeutic strategies (Boote et al., 2019).

1.2. Myopia

1.2.1. Myopia definition and classification

Myopia (near-sightedness) is a refractive error in which image of a distance object is formed in front of the retina. Its most common form is characterized by excessively long eyeball (see figure 9). In myopia, the eye's axial length (AL) is incompatible with the focal length of its optical components resulting in blurred vision (Zadnik, 1997, Flitcroft et al., 2019b). The onset and progression of myopia has been linked with abnormal alterations in the human retinal, choroidal and scleral ocular tissues (Braun et al., 1996, Norton, 1999, Wu et al., 2016, Metlapally and Wildsoet, 2015). These changes eventually cause high myopia and its complications (SER of -5.00D or less) (Wu et al., 2016, Holden et al., 2016).

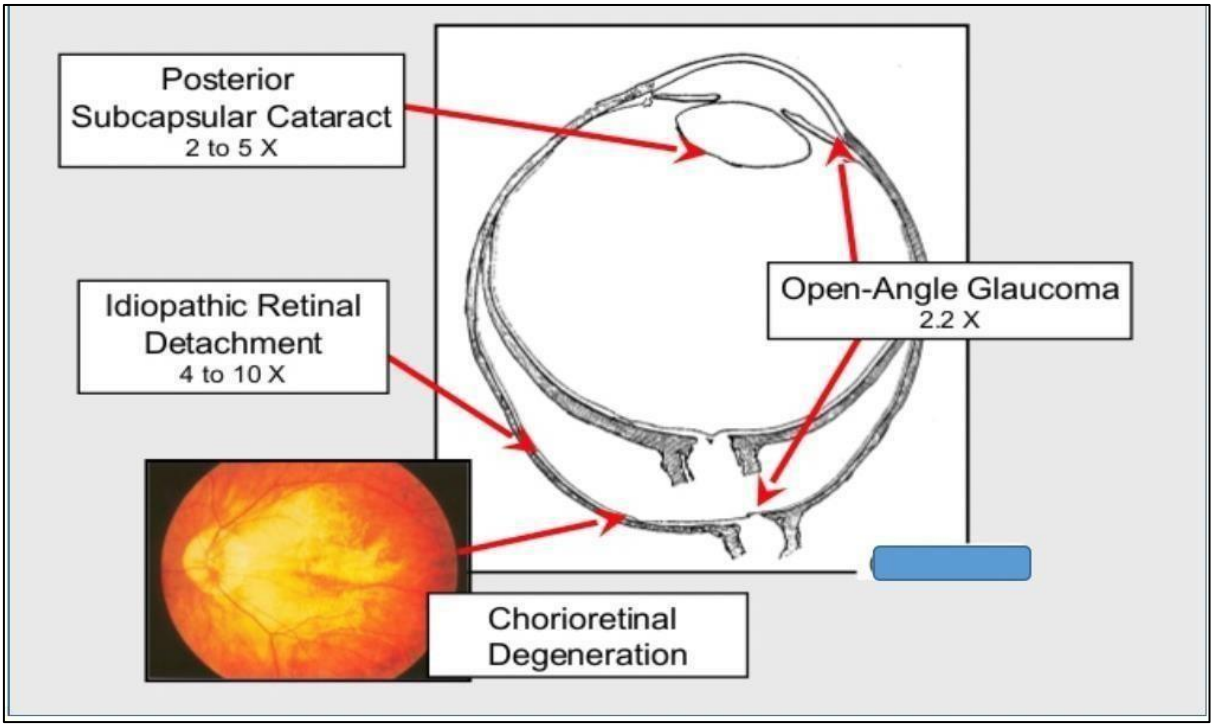


Figure 9 showing the complications of myopia and high myopia adapted from (Curtin, 1985). X = odds ratio or OR (for instance OR of posterior subcapsular cataract (PSC) in low myopia; -1 to -3.5D (OR 2.1; 95%CI 1.4, 3.5), moderate myopia; -3.5D to -6D (OR 3.1; 95% CI 1.6,5.7) and high myopia; <6D (OR 5.5; 95% CI 2.8, 10.9) Saw et al., 2005, Flitcroft, (2012).

Myopia is a serious public health challenge and a major issue in global epidemiological research because its prevalence rate is increasing rapidly and steadily (Holden et al., 2016).

It is believed to result from genetic susceptibility and relatively stronger environmental influence, see figure 10 below (Holden et al., 2016, Wolffsohn et al., 2019, Zhou et al., 2017).

Several possible mechanisms have been postulated and potential interventions are currently being explored using animal models and human subjects (Liang et al., 1995, Liang et al., 1996, Mcbrien et al., 1999, Smith et al., 2009a, Schaeffel and Feldkaemper, 2015). Environmental, pharmacological, surgical, and optical treatment options are in use but their efficacy in myopia control is short-term, albeit some evidence refute this fact. Focusing on scleral role may be worthwhile for a long-lasting effect (Wildsoet et al., 2019, McBrien and Gentle, 2003). This review focuses on sclera changes associated with myopia, the mechanisms that may account for myopia development and important for its control.

1.2.2. Myopia prevalence

Holden et al. (2016) reports that 1.406 billion people (22.9%) were myopic in year 2000 worldwide, and 1.89 billion (27%) in 2010, and myopia will affect 2.56 billion individuals (33%) in 2020. In the next three decades, the affected people worldwide will double if current trends continue, making myopia the most prevalent refractive error and a leading cause of blindness (Holden et al., 2016, Flitcroft et al., 2019b, Tedja et al., 2019). Currently, it has been estimated that the worldwide annual potential lost productivity due to visual impairment from uncorrected myopia is almost US$250 billion (Naidoo et al., 2019, Smith et al., 2009b). High or pathological myopia stated to affect 2.7% of the global population in 2000 was projected to affect 6.1% in 2030 (Holden et al., 2016). It affects mostly working-age patients, is a major cause of visual impairment and irreversible blindness from presenile cataract, glaucoma, retinal detachment, macular atrophy, and other complications which significantly affect the socioeconomic wellbeing of its sufferers by reducing their quality of life substantially, see figure 9 (Wu et al., 2016,
Holden et al., 2016). Hence, it is important to understand how myopia can be controlled.

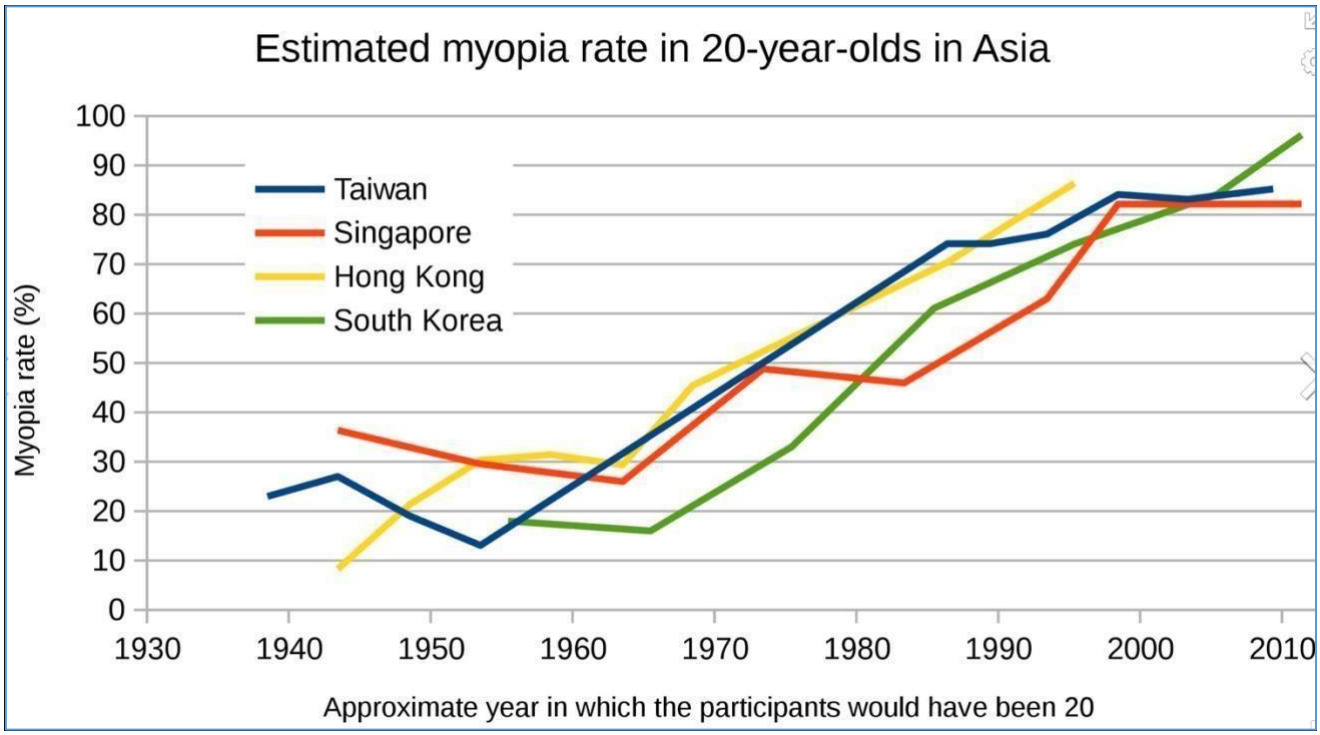


Figure 10 showing rapidly growing myopia prevalence rate since the 1950's, especially in some Southeast Asian countries. Adapted from Dolgin, (2015).

Moreover, myopia prevalence has risen dramatically over the past 50 years especially in certain rich, technologically advanced regions of East Asia including China, the Republic of Korea, Singapore and other areas with significant economic transition. The prevalence rate is lower in Australia, Europe, North and South America and Africa (Holden et al., 2016). However, myopia may account for preventable blindness in many developing countries including urban regions of Africa and India (Rudnicka et al., 2016, Holden et al., 2016, Dandona et al., 2001, Opubiri et al., 2013, Ogbonnaya et al., 2013). Explanation for this so far has been the effect of certain environmental factors (Ip et al., 2008). Also, it was stated that higher rate of urbanization and education are responsible for the rural-urban differences, but the explanation is not clear or conclusive (He et al., 2009, Holden et al., 2016).

Environmental factors are a major focus of epidemiological studies as they are controllable and the dramatic rise in myopia prevalence cannot be explained by genetics alone (Morgan et al., 2018, Tedja et al., 2019). The environmental influences will be discussed shortly. In the Jewish population, researchers have stated that myopia prevalence and progression can be accounted for by genes (Bez et al., 2019, Simpson et al., 2011) and

sustained close work from orthodox education (Zylbermann et al., 1993). Although the findings have still been verified (Tedja et al., 2019, Wojciechowski, 2011, Flitcroft et al., 2019b), over 150 loci of myopia candidate genes have been reported (Hysi et al., 2020). Studies have also shown that environmental factors combine with genes to increase susceptibility to myopia (Tedja et al., 2019). However, genetic factors account for only a small percentage of myopia cases (Ip et al., 2008, French et al., 2013b, French et al., 2013a, Smith et al., 2012, Morgan and Rose, 2005, Morgan and Rose, 2019). This has been further highlighted by animal experiments. Animal model studies have also helped reveal the more developed cues that guide emmetropization, the vision-dependent nature of eye growth and the biochemical signal cascade occurring in the retina, choroid (Schaeffel and Feldkaemper, 2015).

1.2.3. Interplay of Myopia Risk Factors and Myopia Mechanisms (Animal Models)

The first animal model for 'environmentally' induced myopia was described by Wiesel and Raviola in 1977, using monkeys. They reported that visual deprivation (or defocus) is the chief cause of AL elongation and myopia, see figure 3 (Wiesel and Raviola, 1977, Norton, 1999).

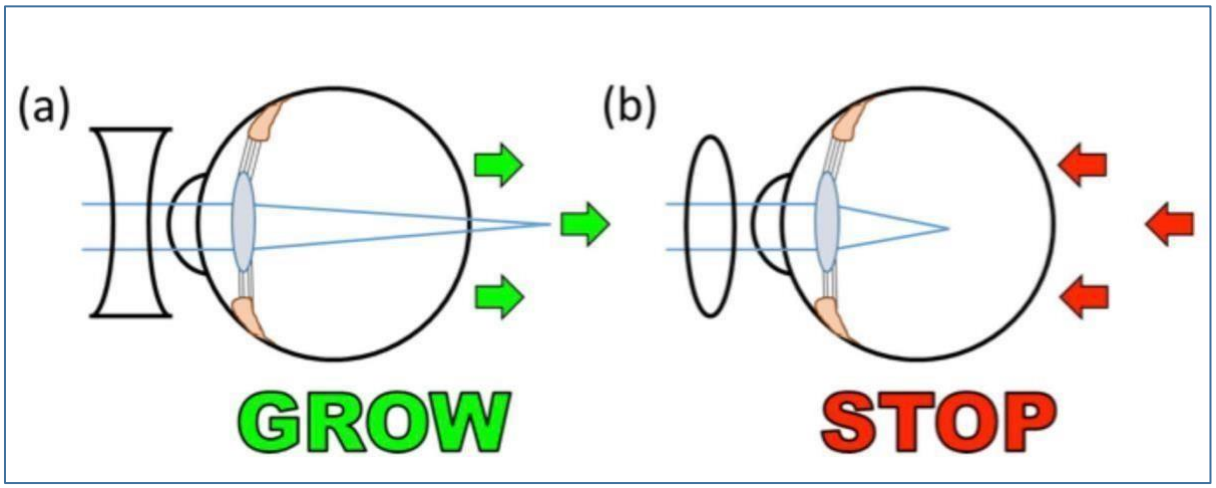


Figure 11 explaining the process of AL elongation/reduction in response to peripheral hyperopic/myopic defocus inductions **using minus lens (a) and plus lens (b). Adapted from Carr and Stell, (2017).**

The AL and scleral ultrastructural features of a mutant lumican (L199P) transgenic murine model were examined using microscope and TEM. AL increased in transgenic mice

compared to wild-type and disrupted scleral fibril lamellar orientation was discovered. Lumican regulates collagen fibril diameter, formation, and ordering. Mutation in lumican gene resulted in ultrastructural alterations that affects scleral elasticity and eye growth (Song et al., 2016).

Although the biological link between abnormal eye growth in animal myopia and less time spent outdoors or less light intensity (Zheng et al., 2018) or the spectral components of light (Mehdizadeh and Nowroozzadeh, 2009) is not clear, it has been postulated that illumination-dependent retinal neurotransmitter (NT) inhibits eye elongation using chick's eye (Mccarthy et al., 2007). Chick's eyes give a relatively more rapid and robust response to alterations in visual environment (Norton and Mcbrien, 1992). Dopamine is a NT found in the inner plexiform layer of the human retina produced by the amacrine cells after light stimulation. Dopamine acts via two major groups D1-like (D1 & D5) and D2-like (D2, D3 and D4) receptors. These receptors are found in various ocular tissues (Mccarthy et al., 2007, Jiang et al., 2014, Forrester et al., 2015). Light-related factors may have a stronger link to myopia than air pollution or diet, albeit greater insight on the link between dopamine and myopia is still explored (Wolffsohn et al., 2019).

Furthermore, Cohen and colleagues (2006) reported that bright light (10,000 lux) hampered myopia development in chicks while dim ambient light (50 lux) promoted myopia progression (Cohen et al., 2006). Six years later, Cohen et al. (2012) using the same chick model proposed an underlying mechanism by showing a link between exposure to light-dark cycles and continuous light, and vitreal dihydroxyphenylacetic acid (DOPAC) and dopamine concentrations. Low vitreal DOPAC concentrations, flat cornea, eye elongation and myopia development were associated with light-dark cycles (Cohen et al., 2012). In the same year, Siegwart et al, reported that juvenile tree shrews with FDM and LIM had statistically significant reduction in FDM by 44% and LIM by 39% after an approximately 8 hrs/day exposure to ~16,000 lux light (Siegwart Jr et al., 2012). Moreover, recent studies by Zheng et al. (2018) have revealed an additive relationship between optical defocus and bright illumination using chicks in inhibiting myopia development and highlighted the dose dependent nature of ambient light effects (Zheng et al., 2018).

In 1996, Kröger and Wagner (1996) discovered that the eye size of blue acara was dependent upon the wavelength of light used to rear them during development (Kröger and Wagner, 1996). In 2013, Park and colleagues stated that low levels of dopamine in mice caused increase susceptibility to FDM (Park et al., 2013). The following year, Jiang et al, (2014) using 2 weeks old albino guinea pigs, reported that Apomorphine, a dopamine antagonist, inhibited myopia development at a higher dose (250ng per injection) via lower affinity D1-like receptors

and promoted myopia progression at a lower dose (25ng per injection) by stimulating the higher affinity D2-like receptors (Jiang et al., 2014). Later in 2015, Smith et al, proposed that exposing infant rhesus monkeys to long wavelength lighting (red filters) under certain conditions, may promote a hyperopic shift (Smith et al., 2015).

