



Keratoconus in Down's syndrome

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For James, Gareth, Alex, Sian, Hollie,
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Abstract

Keratoconus is a primary cause of visual impairment in young people in the UK. Corneal cross-linking is a recently-introduced treatment for halting progression of keratoconus, which is more effective in early cases. It has long been observed that keratoconus is significantly more prevalent in those with Down's syndrome (DS) when compared to the general population. Moreover, young people with Down's syndrome are less able to report early symptoms of keratoconus, often presenting late to eye clinics when cross-linking is no longer possible.

A cohort of children and young people with DS were examined with the aim of discovering optometric correlates of keratoconus and to establish the utility of these parameters as risk factors for identifying keratoconus in primary care. An abnormal retinoscopy reflex was found to be the earliest indicator of keratoconus, showing greater potential as a screening test than either refractive error or objective vision measurement.

The cornea of individuals with DS is known to be thinner and steeper than usual. Despite this, the high prevalence of keratoconus in DS has long been attributed to eye-rubbing, despite the inherent difference in baseline shape. The current work revealed no relationship between eye rubbing and the development of keratoconus in DS eyes. *In vivo* biomechanical analysis demonstrated an increased deformation tendency in DS eyes vs. controls, largely accounted for by the decreased corneal thickness in the test group. These results suggest that the high prevalence of keratoconus in DS originates from biomechanical weakness, permitting the loss of regular corneal shape in the absence of eye rubbing. However, ultrastructural analysis of the cornea of the Tc1 mouse model of DS revealed an unaltered collagen and proteoglycan structure.

Topographical examination of 'cone' morphology in individuals with and without DS demonstrated a similar phenotype at all stages of the disorder, indicating that people with DS and keratoconus may be a useful cohort for future genetic studies into keratoconus as a whole.

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Chapter 1

Introduction to thesis

1.1 Keratoconus

Keratoconus is a complex disease process affecting the cornea, the transparent shell of the anterior ocular surface. Typically, the cornea thins paracentrally, causing subsequent steepening and protrusion of the surfaces, towards a cone-like shape. The corneal distortion inevitably causes a significant decrease in vision, impacting young people ‘during their prime years’ (Kymes et al. 2004). While the prevalence of keratoconus (KC) in the typical population is 0.05% (Rabinowitz, 1998), it appears to be much more common in Down’s syndrome: 5.5% (Cullen & Butler, 1963). The majority of the corneal thickness is composed of a collagenous stromal layer, considered to bear the most significant change in keratoconus.

1.2 Collagen

The cornea is a remarkable part of the human body. While many tissues comprise a structural scaffold of collagen, matrix and cells, it is the unique transparency of the healthy cornea that sets it apart, provided for by the intricate organisation of collagen at the ultrastructural level (Figure 1. 1).

Other key functions of the cornea are:

- Refraction of light to contribute to retinal focus
- Biomechanical strength and protection from physical trauma
- Protection from chemical and biological trauma

Each one of the crucial functions are compromised in the ectatic disease keratoconus. This chapter provides insight into the physiology of the normal cornea, and the aetiologies and impact of keratoconus on the eye and on vision.

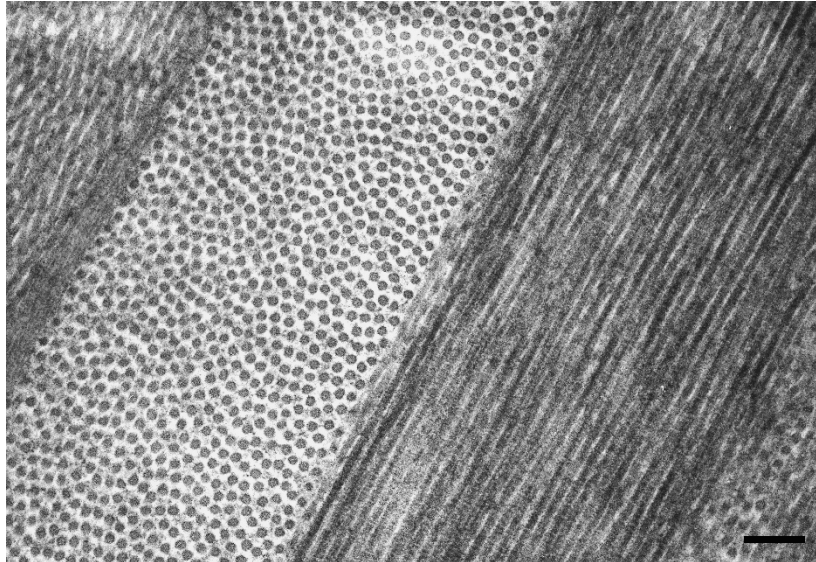


Figure 1. 1 Arrangement of collagen fibrils seen by transmission electron microscopy in the corneal stroma, both in transverse and longitudinal section. Note the highly ordered arrangement of collagen fibrils. Image courtesy of Dr RD Young.

1.2.1 Collagen Fibrils

A single collagen molecule comprises 3 polypeptide chains entwined as a triple helix. It measures just under 2nm in diameter and is around 200 times as long (Shoulders and Raines 2009). Electrostatic forces attract five collagen molecules, such that they align in parallel in order to form a microfibril (Figure 1. 2).