Ultrastructural alterations, after myopia was induced in chicks following two weeks of translucent occlusion, were observed using electron-micrographs. 60% thinning of the choroid, 20% thinning of the retina, lengthening of the photoreceptor mainly the rods outer segment closely adjacent to the retinal pigment epithelium (RPE) basement membrane were reported (Liang et al., 1995, Liang et al., 1996). Rada and colleagues in 2002 then proposed that axial elongation due to form-deprivation is the outcome of PG production and accumulation in the chick's sclera (Rada et al., 2002). In 2003, Wiechmann and Rada suggested that refractive errors mainly myopia are linked to melatonin and melatonin receptors localized in cornea and sclera of Xenopus laevis. They further proposed that these nonneural ocular tissues exhibit circadian rhythms in cellular proliferation, ECM turnover and wound healing (Wiechmann and Rada, 2003). In 2007, after inducing myopia in 2-day old chicks with translucent plastic googles for 10 days, suprachoroidal fluid showed an upregulation of the GAG synthesis. During recovery to emmetropia, GAG synthesis declines as the choroidal permeability increases (Rada and Palmer, 2007). Although the mechanism is still vague, alterations in retinal and choroidal retinoic acid (RA) production may cause a drop-in scleral GAG synthesis rate that accompanies increase in AL extension rate using the eyes of common juvenile marmosets and *in vivo* and *in vitro* analyses (Troilo et al., 2006).

Earlier, Mertz and Wallman (2000) proposed that retinal signals including dopamine, glucagon, acetylcholine, et cetera, can stimulate the RPE to releases a biologically active modulator that regulates RA secretion from the adjacent choroid which in turn guides scleral growth/reduced PG synthesis and axial myopia (Mertz and Wallman, 2000, Metlapally and Wildsoet, 2015). Other factors identified in studies responsible for a weak and thin myopic sclera include disorganized collagen fibrils, decrease in fibril diameter, altered expression of the sclera genes including genes for collagen type 1 and Matrix metalloproteinase (MMPs) (Metlapally and Wildsoet, 2015, Tao et al., 2013).

Although animal studies have helped clarify reports from epidemiological studies, propose myopia mechanisms and reveal potential treatment strategies (Liang et al., 1995, Liang et al., 1996, Mcbrien et al., 1999, Smith et al., 2009a, Schaeffel and Feldkaemper, 2015), the speed of response to visual deprivation and underlying mechanisms vary amongst the animal

models (Norton and Mcbrien, 1992). Extrapolation of animal studies to clinical application for humans is beset with the issue of difference in magnitude of visual deprivation, difference in the underlying mechanisms and different sensitivity periods for inducing myopia in animals and children (Zadnik and Mijtti, 1995).

1.2.4. Interplay of Myopia Risk Factors (Human Subjects) The onset, progression and prevalence of human myopia is the result of an interplay of certain risk factors. Some play more significant roles than others in myopia development (Goss and Jackson, 1996).

Age, though not a direct risk factor, is a major determinant factor at least within the first three decades of life. Younger age of onset increases the risk of high myopia (Akova-Budak et al., 2015, Rudnicka et al., 2016, Grosvenor and Goss, 1999). However, early onset myopia is less common but more familial while school-age myopia is most prevalent especially in developed Asian populations and is likely to be 'environmentally' driven (dopamine related abnormal AL elongation) (Spillmann, 2019, Hopper, 2019, Morgan and Rose, 2005).

Myopia prevalence in UK born white children (10-11 years age range) was 3.4% based on reports from the Child Heart and Health Study in England/CHASE (Rudnicka et al., 2010) 9.9% in 6-7-year-olds and 29% in 12-13-year-olds in the UK (Shah, 2007). Other cross-sectional studies such as the Northern Ireland Childhood Errors of Refraction /NICER study in 2010 (prevalence of 2.8% for 6-7year old's and 17.7% for 12-13 year olds) and the Aston Eye Study/AES in 2011 (myopia prevalence of 9.4% in 6-7 year old's, and 29.4% in 12-13 year old's) have established that myopia prevalence rate is higher in the early adolescence age though the values vary with geographical location (Logan et al., 2004, Logan et al., 2011, O'donoghue et al., 2010). Moreover, a recent survey revealed that 74% of UK children spent less than an hour daily outside (Carrington, 2016). This shows the role of environmental factors (Rudnicka et al., 2016).

Myopia prevalence in adults (44-45 years) is 49% (Rahi et al., 2011). The Beaver Dam Eye Study of 1994 involving 4926 Americans revealed that the total frequency of myopia in adult males and females (43-54years of age) was 42.9% compared to 14.4% in the over-75-year-olds.

Some studies have stated that AL elongation in myopia may be linked with higher birth weights. Findings are inconsistent (Grosvenor and Goss, 1999, Akova-Budak et al., 2015, Chua et al., 2015). Stone and colleagues in 2004 discovered a daily fluctuation in the eye's AL of 17

human participants using partial coherence interferometry to measure the distance from the cornea to the retinal pigment epithelium (RPE). Although they conjectured that the highest AL is present at midday, they measured from cornea to choroid, leaving the sclera (Stone et al., 2004).

Also, heredity plays a key role in early myopia development, with children of two myopic parents being 6.4 times more likely to have juvenile myopia (Pacella et al., 1999). Although family history (sibling and parental myopia) has been linked with higher juvenile myopia prevalence (Chua et al., 2015, Shah, 2007), genetic and environmental interactions cannot be overruled (Morgan and Rose, 2005, Hopper, 2019). A recent study has also stated that combining parental myopia history and genetic risk score helped in predicting children at risk of myopia (Ghorbani Mojarrad et al., 2018).

There are marked variations in prevalence based on ethnicity and urbanization with higher rate in East Asian population (Zhao et al., 2000, Rudnicka et al., 2010, Rudnicka et al., 2016, Grosvenor and Goss, 1999) and education (Morgan et al., 2018, Spillmann, 2019). A population-based study (called Multi-Ethnic Pediatric Eye Disease Study/ MEPEDS) carried out in California on pre-schoolers (6-72 months old) showed a higher prevalence of myopia in African Americans (6.6%) compared to age-matched Hispanics (3.7%). Also, not statistically significant/ SS gender difference was observed (Group, 2010). However, in the Jewish population, researchers have stated that myopia prevalence and progression can be accounted for by genes (Bez et al., 2019, Simpson et al., 2011) and sustained close work due to orthodox education (Zylbermann et al., 1993). A study has also given that young Asian female population than males, in urban regions have high myopia progression rates compared to their European equals (Lam et al., 1999, Lam et al., 2012, Donovan et al., 2012b).

Following a meta-analysis cross-sectional study from E^3 Consortium, it was reported that myopia prevalence across Europe has amplified significantly (mostly across western and northern Europe), similar to the level reported in North America but lesser than the proportion in Southeast Asian regions (Williams et al., 2015, Rudnicka et al., 2016). Formal education and higher educational level have a stronger influence that played an additive role rather than a causal role (Williams et al., 2015). The prevalence in schoolchildren in Taiwan is up to 70%, and 62% in Hong Kong with earlier myopia onset and higher myopia progression compared to their European counterparts. However non-cycloplegic refraction was used and prevalence in the 1990s is similar to 2012. The researchers postulate that the environmental factors have

reached a maximum and stable level (Lam et al., 1999, Lam et al., 2012). In Japan, a 6-year longitudinal study of same high school students showed heightened myopia prevalence from 35.5% in 1985 to 58.1% in 1991 (Hirai et al., 1998, Lam et al., 1999).

Subjects with higher income and better educational levels were more myopic as reported in the National Health and Nutrition Examination Survey/NHNES carried out in the US from 1971-1972 and 1999-2004 analyzed by Vitae et al. in 2009. At the same ages ranges (12-54 years) the prevalence rate increased from 25% to 41% within the 30 years. Other factors involved were gender and race; prevalence is lower in males than females, higher in white than black Americans as reported (Sperduto et al., 1983, Vitale et al., 2009).

In 2003, Gwiazda et al. (2003) showed that peripheral myopic defocus controls central myopia progression rate if induced by multifocal lenses, mostly bifocal soft contact lenses (Aller et al., 2016, Berntsen et al., 2013, Donovan et al., 2012b, Wallman and Winawer, 2004), due to the reduction in accommodative demand during near work (Gwiazda et al., 2003, Smith III, 2013). The following year, George and Rosenfield (2004) discovered that sustained blur (2hrs) improved the visual resolution of myopes due to perceptual adaptation/neural deblurring in the visual cortex (George and Rosenfield, 2004). Three years later, Adler and Millodot, (2006) revealed that peripheral hyperopic defocus is myopiagenic (Adler and Millodot, 2006). It was concluded that peripheral myopic defocus must be of a large degree and cover wider retinal area to cause a long term, beneficial and significant myopic control (Smith III, 2013). Jones-Jordan and colleagues in 2012 followed up myopic children in a Collaborative Longitudinal Evaluation of Ethnicity and Refractive Error/CLEERE survey from 1989-2009. They reported that near work (mainly reading) with other covariables controlled, was responsible for the slightly significant annual myopic progression (0.08D/year) in boys (Jones-Jordan et al., 2012). The results are not consistent with Parssinen and Lyyra's report in 1993 which stated that near work was associated with female juvenile myopia (Pärssinen and Lyyra, 1993). CLEERE study also stated that outdoor activity may be more important for myopia onset than for its progression in children. This is however inconclusive (Jones-Jordan et al., 2012). The evidence for the role of near work in myopia onset and progression may be deficient and inconsistent, as poor correlation was reported between near work and SER and axial length (Ip et al., 2008, Lu et al., 2009). Near work may play an additive role, see figure 12 (Saw et al., 2002, Tasneem et al., 2015, Spillmann, 2019, Huang et al., 2020).

Outdoor time of 14 hours per week may protect the growing eyes from developing myopia based on reports, see figure 12 (Rose et al., 2008a, Rose et al., 2008b).

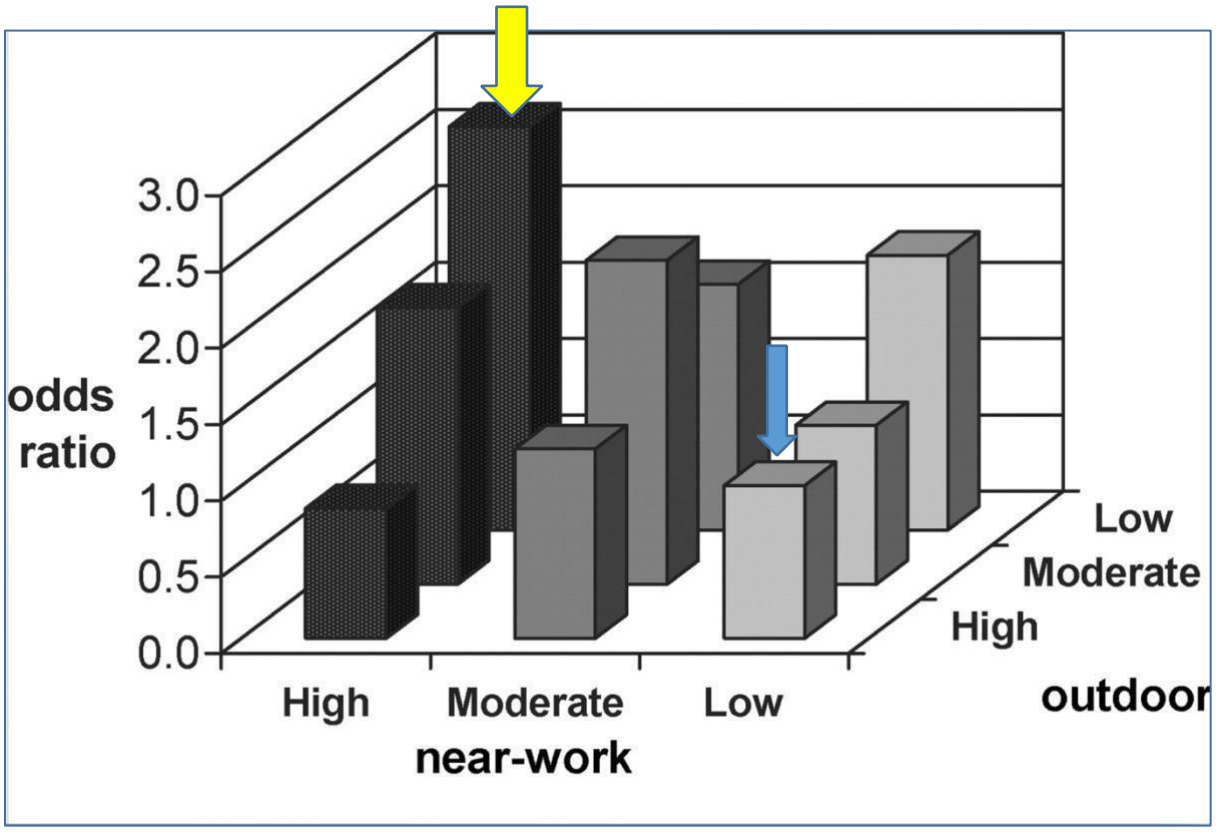


***Figure 12 showing multivariable-adjusted odds ratios (adjusted for education, ethnicity, gender, parental myopia and employment) for myopia** by reported average daily hours spent on near-work versus outdoor activities in 12-year-olds. Activities were divided into columns of high, moderate, and low levels of activity. The reference group is the bar with (blue arrow) high levels of outdoor activity and low levels of near work. The yellow arrow denotes subjects with high near work and low outdoor activity. Adapted from **Rose et al., (2008a)**.*

A Taiwan study by Wu et al. (2013) showed a 50% reduction after 80 minutes of outdoor time and a more recent China study by He et al. (2015) reported a 23% reduction in myopia incidence rate after 40 minutes of outdoor time revealing a dose-response association between outdoor time and myopia (Wu et al., 2016, Wu et al., 2013, He et al., 2015) similar to animal model research (Zheng et al., 2018). There is growing evidence supporting the protective role of time outdoor on human myopia (Guggenheim et al., 2014) but mixed results on myopia development (Hagen et al., 2019).

Although various cross-sectional, longitudinal, questionnaire- and intervention-based studies reveal the protective importance of time outdoors on incident juvenile myopia prevalence, not outdoor sports, clinically relevant effect may be obtained by larger exposures to outdoor light (Morgan et al., 2012, Rose et al., 2008a, Rose et al., 2008b, Dirani et al., 2009, Lu et al., 2009). In 2015, He et al. (2015) conducted a similar study in same Guangzhou city of

China as Morgan et al. (2012), and revealed that a 40-minutes outdoor photopic light exposure for 6-year old's reduced cumulative myopia incidence rate by 9% and SER difference of -1.42D vs -1.59D in intervention and control groups respectively after 3 years. No change in AL observed and more research was recommended for longer study period (He et al., 2015). In 2019, a longitudinal study monitored 82 children aged 6 to 15 years for two years and reported that outdoor also exposure slowed the rate of myopia progression
(Sánchez-Tocino et al., 2019). However, most of these outdoor studies are based on subjective responses of participants (Wildsoet et al., 2019).

Recently, researchers have also reported a potential protective role of scotopic light exposure (<1-1lux) on myopia development in Australia using 10-15-year old myopes and nonmyopes. They suggested the existence/role of photoreceptor rod signaling in myopia development (Landis et al., 2018). Monozygotic twin study has also stated that certain epigenetic/genetic variations/unknown environmental factors might play more significant role than outdoor time or near work (Ding et al., 2018).

Seasonal variations is another factor associated with increased rate of juvenile myopia progression (Grosvenor and Goss, 1999, Gwiazda et al., 2014). Donovan and colleagues (2012) in a Chinese children study (6-12 years) showed that mean myopia progression rate in summer is roughly 60% (−0.31 ± 0.25 D for summer and −0.53 ± 0.29 D for winter) of the winter rates and AL elongation is significantly lower (0.17 ± 0.10 mm for summer and 0.24 ± 0.09 mm for winter) ($p < 0.001$) (Donovan et al., 2012a).
The role of diet on refractive status was first reported in 1956 (Gardiner, 1956a,
Gardiner, 1956b). High blood cholesterol and high blood insulin has been linked with myopia (Cordain et al., 2002,(Bu and Wang, 2017, Mcbrien et al., 2009, Galvis et al., 2016). Insulin is a known growth factor that may have direct and indirect effects on the AL of the human eye (Cuthbertson et al., 1989)Cordain et al., 2002). Trier et al., 2008 reported the role of caffeine metabolite, 7-methylxanthine administered systemically in reducing myopia progression (Trier et al., 2008).

Lim, et al. (2010) showed a link between long AL and high saturated fat and cholesterol (Lim et al., 2010). A recent meta-analysis in 2019 showed a link between high blood concentration of 25-hydroxyvitamin D (25[OH]D) and low myopia risk (Tang et al., 2019).
Tang et al. suggested a link between blood vitamin D and children's outdoor time, but not significant link with genetic factors (Tang et al., 2019). In 2014, the Western Australian Pregnancy Cohort (Raine) Study (946 subjects) revealed that low serum 25-hydroxyvitamin D_3

(25[OH]D_3; determined with mass spectrometry) increased the risk of myopia within different populations (P = 0.003) (Yazar et al., 2014). In the same year, a prospective study (with 49 submariners) suggested that MMP9 (a scleral ECM protein) and serum vitamin D concentration have an inverse correlation ($r=-0.41$, $p=0.01$). The findings were however linked to sun exposure (UVB boosts vitamin D levels) (Baker et al., 2014). More recent study showed an insignificant influence of 25[OH]D on myopia using Mendelian randomization (MR) (Cuellar-Partida et al., 2017).

Most environmental factors determine myopia prevalence rate more, compared to genes. They also cause the variations in regional myopia prevalence (Zhao et al., 2000, Hashemi et al., 2018, Bourne et al., 2013, Young et al., 1969, Holden et al., 2016, He et al., 2009). Although gene-environment combination has been investigated, understanding the link between different environmental factors is also vital (Tang et al., 2019, Morgan and Rose, 2005, Hopper, 2019). This has been overlooked in most studies (Holden et al., 2016, Wolffsohn et al., 2019). Also, the time in the year when subjects are recruited for the study must also be considered as myopia progresses more in summer (Gwiazda et al., 2014).

1.3.The "Myopia Cycle" - Summary

The "myopia cycle" is a model summarizing causal and additive factors involved in human myopia development based on research (Gifford et al., 2019) and suggests points (circles) where a particular intervention should target (Goss and Jackson, 1996, Ip et al., 2008, French et al., 2013a, Smith et al., 2012)Chua et al., 2015). Juvenile myopia results from the interplay of genetic susceptibility and visual environment (blur, illumination) (Pacella et al., 1999). Outdoor time of 2 hours daily and less near work may protect growing eyes from developing myopia (onset) (Rose et al., 2008a, Rose et al., 2008b, Saw et al., 2019).

Moderate progression of myopia occurs as some other factors such as near work and education contribute their effects (Williams et al., 2015). Further progression into sight threatening high and pathological myopia in young adulthood and beyond, occurs at a more molecular level as the scleral biomechanical properties are altered abnormally and significantly (Holden et al., 2016, Rada et al., 2006, Gentle and Mcbrien, 2002, Gentle et al., 2003). Scleral cross-linking (SXL) or a combination with posterior scleral

reinforcement surgery is the remedy at this later stage of myopia progression (Zhu et al., 2018, Xue et al., 2018, Saw et al., 2019).

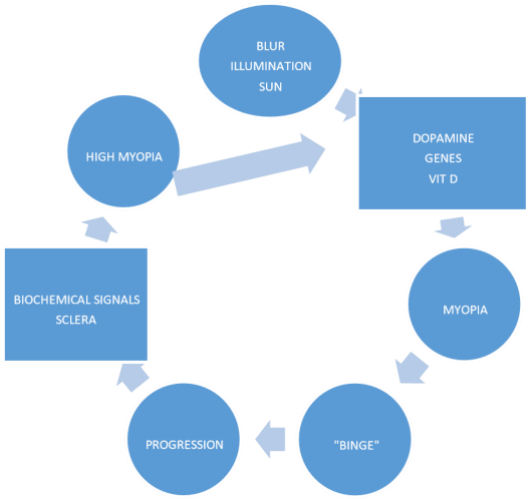


Fig 13 Flow chart showing the combination of major myopia risk factors. "BINGE" means blur, illumination, near-work, genetics, education. Myopia development and progression occurs from the combination of these factors (BINGE) (Gifford et al., 2019).

1.4. Myopia Control

Currently, the following pharmacological interventions are explored: topical atropine, topical timolol, oral 7-methylxanthine, cross-linking and the use of biopolymers for scleral remodelling. The optical interventions are overnight orthokeratology (OK), bifocal spectacles, contact lenses et cetera. Corneal reshaping contact lenses reduced myopia progression (Walline et al., 2009). A combination of optical, pharmacological and environmental intervention is also being investigated (Kinoshita et al., 2020)(Wildsoet et al., 2019). The myopia treatment effects were found to be incomplete, short-term and did not significantly affect the clinically relevant AL (Metlapally and Wildsoet, 2015, Wolffsohn et al., 2019, Wildsoet et al., 2019).

Previous studies have focused on refractive changes as a yardstick for myopia control effectiveness, while AL changes are being looked at recently. Refractive error and AL values have a robust correlation (Wildsoet et al., 2019). However, most AL measurements are being done with a contact biometric technique (ultrasound) which has low resolution and limited ability to identify slight magnitude of AL changes (Santodomingo-Rubido et al., 2002, Wolffsohn et al., 2019).

Also, these treatment methods are associated with adverse effects and the issue of duration of treatment effect and thorough risk-benefit analysis besets these control interventions (Wolffsohn et al., 2019).

1.4.1. Other advances in treatment

Stem cell-based therapy and miRNAs-based strategies for childhood myopia progression have also been explored (Janowski et al., 2015, Tanaka et al., 2019). A recent study showed that certain important miRNAs (microRNAs – small non-coding RNA molecules) are upregulated/downregulated in LIM (Tanaka et al., 2019). Although the study showed these molecules can be potential therapeutic agents, only three murine scleras were used.

The sclera has been proven to be a safe and reachable target for myopia reduction and control, however a better knowledge of its micro and macrostructure using 3D imaging technologies and unique biomechanics analyses will be beneficial in finding the best intervention in future (Metlapally and Wildsoet, 2015, Boote et al., 2019)

These findings and other studies underway provide better understanding of the link between a weak and thin sclera and myopia and possible strategies to remedy the myopia issue (Metlapally and Wildsoet, 2015). Some studies have not reported a major change in the human posterior sclera (its different layers) but an overall enlargement of the myopic eyes (Curtin, 1969, Cheng et al., 1992, Metlapally and Wildsoet, 2015). This warrants investigation of the myopic scleral biomechanics and its ECM proteins using more novel techniques with higher precision such as the Atomic Force Microscope (AFM). More studies will widen the current understanding of the emmetropization process and suggest novel therapeutic methods for myopia onset and progression (Rada et al., 2006).

1.5. Scleral Cross-Linking or SXL; Background

Crosslinking procedure was applied first in the 90s to treat keratoconus using UVA radiation and riboflavin. It helps form covalent bonds between corneal collagen molecules making it stiffer and halting further abnormal protrusion (Elsheikh and Phillips, 2013). It has been applied to sclera to treat myopia. This is termed scleral cross-linking or SXL. Although UV irradiation is efficient in SXL, it is cytotoxic (damage to cells) at high doses (Zhang et al., 2014b, Wollensak and Spoerl, 2004). Recently, SXL was combined with posterior scleral reinforcement surgery to treat high myopia-related macular hole in 19 patients in China (Zhu et al., 2018). Collateral and primary scleral surgeries on its own, produce debatable and inconsistent clinical results even in high myopia treatment (Boote et al., 2019), whereas SXL offers long-term safety and efficacy in strengthening scleral ECM and reducing progressive

myopia, based on animal and human experiments (Boote et al., 2019, Wang and Corpuz, 2015, Levy et al., 2018).

SXL boosts scleral rigidity by forming collagen fibrillar cross-links and is a promising intervention for myopia progression (Wollensak and Spoerl, 2004, Saw et al., 2019). It could be physically (using riboflavin/ultraviolet A light, riboflavin/blue light and rosebengal/white, etc) or chemically (using glucose, glyceraldehyde, glutaraldehyde, genipin et cetera) performed on the scleral tissue (Metlapally and Wildsoet, 2015). In 2019, Kim et al. (2019) utilized the AFM and other procedures to show a superior increase in fibril diameter (30%) and biomechanical stiffness after cross-linking rabbit sclera with 0.4M ribose (Kim et al., 2019). Recently also, researchers have revealed that natural cross-linker genipin at a certain concentration (150mM) made sclera of few Norway rats stiffer *in vivo* (retrobulbar injections) and the effect was sustained for a month (Hannon et al., 2019). In the same year, Carriel and colleagues, based on macroscopic and histological analyses, revealed that genipin and glyceraldehyde are capable of stiffening New Zealand rabbit sclera and repairing structural defects after 40 days of treatment (Carriel et al., 2019). Another study followed young high myopes for 2-3 years after combining scleral surgery with SXL (using low cytotoxic genipin). Myopia progression was significantly controlled (AL in fellow eyes vs treated eyes; 0.82mm vs 0.32mm) though it was not a randomized clinical study, and the scleral tightening was not quantified clearly (Xue et al., 2018). Genipin was be discussed in the next section.

Recent studies using animal models report that chemical SXL is more effective in scleral strengthening and less difficult to regulate than the physical SXL (Wollensak and Spoerl, 2004, Zhang et al., 2014b). The safest and best SXL on *in vivo* human scleras is yet to be identified although genipin and glyceraldehyde are stated to be least cytotoxic (Wollensak and Spoerl, 2004, Boote et al., 2019). In addition, porcine scleras have similar biomechanics to human sclera (Zhang et al., 2014b). This current study focuses on the effect of chemical crosslinkers such as genipin and glutaraldehyde on scleral structure and biomechanics. **Structure:** More recently, an *in vivo* study using genipin on Norwegian rat sclera showed stiffened sclera (Hannon et al., 2019). Chemical crosslinking has been used in humans to treat macular hole due to high myopia (Zhu et al., 2018).

Biomechanics: Chemical crosslinking using glutaraldehyde and glyceraldehyde significantly affect scleral stress-strain response in human and porcine eyes (Wollensak and Spoerl, 2004). Genipin (0.10mL of 0.50%) administered *in vivo* increased Young's modulus and ultimate stress substantially in guinea pig sclera with 21-days induced FDM (Wang and Corpuz, 2015).

It caused no irreversible damage to surrounding tissues.

1.5.1. CHEMICAL CROSS-LINKING AGENTS

Genipin (GN or GP) is a natural concentration-dependent cross-linker derived from *Gardenia jazminoides* plant with medical and non-medical applications (Gharaibeh et al., 2018). Genipin cross-linked biospheres have been reported to aid in drug administration (Liang et al., 2003). GN may help in reconstructing faulty cardiac tracts in dogs (Chang et al., 2001) and is a component of Japanese herbal medicine used to remedy liver apoptosis in murine model (Yamamoto et al., 2000). It has also been used to cross-link ocular (Dias et al., 2015) and nonocular tissues rich in chitosan and collagen (Dimida et al., 2017, Hannon et al., 2019). Within 0.1-0.5% concentration, GN is capable of nanostructuration and biomechanical stabilization by reducing fibril scaffold porosity and increasing fibre density. This is achieved through interaction with the functional groups of fibrin and agarose gel in the fibrin-based tissue model used by researchers in 2018 (Campos et al., 2018). GN's 2 carbonyl groups react with free amines in these macromolecules and stablish covalent bonds (Fig 14). These bonds provide a strong scaffold for the collagen fibrils. Beyond 0.5% concentration, cell viability is compromised. See figures 15 and 16 (Campos et al., 2018, Ninh et al., 2015). However, higher concentrations of genipin (up to 0.6%) prepared with Phosphate buffered saline, after incubating for more than 24 hours, have been shown to cause a two-fold increase in elastic moduli of collagenous tissues, reduce the swelling ability of the fibrils and boost the biostability of fibrillar scaffolds (Zhang et al., 2014a, Nair et al., 2019). Some of these studies were *in vitro* investigations. Although Zhang and colleagues suggested that 0.30% genipin concentration, incubated at 37^{o}c, is optimal to obtain the stated results, they concluded that higher concentrations and temperatures influence genipin cross-linking ability (Zhang et al., 2014a). Although genipin has similar cross-linking ability and effect on tensile strength of collagen fibrils as glutaraldehyde cross-linking reagent, it is significantly less cytotoxic (over 5000 times) (Yoo et al., 2011, Tomasula, 2009).

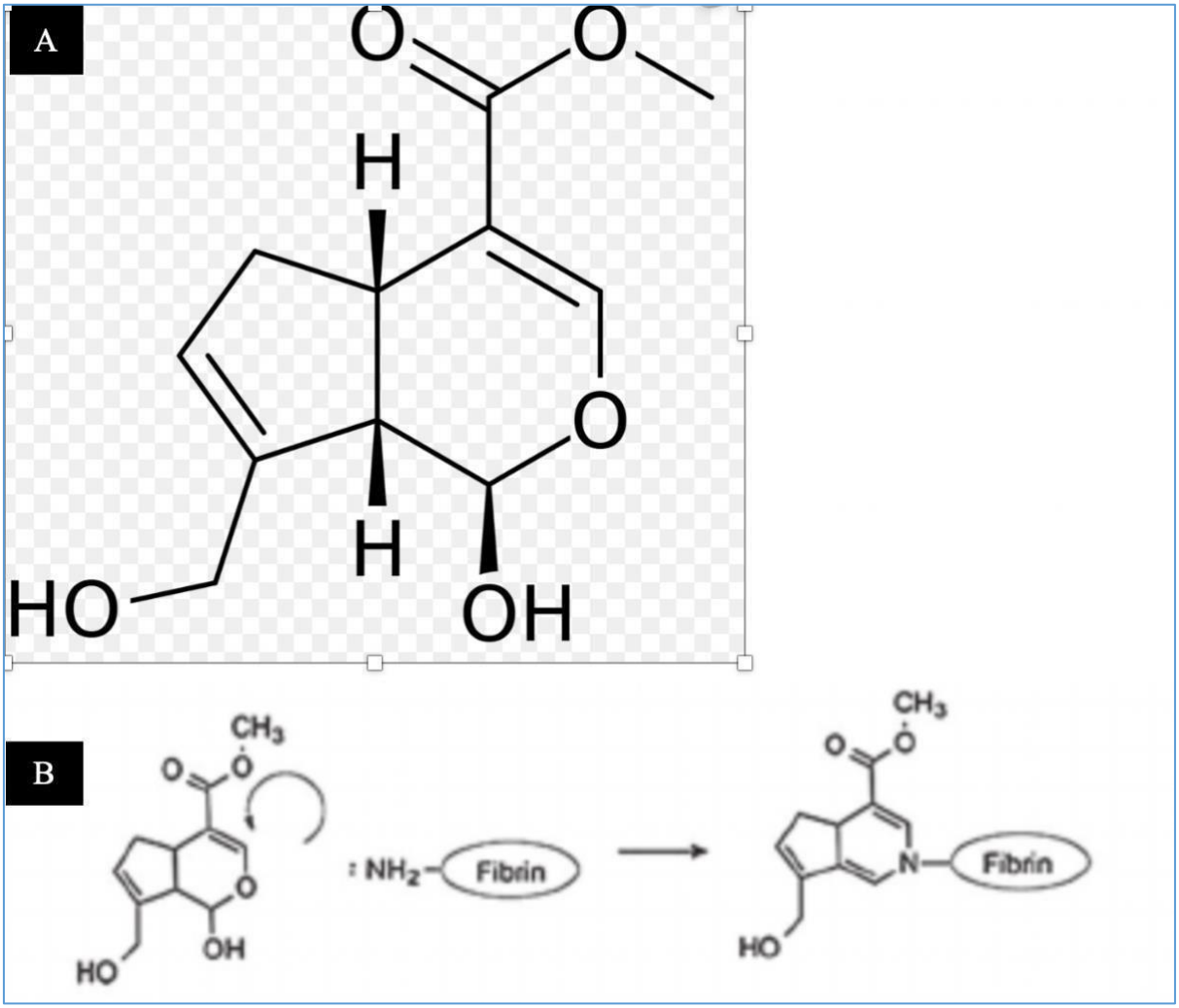


Figure 14 chemical structure of genipin (A), reaction with fibrin (B) adapted from Ninh et al., (2015)

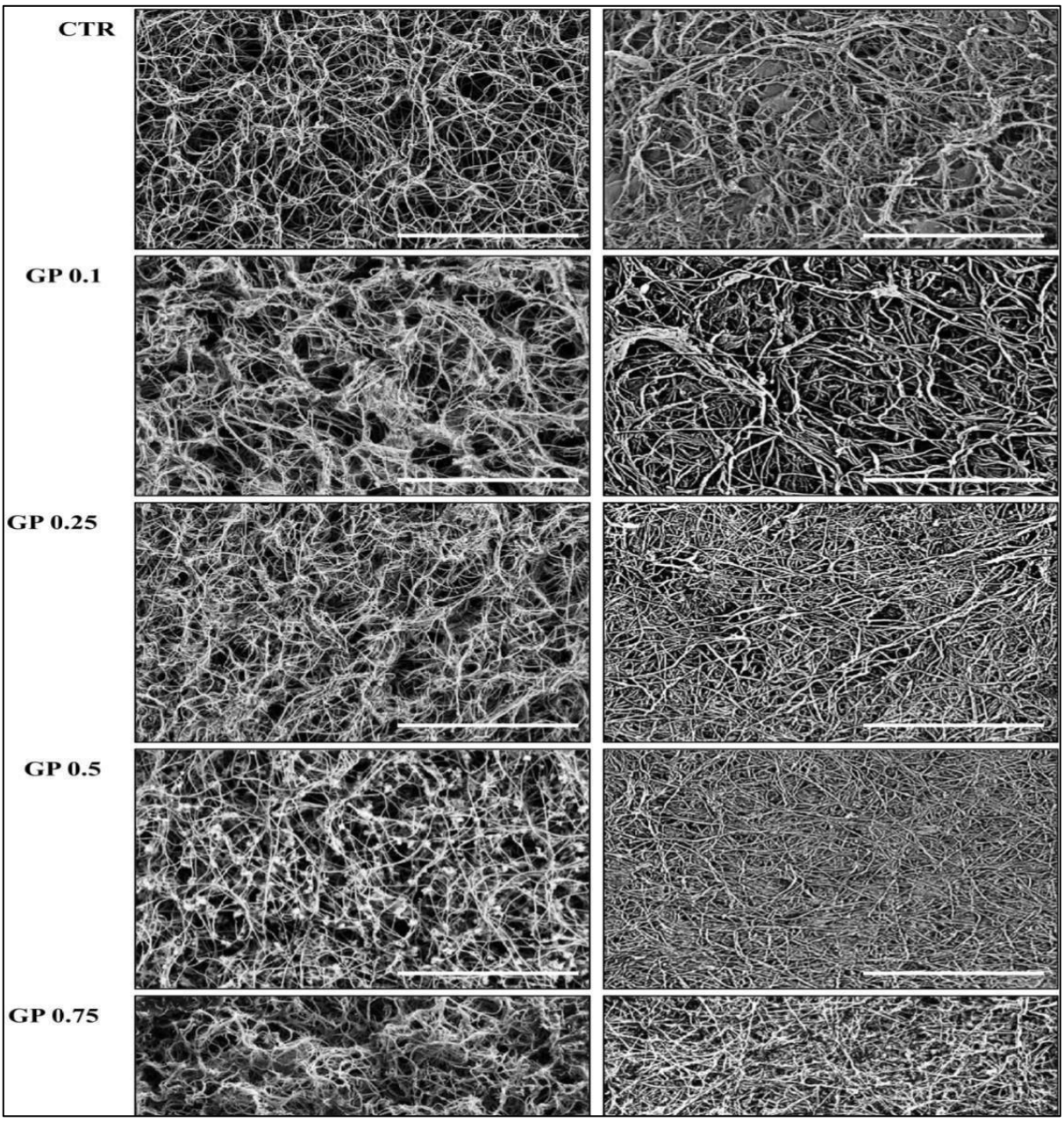


Figure 15 illustrating the nanostructuration/porosity of fibrils in agarose gel models crosslinked with genipin at 0.1% (GP 0.1), 0.25% (GP 0.25), 0.50% (GP 0.5) and 0.75% (GP 0.75) concentrations, left panel (FAH; Fibrin agarose hydrogel), right panel (NFAH; non-fibrin agarose hydrogel), adapted from Campos et al., (2018)

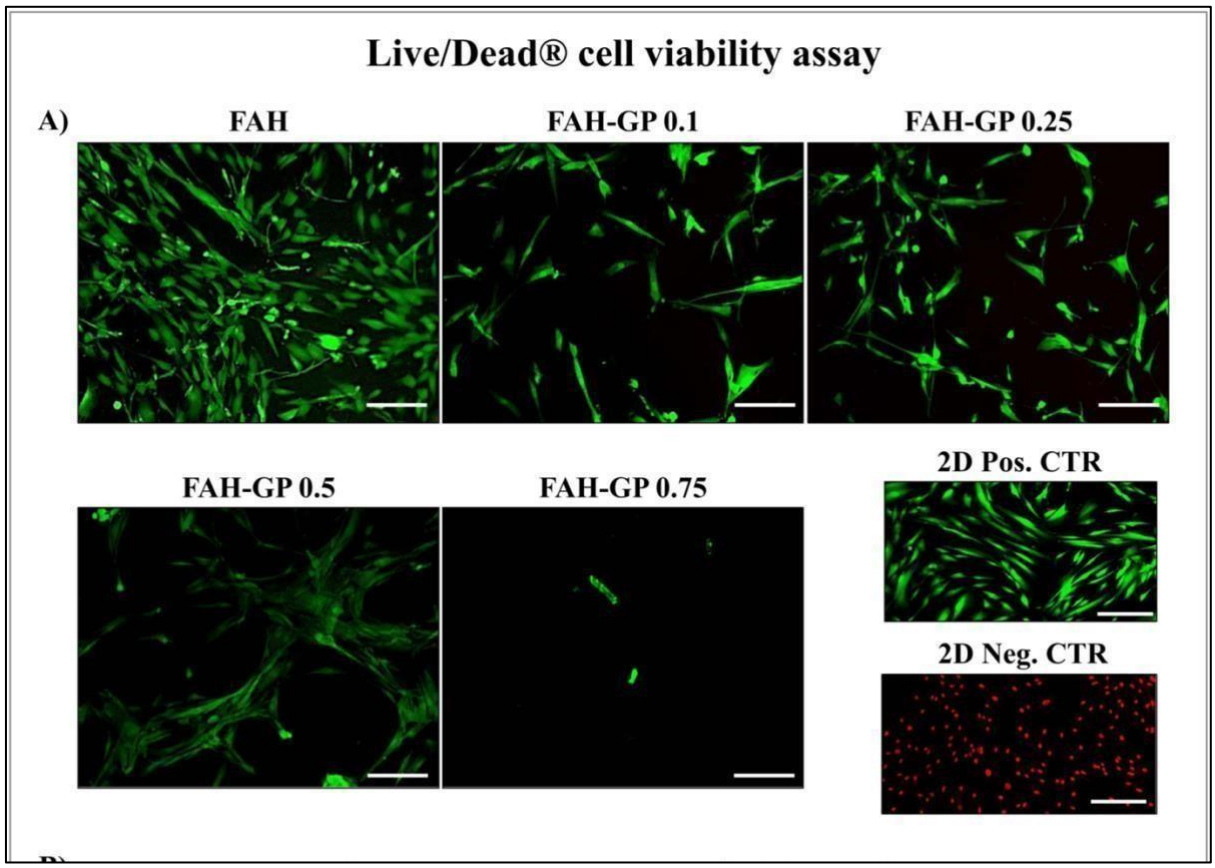


Figure 16 showing cell viability assay of fibrils in the agarose gel model cross-linked with genipin at 0.1% (GP 0.1), 0.25% (GP 0.25), 0.50% (GP 0.5) and 0.75% (GP 0.75) concentrations adapted from Campos et al., (2018).

Glutaraldehyde is a disinfectant and fixative which contains an aggressive carbonyl group (-CHO) that condenses amine ($R\text{-}NH_2$) functional group through Mannich chemical reactions (Plodinec and Lim, 2015). It has been successfully used to cross-linking collagen rich tissues such as sclera in animals and human and significantly affects the scleral stress-strain response to intraocular pressure (Wollensak and Spoerl, 2004, Coudrillier et al., 2016)

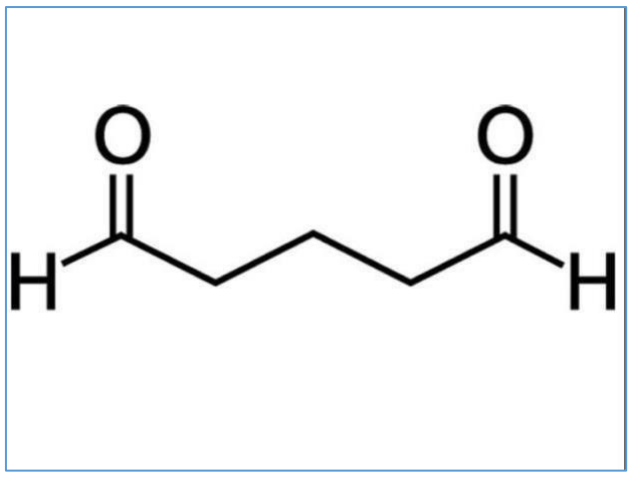


Figure 17 Chemical structure of glutaraldehyde adapted from Sehmi et al., (2016)

Table 2 Summarising the roles of the two Chemical Sxl Agents

Property/Sxl Agent	**Genipin (0.25% - 0.5%)**	**Glutaraldehyde (0.1%)**
Young's modulus	Increase (animal model)	Increase (human and animal) (***P*** = 0.02)
Ultimate stress/strain	Increase ($P < 0.05$) (animal)	-
Ability to repair scleral defects (after 40 days)	Increase (animal)	-
Cytotoxicity	None	Moderate
References	(Xue et al., 2018, Hannon et al., 2019, Wang and Corpuz, 2015, Carriel et al., 2019)	(Wollensak and Spoerl, 2004)

Aim and hypothesis:

The project sets out to demonstrate the role of the sclera in myopia by showing the effect of genipin and glutaraldehyde on human *ex vivo* sclera collagen fibril microstructure and micromechanics using the Atomic Force Microscope (AFM).

Based on research findings reviewed previously, chemical cross-linking agents alter the structural and mechanical properties of human sclera. Using the AFM, the collagen fibril structure and packing will be measured, and the local stiffness (biomechanics) probed.

CHAPTER 2. METHODOLOGY

In this laboratory study, three groups of post-mortem longitudinally oriented scleral strips 7mmx3mm of healthy human scleras (average age of 54.5 years) were dissected. The first group of strips (from near-equatorial and posterior regions) were untreated (control), see table 3. The other two groups from male and female donor scleras (total number of strips; 12; same scleras/regions) were cross-linked/treated with genipin (Sigma-Aldrich, Steinheim, Germany) and glutaraldehyde (Sigma-Aldrich, Steinheim, Germany), both mixed with Phosphate Buffered Saline Gibco™ Dulbeccos Phosphate-Buffered Saline (DPBS) with calcium, magnesium for specific number of hours at room temperature). The strips were dissected with sterilized scalpel from scleral bands removed from eyes of donors within 12 hours of death and placed in organ culture media at 37^{o}C, then frozen (at -40 ^{o}C). The donors were free from diabetes mellitus or HIV/MRSA. Other medical histories/refractive statuses of donors were not obtained. The strips were then thawed by placing tube containing sample in cold tap water at room temperature. Then the strips were dissected in a class II microbiological (class II MSC) safety cabinet. After dissection, samples were washed with sterile distilled water, dried and stored in the spark-free fridge (4^{o}C) overnight to stick onto a sterilized slide for AFM (Bruker Catalyst AFM (Bruker, Santa Barbara, CA. USA) equipped with BioScope Catalyst, EasyAlign, MIRO, NanoScope, PeakForce Capture et cetera for biomechanics (US4724318). Already crysectioned human cornea was used to optimize the AFM imaging techniques. This study was approved by the Committee on the Ethics of Research on Human Beings of the University of Manchester and followed the terms of the Declaration of Helsinki. Cornea and scleras were obtained from the Manchester Eye Bank/repository. All the scleras had research permission from the donors' relatives where transplantation was not done.

2.1. PROTOCOL

Title: Laboratory study of chemical cross-linking on *ex vivo* human scleral biomechanics and microstructure using the AFM.

2.1.1. TISSUE PREPARATION

Four human *ex vivo* scleras were obtained from the donors. Three parallel strips were dissected from near-equatorial and posterior regions of scleras for each group, to serve as: control (untreated), treated with genipin for 3.5hrs, treated with glutaraldehyde for 1 hr. See table 3 below. The exposure times/conditions were selected based on previous findings (Zhang et al., 2014a, Wollensak and Spoerl, 2004).

Experimental sequence:

In a class II microbiological (class II MSC) safety cabinet, after dissection, the scleral strips (control and treated) were washed with sterile distilled water and stored in the fridge (4 ^{o}C).

Treatment and imaging: The strips (treatment group) were then incubated in genipin and glutaraldehyde solutions in falcon ^{TM} 15mL conical centrifuge tubes at 18-20 ^{o}C for 1 – 3.5 hrs depending on the sxl treatment group as described above. Also, the samples were left overnight (12 hours) in the same incubator. All washing-off of strips were carried out with sterile distilled water. The strips were dried and observed using the AFM. The remaining scleras were stored at -40 ^{o}C, for future use.

STEPS:

Samples were defrosted by placing test tube in sterile distilled water for 20 minutes at room temperature. They were dissected longitudinally with sterile scalpel; three 7.0 mm × 3.0 mm scleral strips per eye/sample. The tissues adherent to the scleral strips internally were carefully peeled off/excised: anterior/posterior segment structures. Strips from equator or near equator and posterior (central middle of tissue) were dissected from same eye for treatment and corresponding adjacent part for control strips. Tissue dimensions were measured with a ruler. Chemical sxl solution was introduced in falcon ^{TM} 15mL conical centrifuge tubes, 3.5hrs for genipin and 1hr for glutaraldehyde at room temperature = 18-20 ^{o}C. In class II microbiological (class II MSC) safety cabinet, using sterile tweezers, strips were palced onto sterilized non-charged glass calibration slide. Washing off cross-linking agent was done with distilled water to remove excess fixative (sxl agent). Control and treated strips were placed onto slides and then in a Petri dish to minimize tissue contamination, allowed to dry overnight. They were stored in the fridge. Strips were observed using the AFM. The biomechanics was obtained after rehydrating all strips with sterile distilled water for 20 minutes. AFM imaging and biomechanics analysis were performed, and analysis data was saved in excel sheet. The remaining scleras were stored in freezer (-40^{o}C) until usage. Samples treated with glutaraldehyde and remnant fixative were discarded after rinsing with 23% glycine to prevent any chemical-related hazards. Sterilization of slides and other items such as scalpel was done with 70% alcohol.

2.1.2. CHEMICAL PREPARATION STEPS:

Cross-linking solution with concentration of 0.5 mM (w/v) of genipin and 0.1mM (v/v) of glutaraldehyde was prepared within 30 mins. All dilutions were made with sterile distilled water. Proper PPE (personal protective equipment) was worn while working in the fume hood, to make 50mM stock solution, 20 mg genipin (molecular weight of 226.23g/mol) in 1.77 ml PBS+ (gibco Dulbecco's Phosphate Buffered Saline: Calcium chloride and Magnesium chloride). To obtain 0.5 mM working solution, 1ml stock was added into 99ml PBS+ (1/100). In the fume hood, since molecular weight of glutaraldehyde is 100.12g/mol, 10mM stock solution had 1% glutaraldehyde concentration. Therefore, 1mM gave 0.1%. To get 0.1mM working solution, 1ml of glutaraldehyde was added into 9ml of PBS+ (1/10) and stored in fridge (4
^{o}C).

Table 3 SUMMARISING THE PROTOCOL

DISSECTION/TREATMENT	**GP 1**	**GP 2***	**GP 3***	**PROCEDURES**
3 parallel (longitudinal sections) strips; 7x3mm, (Male scleras)	Control	Treated with GENIPIN (0.5mM) 3.5hrs/12 hrs	Treated with GLUTERALDEHYDE (0.1mM) for 1hr/12hrs	Culture,incubate*, wash, dry, (attach) AFM imaging, wet, AFM biomechanics
3 parallel strips (Longitudinal sections); 7x3mm, (female scleras)	Control	Treated wt GENIPIN (0.50mM) 3.5hrs/12hrs	Treated with GLUTARALDEHYDE (0.1mM) for 1hr/12hrs	Culture,incubate*, wash, dry, (attach), AFM imaging, wet, AFM biomechanics
3 parallel strips (Longitudinal sections); 7x3mm, (male scleras)	Control	Treated with GENIPIN (0.50mM) 3.5hrs/12hrs	Treated with GLUTARALDEHYDE (0.1mM) for 1hr/12hrs	Culture,incubate*, wash, dry, (attach), AFM imaging, wet, AFM biomechanics

3 parallel strips; 7x3mm, (Female scleras)	Control	Treated with GENIPIN (0.50mM) 3.5hrs/12hrs	Treated with GLUTARALDEHYDE (0.1mM) for 1hr/12hrs	Culture,incubate*, wash, dry, (attach), AFM imaging, wet, AFM biomechanics
*= treated, GP = Group				

2.2. Atomic Force Microscopy (AFM)

AFM gives surface topography images and pseudocolor plot and can show accurate biomechanical properties of scleral samples such as Young's modulus measure of stiffness and adhesion strength (Fang et al., 2012; Graham et al., 2010).

AFM PeakForce tapping-mode imaging and biomechanics was carried out in 35% relative humidity at room temperature using a silicon cantilever with an integral pyramidal shaped tip (SICONG, Santa Clara, CA). Nominal tip radius (<10 nm) and height (12–16 μm) was utilized (Choi et al., 2016).

2.2.1. Optimization of AFM Imaging Technique using corneal samples

The AFM Scanasyst Peak force Tapping-mode was used in this study. It is more efficient than the basic tapping mode because it identifies and measures surface features as the oscillation amplitude reduces during scanning (Monitor, 2019, Choi et al., 2016). It is easier to perform and saves time. The adjustment of probe and critical imaging parameters are automatic; constantly optimizing and adjusting 'gain' within a predefined noise level based on the condition (hard or soft) of the sample (Monitor, 2019).

Best resolution: Peakforce Tapping-mode, scan size 2um or 2000nm and samples/line of 512, and scan size of 10um & samples/line of 2056, 'height' and Peakforce images.

Also, the 2-dimensional Fast Fourier Transform (FFT) analysis and 3-dimensional AFM imaging allows for quantitative collagen D-spacing analysis at micrometer and submicrometer levels. It is important to note that high resolution imaging (similar to SEM resolution) is directly obtained in air (naked collagen fibrils), to reveal the collagen 'overlap' and 'gap' zones (Zhong et al., 1993, Revenko et al., 1994, Garcıa and Perez, 2002, Erickson et al., 2013). The AFM is capable of showing the 67nm periodicity banding organisation and the twisted microfibrillar arrangement of collagen fibrils (Fang et al., 2012; Graham et al., 2010).

2.2.2. AFM PROCEDURE:

Good laboratory practice was observed at all times when dealing with laser equipment; nanoscope software was shut down before tilting the scan head and an infra-red sensor card was used to confirm; the indicator lights was 'off'. The right cantilever holder was selected and clamped unto a round stand – probe was loaded using the forceps and positioned properly. 2-3 drops of distilled water were instilled onto the slide on the sample to rehydrate it. Probe holder was slowly pulled out and scanner head was lifted up and well placed under scanner head. Head was mounted and EasyAlign unit was switched on using the front button.

On PC monitor, the Nanoscope V9 icon was clicked twice. The QNM (Quantitative Nanomechanical) mapping and Peakforce standard amplitude were highlighted. "Mechanical properties" mode (because of the tissue being studied) and 'load experiment' buttons were selected.

Using the knobs and dials on the sides of the AFM head and Easyalign unit, 'SUM' was set at highest value; Z ≤-4,900 (on the bottom of the computer monitor) to prevent breakage of cantilever when in contact with sample. On the Easyalign screen, the laser was made to be exactly on the cantilever tip; red dot on the center of 'crossed' square on computer monitor.

The AFM head was carefully placed on the microscope and the microscope was turned on. On the PC monitor, the light of the microscope reduced to 0.3 to enable visualization of sample. The sample video on the monitor were enlarged/minimized by using 'dock & undock' icon while the cantilever tip position was maintained on the center of the cross thoughout. The calibration, biomechanics and imaging procedures were not added here.

2.3. OPTIMISATION OF MAIN EXPERIMENT WITH SCLERAL SAMPLES

Method: Tissue was prepared with the procedure described above: (Electronic Test Requesting (ETR) 137 = 68-year-old male, see appendix).

Imaging with QNM in peak force TappingMode using probe tip-air (Silicon tip on Nitride Lever; Bruker) to image 2x2um areas recording surface topography, dissipation, deformation, adhesion and DMT Modulus of samples in dry conditions. Biomechanics with QNM in Peakforce TappingMode with spherical probe tip (in fluid; CONT-Silicon-SPM-Sensor with colloidal particle; SQUBE product) after calibration was carried out as stated above.

2.3.1. Biomechanics:

The biomechanics was done at AFM deflection sensitivity of 70.28nm/V, spring constant (K): 0.3600 N/m, thermal tune range = 1-100KHz, spherical glass probe (radius of curvature: 2500nm), ramp size of 2.000um and trigger threshold of 20.00nm. CONT-Silicon-SPM-Sensor with colloidal particle and spherical glass probe with diameter of 5um were used.

For each set of measurements (n = 3), a single probe tip was used for which deflection sensitivity was calibrated on a non-charged slide and AFM parameters set to fit QNM peakforce standard. NanoScope ScanAsyst was used to optimise gain, scan rate and set point.

Table 4 showing biomechanics results of optimization of AFM techniques using scleral samples (control, ETR 137) at XYZ positions

Sample position/values	**Control**		
Z µm	-4401.1	-4401.1	-4401.1
X µm	-2253.0	-2653.0	-2653.0
Y µm	2892.6	2892.6	3192.6
Reduced YM (average) MPa	0.36	0.29	0.11
Standard Dev.	0.16	0.32	0.05
Total av. and sd.	0.25 ±0.18 MPa		

YM = Young's Modulus in Megapascals (MPa), av. = Average, sd. = standard deviation

2.3.2 Imaging:

At AFM deflection sensitivity of 18.18nm/V and Calculated Spring constant (K) of 0.6427 N/m, Thermal tune range = 1-100KHz, probe radius of curvature: 5.00nm

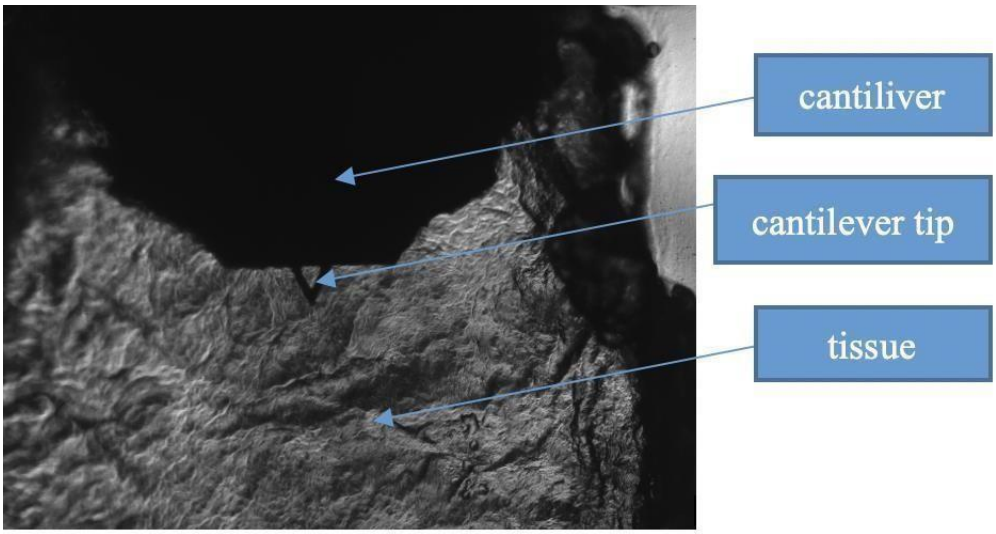


Figure 18 showing scanasyst probe tip in air during AFM optimisation

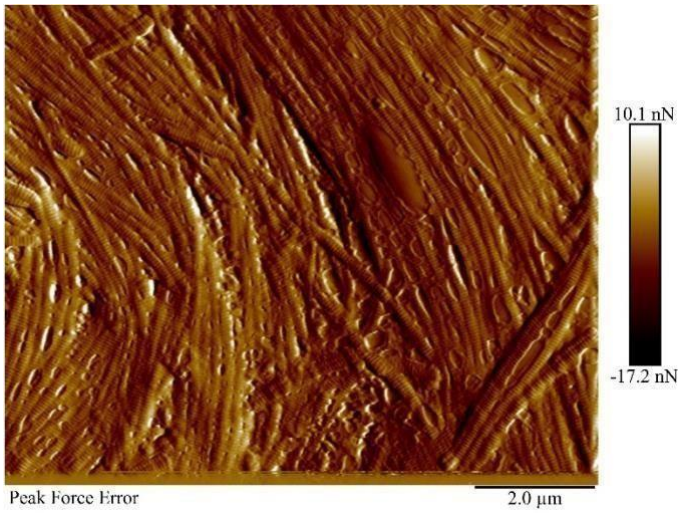


Figure 19 showing peakforce error optical image at near edge position of control scleral strip during optimisation

CHAPTER 3: RESULTS

3.1. Increased scleral stiffness from chemical crosslinking observed with the AFM

The reduced-Young's modulus and images of *ex vivo* human scleral strips (treated and untreated) from four healthy subjects obtained using the AFM were analysed for stiffness, micro and nano-structure/collagen fibril D-periodicity and mechano-structural relationship. The analysis was also carried out to compare the difference in arrangement and packing of the fibrils before and after cross-linking and at different cross-linking times. Initially, human corneal samples were used for optimization purposes (see Materials and Methods section).

3.1.1. Mechanical stiffness (reduced-Young's modulus; YM) in MPa

From results below, in three (3) out of four (4) of the samples, collagen crosslinking with a significant increase in variability and biomechanical stiffness (higher reduced-YM) by up to 1.3-, 1.2- and 5.2-folds (F_1 = 27.64, p = 2.0323E-11; F_2 = 80.91, p = 1.6161E-28; F_3 = 213.26, p = 1.0364E-46) in samples 1,2 and 3 respectively was obtained using genipin (see figure 20-24). Whereas with glutaraldehyde, 2-fold (F_3 = 213.26, p = 1.0364E-46) rise in the biomechanical strength was observed only in the 3rd sample. A decrease was seen in the rest of the samples.

In the last sample, a reversal in biomechanical stiffness was noted for both genipin and glutaraldehyde crosslinking. Initially, the scleral strip showed stiffness before crosslinking. The time-dependent nature of genipin crosslinking was shown in figure 25, as a 2-fold ($F_{1,4}$ = 36.88, p = 0.00000001) increase in biomechanical stiffness was recorded after 12 hours of incubation.

From the histogram and box and whisker plots, a higher variability towards increasing stiffness with genipin treatment was observed in samples 1-3.

The F-statistics and p values of AFM reduced-YM for the control, genipin and glutaraldehyde groups using the one-way ANOVA is F_1 = 27.64, p = 2.0323E-11 for experiment 1, F_2 = 80.91, p = 1.6161E-28 for experiment 2, F_3 = 213.26, p = 1.0364E-46 for experiment 3 and F_4 = 2.62, p = 0.08 for experiment 4.

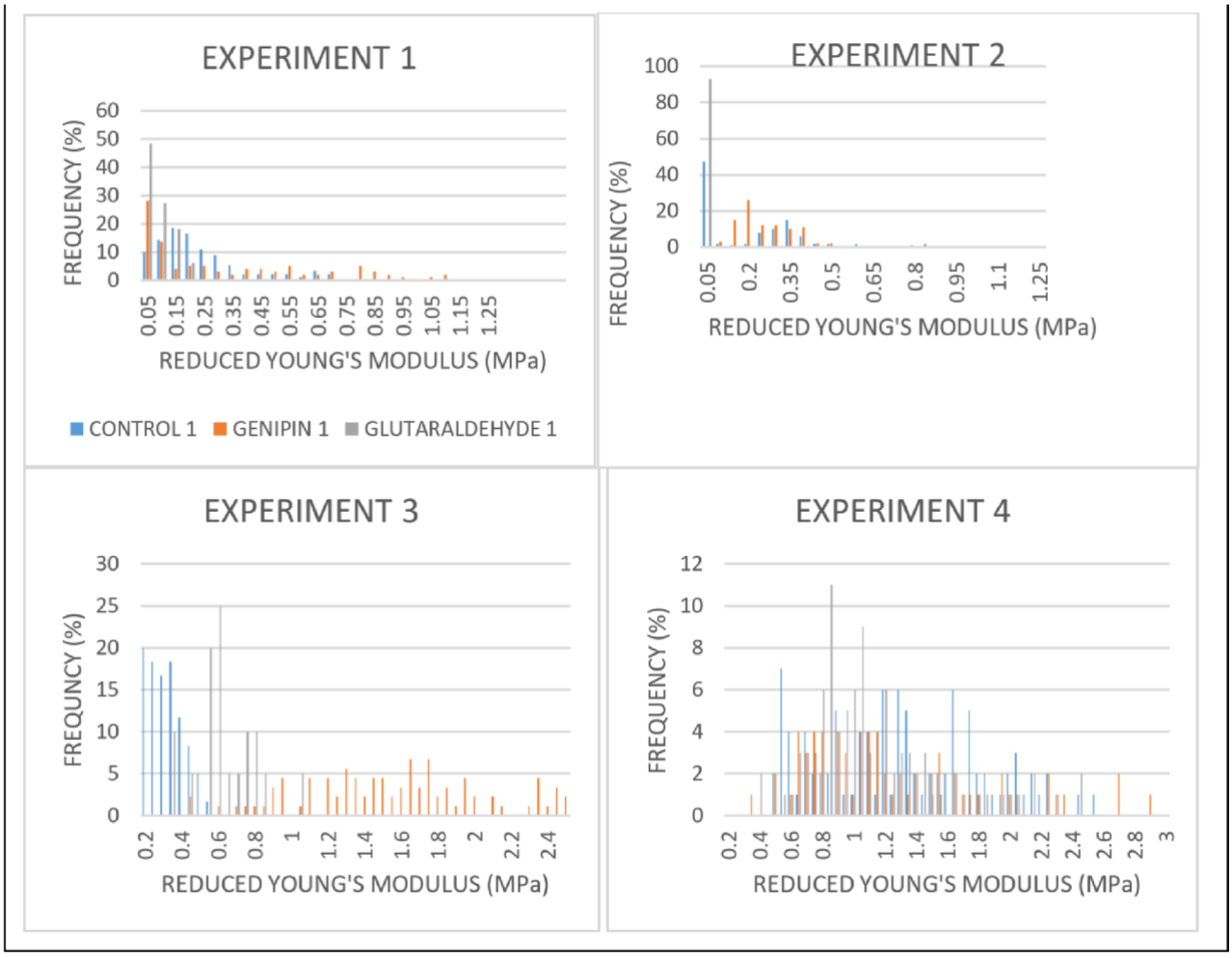


Figure 20. Histograms showing relative frequencies (%) vs the reduced-YM in MPa of the ex vivo scleral strip for the control, genipin and glutaraldehyde groups for experiments 1,2,3,4. Genipin significantly increases stiffness while glutaraldehyde decreases it in experiments 1, 2 & 3, with higher variability with genipin treatment. In experiment 4, a different pattern occurs with both genipin and glutaraldehyde decreasing stiffness.

Time-Dependent effect of Genipin: The average of the AFM reduced-YM of the genipin group for sample 1 at 3.5 hrs and 12 hrs incubation using the one-way ANOVA ($F_{1,4}$ = 36.88, p = 0.00000001) are given as 0.45 MPa and 0.69 MPa respectively. See figure 21 below.

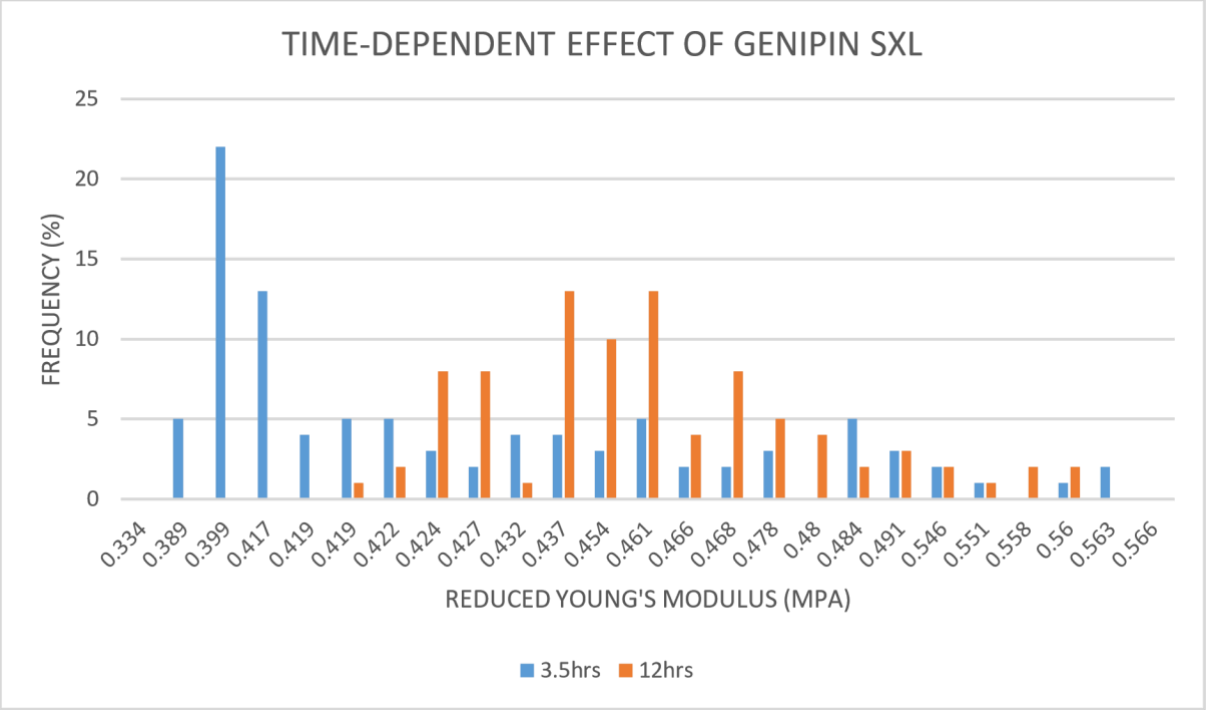


Figure 21. Histograms showing relative frequencies (%) vs the reduced-YM in MPa of the ex vivo scleral strip genipin treatment group at 3.50hrs and 12 hrs for experiment 1. Significant increase in stiffness in the 12hrs incubation compared to the 3.50hrs time.

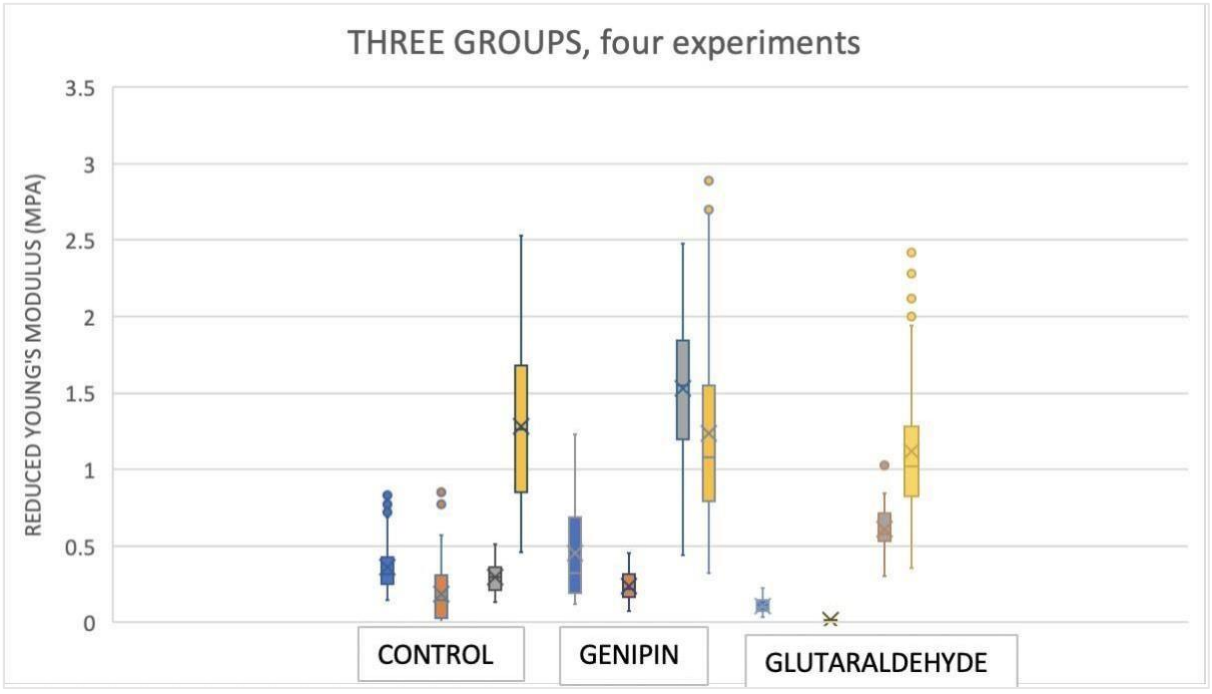


Figure 22. Box and whiskers showing the reduced-YM in MPa of the ex vivo scleral strip for the control, genipin and glutaraldehyde groups for all experiment groups. A similar pattern in the first three experiment groups with stiffness highest in genipin and lowest in glutaraldehyde group. A different pattern is seen in the fourth experiment group with decline

in stiffness towards the treatment groups (genipin and glutaraldehyde) In summary, a higher variability towards increasing stiffness with genipin treatment was observed in the first three samples. This change is time dependent.

3.1.2. Collagen fibril D-periodicity; alterations in D-period mean values after treatment and time-dependent changes

In parallel, it was discovered that for the scleral strip fixed with both crosslinking agents, the topography (D-periodicity) of the heterotypic fibrils did not change significantly ($p>0.05$). However, using 2-dimensional Fast Fourier Transform and individual fibril image horizontal measurement (not shown), mild D-periodicity shortening was observed after incubation with genipin for 12 hours in samples 1 and 2 (see figure 27). This was however not statistically significant (one-way ANOVA: $F_{1,2} = 3.70$, $p = 0.19$)

After 2D FFT analysis using WAXS software, the D-period mean values obtained with the control strips is given in the table below. The 2D FFT D-period for other experiments not listed (x) were not completed due to poor AFM image quality.

In experiment 1, the D-period changed from 66.0nm after 3.5hrs of genipin treatment to 59.1nm after 12 hrs while in experiment 2, it changed from 74.3nm to 63.3nm (see figure 27). The findings were also confirmed by individual fibril measurements using the AFM Nano-imaging analysis program (see appendix).

Table 5 showing the mean values of D-periodicity for all treatment and experiment groups

EXPERIMENTS/ groups	Control (nm)	Genipin(3.5hrs) (nm)	Genipin 12hrs(nm)	Glutaraldehyde(1hr) (nm)	glutaraldehyde 12hrs(nm)
1	64.4	66.0	59.1	63.0	x
2	61.6	74.3	63.3	x	43.6
3	62.9	68.2	x	x	x
4	68.6	68.0	x	64.8	x

X = not completed

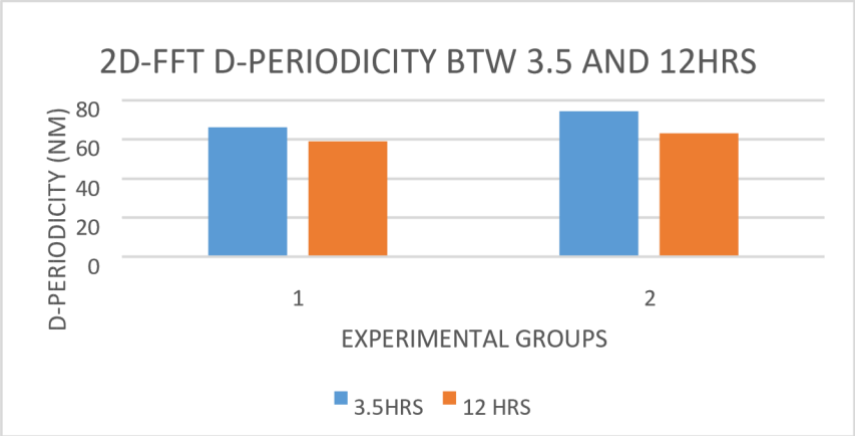


Figure 23. Bar charts showing the 2-dimensional Fast Fourier Transform D-periodicity mean values of collagen molecules of the ex vivo scleral strip for the genipin treatment groups (experiments 1 and 2) at 3.50hrs (blue bar) and 12 hrs (orange bar). Though not statistically significant, the d-periodicity decreased with time (reduced at 12hrs).

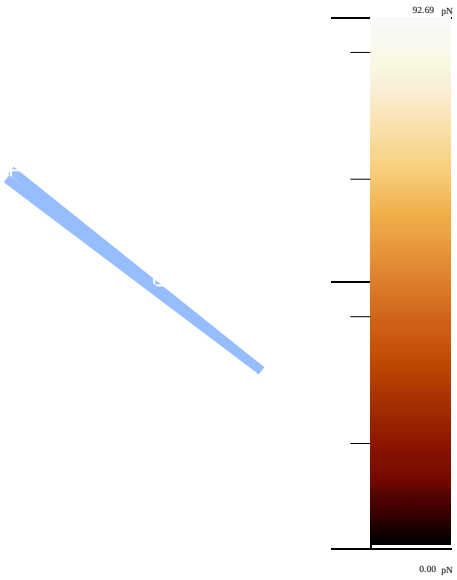


Figure 24. showing 2-dimensional Fast Fourier Transform profile for D-spacing of collagen molecules after 12 hours incubation of sample 1. The mean values obtained here were used to plot the bar chart in figure 23 above.

3.1.3.Mechano-Structural Relationship

A significant finding in the micro- (10 microns) and nano- (2 microns) structures of the present study is the ‘packed’ appearance of the genipin-treated samples and the ‘loose’ or ‘basket-like’ appearance of the glutaraldehyde cross-linked samples (See figures 29 and 30).

This observation correlates with the increase in stiffness discovered in the genipin groups. The 'packing' appears to improve in the 12 hrs genipin incubation sample as seen in the representative images below.

Micro and nanomorphological alterations have a direct link to the biomechanics of the scleral tissue.

REPRESENTATIVE IMAGES:

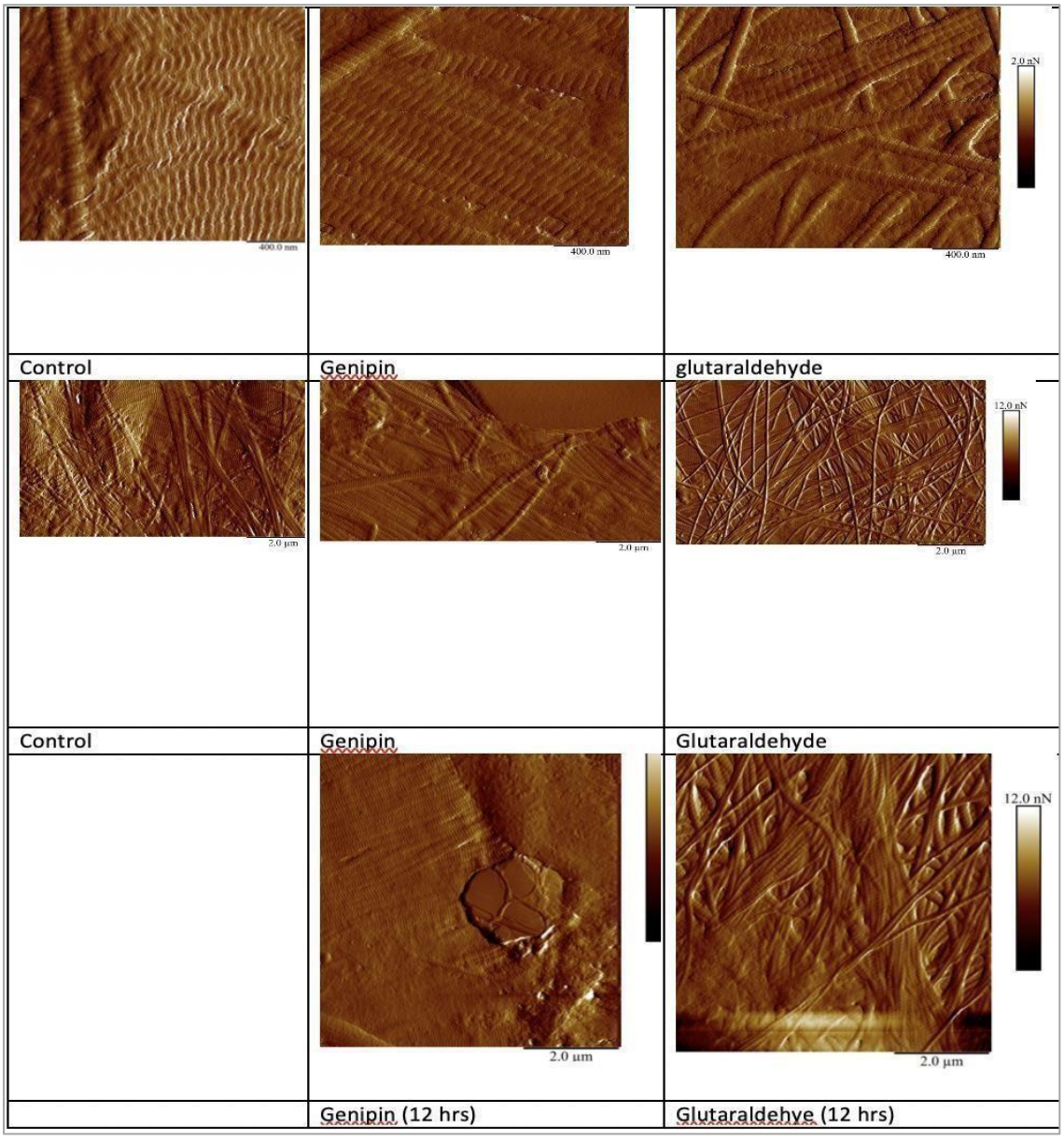


Figure 25: Representative AFM 2D peakforce error images at 2- and 10 microns of the scleral strips for the control, genipin and glutaraldehyde groups. The captured AFM images showed clearly visible D-periodicity banding of the fibrils. It shows the 'basket-like' appreance of glutaraldehyde group compared to the 'packed' appearance of genipin group.

REPRESENTATIVE IMAGES WITH DASHED LINES:

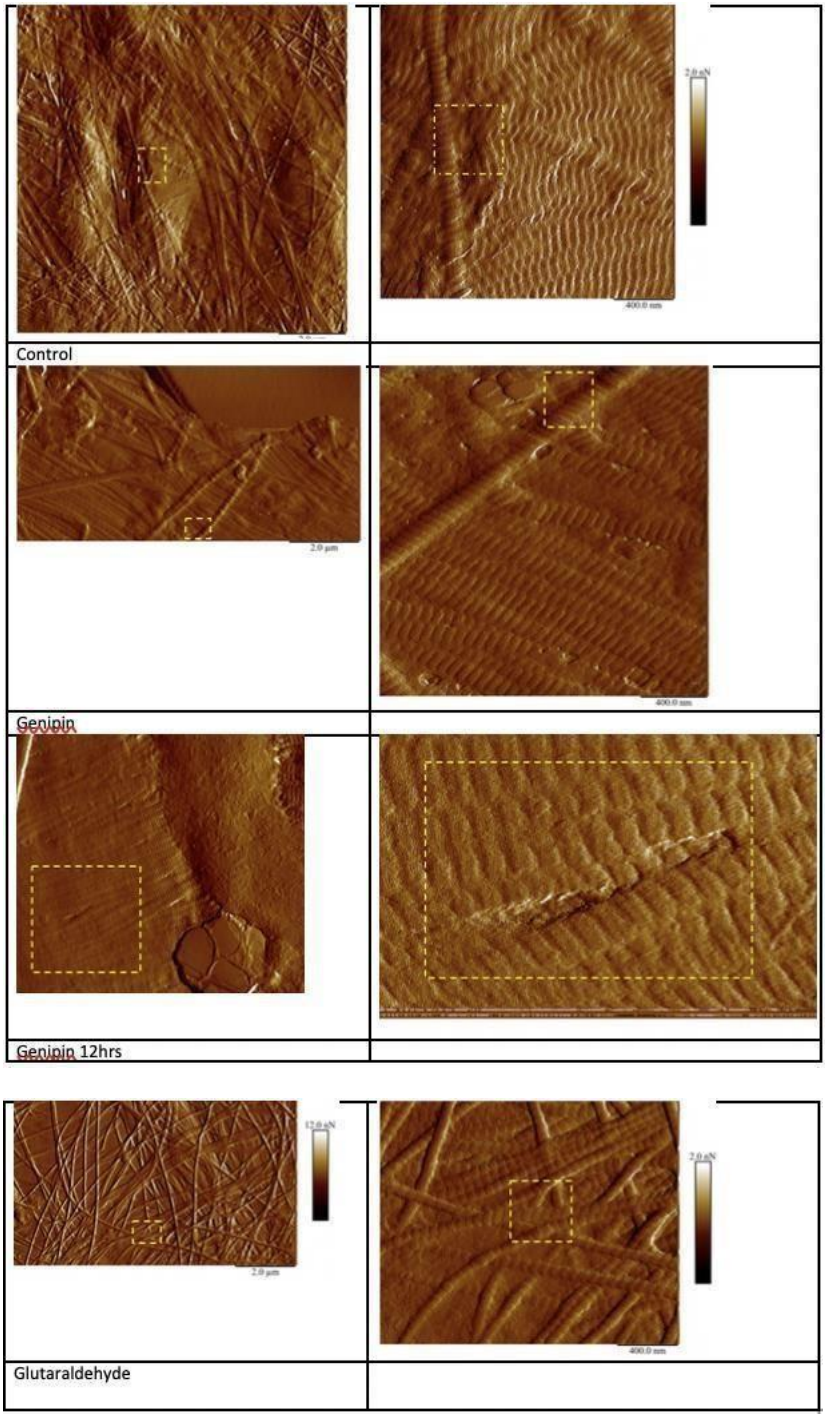


Figure 26: Representative AFM 2D peakforce error images at 2- and 10 microns of the scleral strips for the control, genipin and glutaraldehyde groups. Further shows the

'basket-like' appreance of glutaraldehyde group compared to the 'packed' appearance of genipin group.

CHAPTER 4: DISCUSSION OF FINDINGS

Recent findings have revealed the importance of scleral structure and biomechanics in myopia development and treatment (McBrien and Gentle, 2003, Wildsoet et al., 2019). Understanding the relationship between scleral nanomorphology and biomechanical properties will further explain the emmetropization and myopization process (Ouyang et al., 2019). This study has showed the effect of chemical crosslinking on *ex vivo* human sclera using the Atomic Force Microscope (AFM) to understand scleral role in myopia development and control.

4.1. Mechanical stiffness (reduced-Young's modulus; YM) in MPa

This study suggests that 3.50 hours incubation of *ex vivo* human sclera with 0.5mM genipin can successfully stiffen it. The stiffening effect increased slightly but significantly after 12 hours of incubation (2-fold; 0.36 to 0.69). However, these findings cannot be justified due to the number of missed trials and one of the trials giving different results.

Previous animal model studies reveal a 2-fold increase in stiffness (Young's modulus) in photochemical crosslinking with rabbit and porcine scleras at 4% to 8% strain and a similar result with genipin 0.01% and 0.3% at 30 mins and 15 mins) crosslinking of porcine scleras.

Almost 400% increase in Young's modulus was reported (Kwok et al., 2019, Kwok et al., 2017, Zhang et al., 2014b, Liu and Wang, 2013). Although a recent rat model study stated that a nearly 100% scleral stiffening can be achieved with 1mM genipin and revealed a dose response relationship (Campbell et al., 2017), the magnitude of stiffening required to control myopia-related scleral remodeling and the feasibility in preventing myopia in growing eyes is not fully ascertained (Elsheikh and Phillips, 2013, Hannon et al., 2019). From figures 21, 22, 23, the average reduced-YM increased significantly (F_1 = 27.64, p = 2.0323E-11; F_2 = 80.91, p = 1.6161E-28; F_3 = 213.26, p = 1.0364E-46) after treatment with genipin 0.5mM. This is in tandem with previous findings that genipin at 0.5% concentration is an effective and biocompatible cross-linker and can give a 2-fold increase in elastic moduli and stabilize fibrillar scaffolds (Zhang et al., 2014a, Nair et al., 2019, Campos et al., 2018, Wang and Corpuz, 2015). The mechanism is not fully understood but it has been conjectured that under neutral and acidic pH, genipin forms heterocyclic amines by interacting with primary amino groups. The free heterocyclic amines form intermolecular and intramolecular crosslinks and covalent bonds by further dimerization (Campos et al., 2018, Ninh et al., 2015, Gharaibeh et al., 2018, Chang et al., 2001). Although some studies have stated that it has similar crosslinking ability with glutaraldehyde (Yoo et al., 2011, Tomasula, 2009), our findings reveal that crosslinking with

0.1mM glutaraldehyde did not stiffen the sclera significantly except in experiment 3. The difference in the reduced-YM of all samples (ETR

169, 189, 112, 144) before crosslinking could be due to age (42, 64, 56, 56 years respectively) or other individual differences. Studies have reported the role of age on the stiffness of sclera and fibril density (Yan et al., 2011, Girkin et al., 2017, Wollensak and Spoerl, 2004, Girard et al., 2009, Wang et al., 2018, Coudrillier et al., 2015). Moreover, studies have shown that biomechanical stiffness of collagen-rich tissues such as the human sclera changes under medical conditions such as diabetes mellitus and cross-linking treatment. This occurs by induction of intramolecular covalent bonds through physical and chemical interactions (Fawzy et al., 2012, Wollensak and Spoerl, 2004, Boote et al., 2019, Coudrillier et al., 2015). From experiment 4 (Figure 24), the scleral strip may have been stiff prior to treatment. It may be suggested that the 56-year-old patient may have high cholesterol levels or be on antihypertensive medications such as Candesartan which affects scleral structure (Torres et al., 2014), or sample was obtained from a more equatorial/anterior region (Wang et al., 2018). Also, there was a reverse effect (less stiffening) after crosslinking sample/strip 4. Factors such as individual differences, concentration of crosslinker and poor crosslinking infiltration may be the cause. Stiffening due to cross-linking may have occurred on a gross scale, smaller areas may be more elastic due to a change in tissue structure at the micron length scale (Erickson et al., 2013). Additionally, the cross-linking may have reduced the swelling ability ratio of the tissue without affecting its biomechanical properties significantly (Zhang et al., 2014a, Zhang et al., 2014b, Grytz et al., 2014).

Our genipin crosslinking in experiment 3 showed a 5-fold increase in stiffness and 2-fold with glutaraldehyde. Genipin and glutaraldehyde are known to be strong crosslinkers with genipin being more efficacious and least cytotoxic (Wollensak and Spoerl, 2004, Boote et al., 2019). However, directly comparing the results obtained in our study across the subjects can be challenging due to individual variability (Hannon et al., 2019).

Several animal and human studies conjecture an association between scleral mechanical properties and myopia development (Siegwart Jr and Norton, 1999, Phillips et al., 2000, Metlapally and Wildsoet, 2015, McBrien and Gentle, 2003, Mcbrien et al., 2001, Levy et al., 2018) and the role of collagen crosslinking in long-term experimental myopia progression, even in human scleras as revealed in current study (Lin et al., 2018, Mcbrien et al., 2001).

The type and concentration (pH) of the crosslinker, temperature and time of incubation, individual differences are important factors to consider in our study. For instance, race, an

individual variable, was not considered in the present study (Yan et al., 2011, Girkin et al., 2017, Gharaibeh et al., 2018). Bearing all of the above in mind, although it is not clear what extent an increase in scleral stiffness will prevent myopia onset and progression, we have provided a foundation for more research. Successful crosslinking was confirmed in our research from the AFM imaging, biomechanics and D-periodicity results after incubation in all samples except experiment 4. Methodological improvements from this study will benefit future work. It was also confirmed that AFM is a beneficial tool in showing a classic link between sclera nanomorphology and biomechanics (Boote et al., 2019). Although the use of bulk tissue in present study reduced tissue processing steps and time, cryosectioning will help for better tissue mounting and prevent bias in choosing a portion to scan during AFM as some of the strips folded while drying up (Fang et al., 2012). Nevertheless, an improved tissue mounting method was implemented in the study similar to that by Plodinec and Lim, (2015) (Plodinec and Lim, 2015).

Furthermore, we also noticed a bluish discoloration of the scleral samples (see appendix 13, Pg.78) after incubating with genipin which became deeper after 12hrs of treatment similarly reported by Liu and Wang, (2013) and Chang et al, (2001) (Chang et al., 2001, Liu and Wang, 2013). Chang et al. (2001) stated that the bluish discoloration is the result of polymerization, dehydrogenation of various intermediary pigments and radical interactions involving oxygen (Chang et al., 2001). This may also suggest that treatment with genipin at long-term may be unsafe.

4.2. Collagen fibril D-periodicity; alterations in D-period mean values after treatment and time-dependent changes

The ‘peakforce’ error AFM images in all control and treatment groups showed the scleral collagen fibrils with the characteristic D-banding pattern. In the untreated group (control) across the samples, the mean values of the D-period were comparable to that in previous reports (64 5nm) (Meek, 2008, Shih et al., 2017, Jastrzebska et al., 2017). The Dperiod of sample 4 was slightly higher than the rest. Although it may account for its initial stiffness before crosslinking, this does not agree with previous reports (Zhang et al., 2014a, Zhang et al., 2014b, Grytz et al., 2014). Genipin had no statistically significant effect on the D-periodicity at 3.50hrs, across the samples, but change was mild but not significant ($p = 0.19$) at 12 hrs of incubation (66.0nm to 59.1nm and 74.3nm to 63.3nm). The change in glutaraldehyde group was not shown because the 2D FFT profiling would not complete

probably due to poor image quality. Cross-linking has been reported to slightly alter the Dperiodicity of the collagen fibrils (Zhang et al., 2014a, Zhang et al., 2014b, Grytz et al., 2014). However, some studies stated that crosslinking may prevent air-drying from altering the D-periodicity of the sclera by introducing intra- and intermolecular crosslinks to the fibrillar network (Jastrzebska et al., 2017). At 3.50hrs incubation, unaltered D-spacing agrees with its effective crosslinking and biocompatibility at 0.5mM concentration (Hannon et al., 2019, Wollensak and Spoerl, 2004, Boote et al., 2019). This may also be a positive sign that the treatment will change the structure and function but will not directly or drastically alter the molecular structure of the collagen molecules. In future, it will be important to study the periodicity of a particular spot/individual fibrils of the sample, before and after treatment and have a positive control as well. Genipin may affect biomechanics at short-term (3.50hrs) by reducing the cyclic softening response not by altering microstructure/D-periodicity (Levy et al., 2018, Jastrzebska et al., 2017). Other factors such as ECM components (Mcbrien et al., 2001) and individual differences may play more significant roles in scleral biomechanics (Erickson et al., 2013)(Fang et al., 2012; Graham et al., 2010) although it has been stated that collagen has a direct effect on the biomechanics of the sclera (Wang et al., 2018). In addition, D-periodicity variations in the different samples may be accounted for by estrogen levels of the patients (Erickson et al., 2013)(Fang et al., 2012; Graham et al., 2010). Future work should ascertain D-periodicity spacing role in human myopia onset and progression similarly studied in tree shrews (Mcbrien et al., 2001).

We have demonstrated that genipin 0.5mM significantly (~2 to 5-fold) improved collagen crosslinking after 3.5hrs and 12hrs. Altogether, the results will guide more *ex vivo* studies and subsequent *in vivo* studies to understand scleral remodelling in emmetropization and myopization, and in developing effective and long-term myopia treatment.

4.3.Mechano-Structural Relationship

Quantitatively, on the 2- and 10-micron scales, the collagen fibril bundles were oriented in a wavy pattern and parallel longitudinal direction. Organization seems to be more apparent in genipin treatment than glutaraldehyde analogous to the report by Levy et al., (2018) (Levy et al., 2018).

Although cryo-sectioning will allow for observation of the fibrils in the external (thinner bundle) and internal regions of the sclera (Komai and Ushiki, 1991), our AFM study with bulk

tissue is unique because there were relatively no artificial changes in the microstructure secondary to tissue preparations such as embedding (Markov et al., 2018).

The fibrils are packed irregularly, running parallel and forming lamellar bundles, characteristic of the human sclera. These features keep the sclera opaque, maintains eye shape and help withstand forces such as IOP (Forrester et al., 2015).

The type I collagen fibrils are most predominant while type III and V are sparse in the sclera (Meek, 2008, Shih et al., 2017). Fibril- width regulating collagen type V are few hence the fibril diameter of the sclera is larger than that of the cornea (Jastrzebska et al., 2017). Fibrillar crisscrossing and varied diameter were also observed as reported by Komai and Ushiki, (1991). Fibril diameter, profile, concentration and orientation across scleral regions, though important in scleral biomechanics and myopization, were not measured in this study (Mcbrien et al., 2001, Zhang et al., 2015, Komai and Ushiki, 1991, Wang et al., 2018).

From figures 29 and 30, it could be conjectured that cross-linking improves the packing/organization of the fibrils and affects the fibrillar spacing. This is mostly evident in the genipin-treated samples. For sample (1) ETR 169, genipin (figure 29) improves fibril organization compared to glutaraldehyde (figure 30) and in sample (2) ETR189, fibril packing is better with genipin cross-linking compared to strip incubated in glutaraldehyde even overnight (12hrs). This may be due to their ability to stabilize the inherent intermolecular covalent bonds. (Mayne and Burgeson, 1987, Forrester et al., 2015). This is achieved by the reaction of carbonyl groups in genipin with free amines in the fibrils molecules, see figure 14B(Campos et al., 2018). Also, fibril orientation and thickness difference may depend on the region (outer or inner) of the sclera (Meek, 2008). Myopic sclera has been found to have disorganized collagen fibrils (Metlapally and Wildsoet, 2015, Tao et al., 2013).

Moreover, in some of the representative images, individual fibrils crossing a bundle of fibres was seen as reported by Levy et al. (2018) (Levy et al., 2018). The function of these crossing fibrils is yet unknown.

In conclusion, promising SXL results have been obtained with genipin (0.5mM) than glutaraldehyde (0.1mM) on *ex vivo* healthy human scleras. According to the present study, incubating scleral strips with genipin for 3.5hrs and 12 hrs at 37^{o}c, provided stiffer scleras than the controls. The D-period was slightly shortened after 12hrs incubation with genipin and the fibrils became more ‘packed’ or ‘organized’ compared to incubation with glutaraldehyde. More practical methods to effectively crosslink/stiffen the tissue need further investigation. The

current findings may serve as a guide for future application of genipin in the *in vivo* animal and prospective clinical studies.

FUTURE WORK

Our present study was faced with minimal but notable limitations including low sample size and sclera from healthy patients although their complete refractive status and ethnicity were not known hence conclusions could not be made on the reason for initial scleral stiffness in sample 4. To strengthen the correlations, more samples should be crosslinked and analysed in the future. AFM scanning of some of the strips could not be completed. Incomplete cleaning of samples and probe tip may be responsible (Kirchhofer, 2018).

Additionally, future studies might use the drop method rather than soaking the scleral strips in genipin or glutaraldehyde as this drop method (140 l every 15 mins for 2 hrs) is a more practical and efficient way to clinically crosslink the tissue (Gharaibeh et al., 2018).

APPENDICES:

1. ***Screenshot of biomechanics analysis of corneal samples (10µm thick). STDEV stands for standard deviation***

Force Indentation						
Image File N	R≤		Young's Mod	Reduced Modulus(MPa)		
corneal10um	0.9996		0.672	0.738		0.143
corneal10um	0.9995		0.834	0.916		0.157
corneal10um	0.9979		0.512	0.563		0.194
corneal10um	0.9961		0.331	0.364		0.202
corneal10um	0.9997		0.561	0.617		0.21
corneal10um	0.9998		0.301	0.33		0.228
corneal10um	0.9992		0.527	0.579		0.253
corneal10um	0.9975		0.453	0.497		0.26
corneal10um	0.9993		0.38	0.417		0.269
corneal10um	0.9996		0.426	0.468		0.274
corneal10um	0.9992		0.462	0.508		0.299
corneal10um	0.9987		1.16	1.27		0.3
corneal10um	0.9992		0.435	0.478		0.315
corneal10um	0.9967		0.348	0.383		0.323
corneal10um	0.9998		0.475	0.522		0.33
corneal10um	0.9995		0.612	0.673		0.333
corneal10um	0.9996		0.303	0.333		0.333
corneal10um	0.9996		0.389	0.427		0.336
corneal10um	0.9988		0.422	0.464		0.339

corneal10um	0.996		0.327	0.359			0.705
corneal10um	0.9993		0.411	0.451			0.705
corneal10um	0.9996		0.231	0.253			0.738
corneal10um	0.9996		0.273	0.3			0.745
corneal10um	0.9992		0.244	0.269			0.765
corneal10um	0.9998		0.309	0.339			0.778
corneal10um	0.9999		0.642	0.705			0.805
corneal10um	0.9863		0.422	0.464			0.808
corneal10um	0.9993		0.561	0.617			0.827
corneal10um	0.9998		0.795	0.874			0.872
corneal10um	0.9989		0.306	0.336			0.874
corneal10um	0.9994		0.237	0.26			
corneal10um	0.9992		0.191	0.21			
corneal10um	0.9991		0.287	0.315			
corneal10um	0.999		0.272	0.299			
corneal10um	0.9993		0.249	0.274			
Average	0.998127		0.48696	0.53498			0.5031979 MEAN
Std Dev	0.003254		0.195567	0.214639			0.1640872 STDEV
Maximum	0.9999		1.16	1.27			
Minimum	0.9755		0.13	0.143			

2. SCLERAL SAMPLES SHEET (SCREENSHOT)

		ETR	SEX	AGE	Post-mortem time (hrs)	AMD	Diabetic?
2		ETR30	F	64	63	No	No
3		ETR41	F	67	42	No	No information
4		ETR43	M	60	40	No	No information
5		ETR44	M	64	47	No	No information
6		ETR54	M	67	49	No	No
7		ETR55	M	65	47	No	No information
8		ETR63	M	65	36	No	No information
9		ETR71	F	65	27	No	No information
10		ETR79	M	64	33	No	No information
11		ETR82	F	61	50	No	No information
12		ETR88	F	62	46	No	No information
13		ETR89	M	62	32	No	No information
14		ETR96	M	61	36	No	No
15		ETR100	F	63	30	No	Yes type I (insulin dependent)
16		ETR101	F	54	46	No	No information
17		ETR102	F	66	23	No	No information
18		ETR103	M	65	48	No	No information
19		ETR104	M	68	46	No	Yes type 2 (early/diet controlled)
20		ETR108	M	69	38	No	No information
21		ETR109	M	72	36	No	No information
22		ETR111	M	73	47	No	No
23		ETR112	M	56	35	No	No
24		ETR121	M	65	43	No	No information
25		ETR134	M	64	39	No	No

25		ETR134	M	64	39	No	No		
26		ETR135	M	60	35	No	No		
27		ETR136	M	67	39	No	No		
28		ETR137	M	68	46	No	No		
29		ETR144	M	56	36	No	No		
30		ETR154	M	57	47	No	No		
31		ETR155	F	52	48	No	No		
32		ETR158	F	62	48	No	No		
33		ETR159	M	64	43	No	No		
34		ETR166	F	52	26	No	No		
35		ETR168	M	50	33	No	No		
36		ETR169	F	42	44	No	No information		
37		ETR177	M	65	42	No	No		
38		ETR189	F	64	41	No	No information		
39		ETR190	F	44	30	No	No		
40		ETR195	F	62	41	No	No		
41		ETR207	F	63	44	No	No		
42		ETR212	M	62	40	No	No		
43		ETR218	M	59	32	No	No		
44		ETR240	F	55	48	No	No		
45		ETR272	M	55	41	No	No		
46		ETR291	M	61	34	No	No		
47		ETR301	F	59	43	No	No		
48		ETR304	F	54	41	No	No information		
49		ETR322	F	54	31	No	No		
50		ETR345	F	55	34	No	No		
51		ETR380	M	24	47	No	No	Blind: secondary to optic atrophy	

3. Table showing biomechanics result of main sample 1 (ETR 169) for control and treated (genipin and glutaraldehyde) at XYZ positions

Value/group	Control			Genipin			Glutaraldehyde		
Z µm	-4700	-4700	-4700	-4600	-4600	-	-5100	-5100	-5100
X µm	-3536.1	-4613.1	-4612.2	2507.8	2934.3	-	2934.3	3911.5	5208.2
Y µm	3875.5	3875.5	4874.4	1953.4	1109.9	-	1109.9	1733.9	1711.8
Reduced YM (av.) MPa	0.36	0.22	0.11	0.29	0.085	-	0.051	0.07	0.108
Sd.	0.16	0.21	0.05	0.11	0.087	-	0.018	0.031	0.05
Total	0.23±0.14 MPa			0.19±0.1 MPa			0.08±0.03 MPa		

YM = Young's Modulus in Megapascals (MPa), av. = Average, sd. = standard deviation

4. Table showing biomechanics results at XYZ positions for EXPERIMENT 1 (12 hours)

Value/group	Genipin		
Z µm	-5197.3	-5026.4	-5179.3
X µm	763.0	972.6	972.6
Y µm	532.2	987.7	987.7
Reduced YM (av.) MPa	0.454	0.689	0.486
Sd.	0.309	0.205	0.27
Total	0.543±0.26 MPa		

5. Table showing biomechanics results at XYZ positions for EXPERIMENT 2

Value/group	Control			Genipin			Glutaraldehyde		
Z µm	-5000.2	-5000.2	-5000.2	-4600	-4600	-4600	-4500	-4500	-4500

X µm	4700.0	4746.0	4480.1	4480.1	4117.6	4117.6	-2543.8	-3747.6	-3491.6
Y µm	1000.7	1656.7	1656.7	1955.3	1955.3	2432.7	3691.5	4160.1	3637
Reduced YM (ave) MPa	0.0186	0.053	0.0332	0.0761	0.23	0.101	0.013	0.010	0.031
SD	0.192	0.041	0.0122	0.048	0.1	0.100	0.004	0.005	0.044
Total	0.035±0.08 MPa			0.053±0.1 MPa			0.018±0.04 MPa		

6. Table showing biomechanics results at XYZ positions for EXPERIMENT 3

Position/group	**Control**			**Genipin**			**Glutaraldehyde**		
Z µm	-4892.5	-4892.5	-4892.5	-5128.2	-5114.9	-5119.0	-4884.4	-4639.1	-5173.7
X µm	-1576.5	-1336.3	6013.6	4274.8	4276.7	3762.8	-3262.1	-2446.2	-5223.0
Y µm	-3027.3	-3027.3	-2142.0	-3330.4	-2668.3	-2999.4	529.0	-944.7	236.2
Reduced YM (av) MPa	0.29	0.43	0.104	3.18	1.53	1.298	1.19	0.61	0.60
Sd	0.09	0.19	0.016	0.77	0.49	0.41	0.41	0.17	0.17
Total	0.27 MPa			2.00 MPa			0.80 MPa		

7. Table showing biomechanics results at XYZ positions for EXPERIMENT 4

Position/group	**Control**			**Genipin**			**Glutaraldehyde**		
Z µm	-4931.7	-4980	-4915.2	-5030.4	-5020.8	-5030.5	-5192.3	-5110.4	-5091.8

X µm	-3052.0	2052.0	-2121.3	3762.8	5070.8	5892.0	3185.1	-3106.0	-2061.8
Y µm	-4316.0	-4316.0	-2999.4	-2039.0	-2630.9	-2999.4	-928.3	-2717.8	-2721.0
Reduced YM (av) MPa	1.57	1.28	1.43	1.103	0.87	1.24	0.56	1.12	0.75
SD	0.52	0.53	0.39	0.53	0.27	0.41	0.35	0.43	0.17
Total	1.43 MPa			1.07 MPa			0.81 MPa		

8. ANOVA TABLES FOR EXPERIMENTAL GROUPS

Anova1: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	91	33.031	0.36297802	0.02682953
Column 2				
Column 3				

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.5656	2	1.47020675	27.6361764	2.0323E-11	3.03747228
Within Groups	0.10804848	96	0.45405208	0.09529397		
Total	2.94041349	217	0.05319863			
	11.5441029	219				

Anova3 : Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	96	43.589	0.45405208	0.09529397
Column 2	90	62.015	0.68905556	0.04207963

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.56536613	1	2.56536613	36.8828623	0.00000000703	3.89249438
Within Groups	12.7980135	184	0.06955442			
Total	15.3633796	185				

ANOVA

Anova Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	100	18.820411	0.18820411	0.0366906
Column 2	93	21.7968	0.23437419	0.00907451
Column 3	93	1.18662	0.01275935	1.8355E-05

ANOVA6

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.55547598	2	1.27773799	80.9144948	1.6161E-28	3.02766896
Within Groups	4.46891316	283	0.01579121			
Total	7.02438914	285				

Anova 7: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	60	17.629	0.29381667	0.00863754
Column 2	90	137.696	1.52995556	0.2441235
Column 3	20	12.188	0.6094	0.02930583

ANOVA8

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	58.213438	2	29.106719	213.255518	1.0364E-46	3.05011974
Within Groups	22.7934176	167	0.13648753			
Total	81.0068557	169				

Anova 9: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	97	124.291	1.28135052	0.27726858
Column 2	68	84.138	1.23732353	0.34058622
Column 3	95	106.108	1.11692632	0.18877971

ANOVA 10

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.36752629	2	0.68376314	2.61567389	0.07506446	3.03092512
Within Groups	67.1823534	257	0.26140994			

Total	68.5498797	259

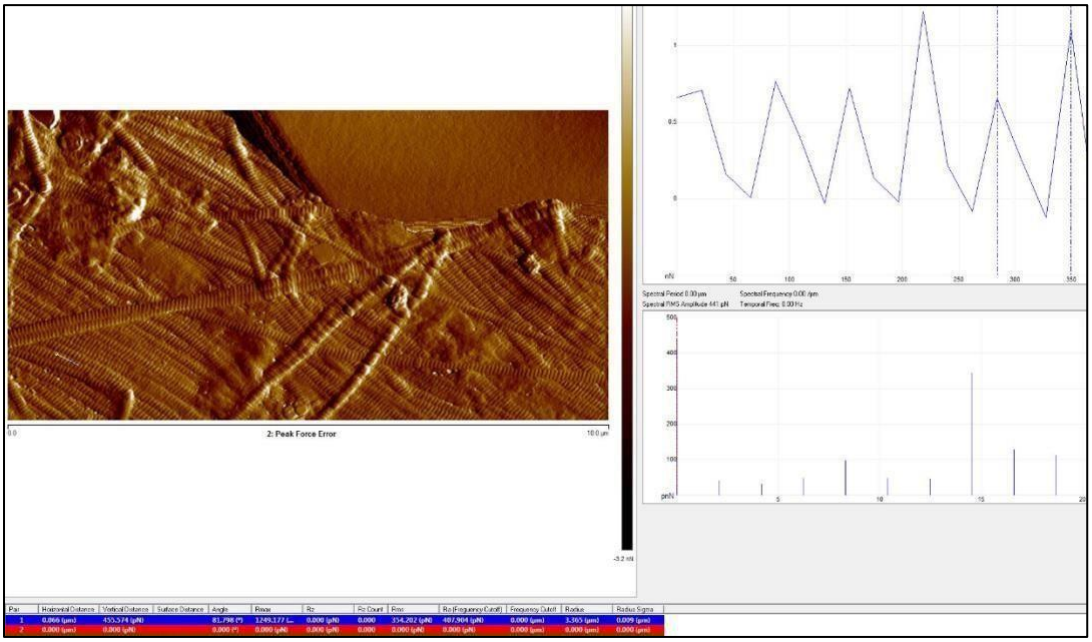
Anova 11

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	2	140.35	70.175	34.19645
Row 2	2	122.36	61.18	8.82

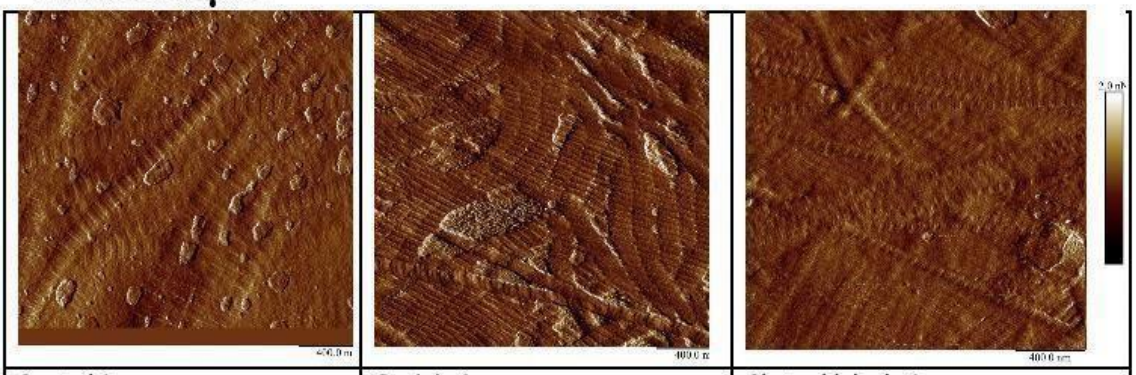
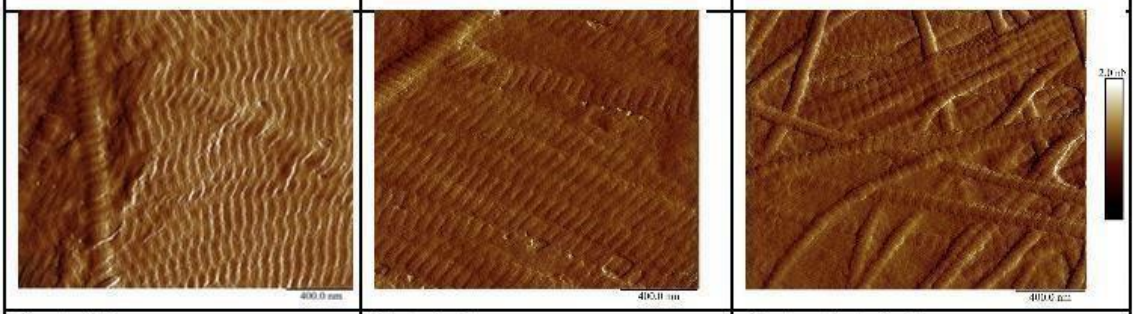
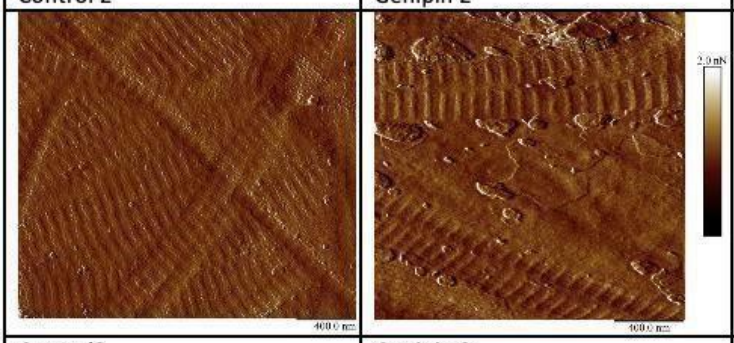
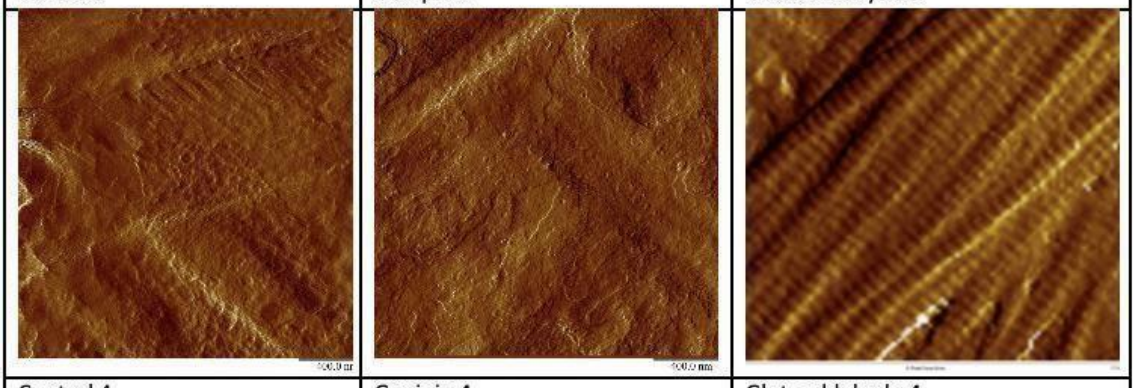
ANOVA 12

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	80.910025	1	80.910025	3.76181786	0.19198557	18.5128205
Within Groups	43.01645	2	21.508225			
Total	123.926475	3				

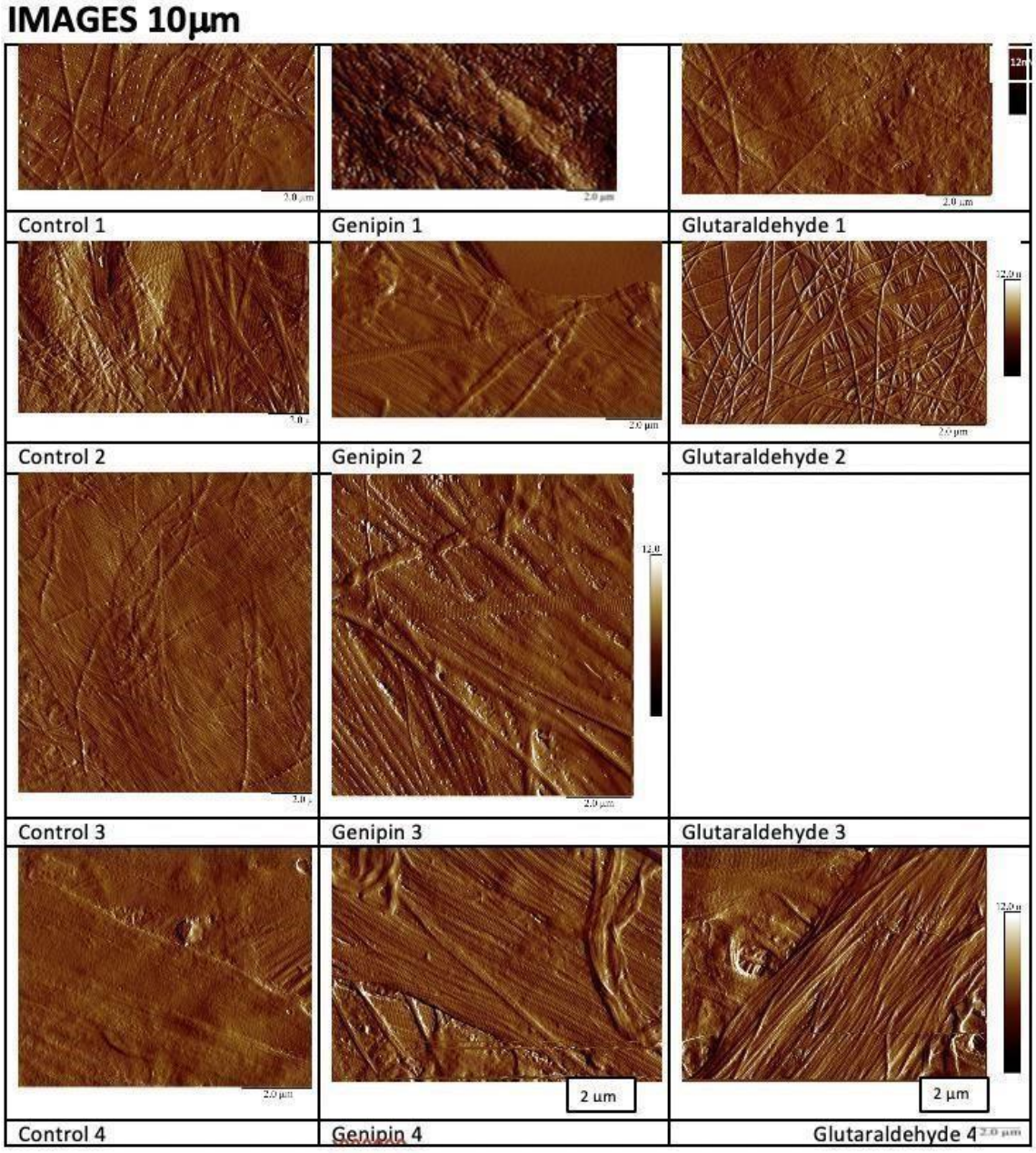


9. Figure showing individual fibril measurement using the AFM Nano-imaging software to obtain horizontal distance of D-period

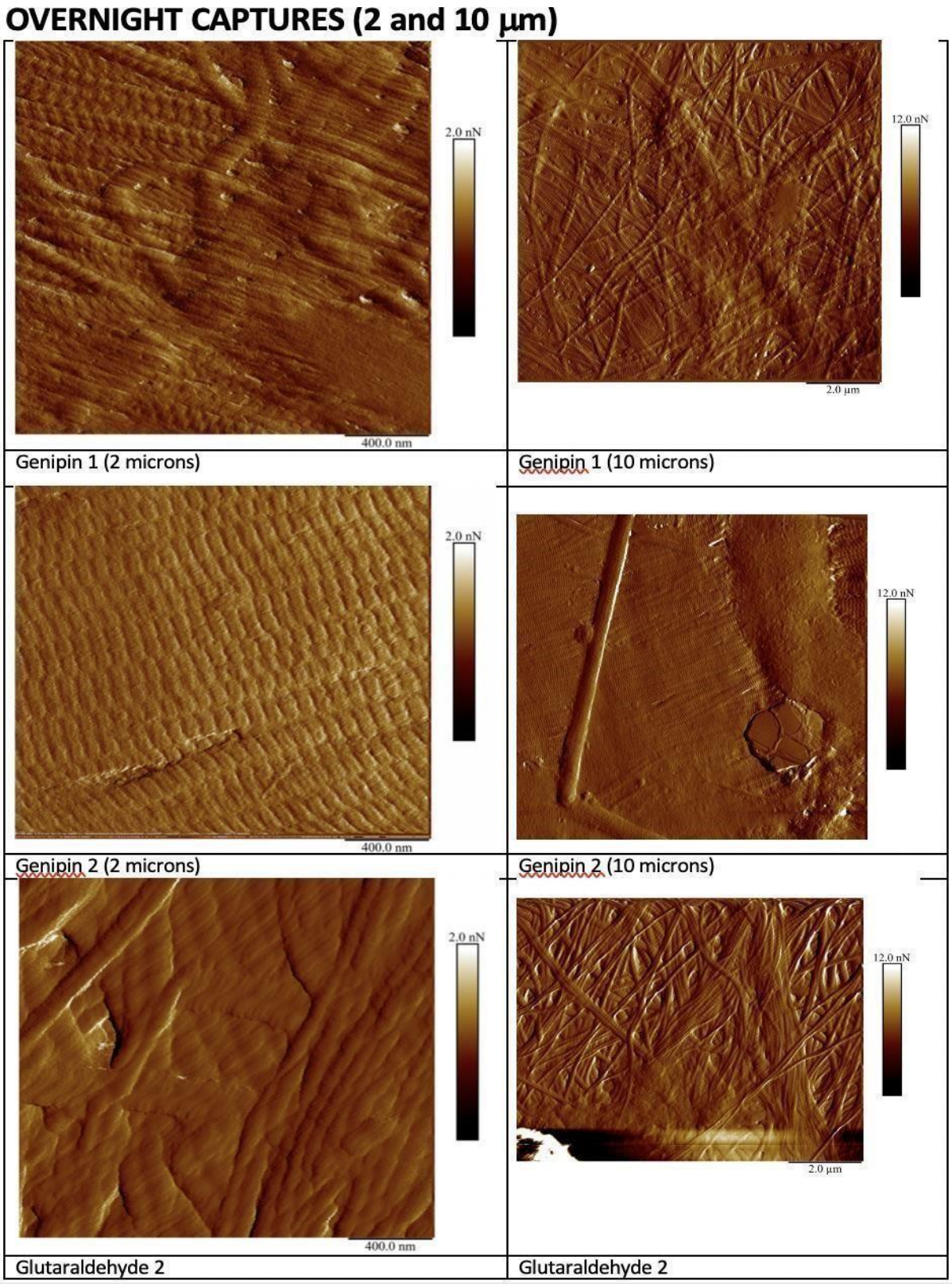
IMAGES 2µm

		
Control 1	Genipin 1	Glutaraldehyde 1
		
Control 2	Genipin 2	Glutaraldehyde 2
		
Control3	Genipin 3	Glutaraldehyde 3
		
Control 4	Genipin 4	Glutaraldehyde 4

10. Figures showing 2 microns representative AFM images



11. Figures showing 10 microns representative AFM images



12. Figures showing 2 & 10 microns representative AFM images after overnight

incubation with both cross-linkers

13. Figure showing genipin treated strip discoloured after 12 hours of incubation

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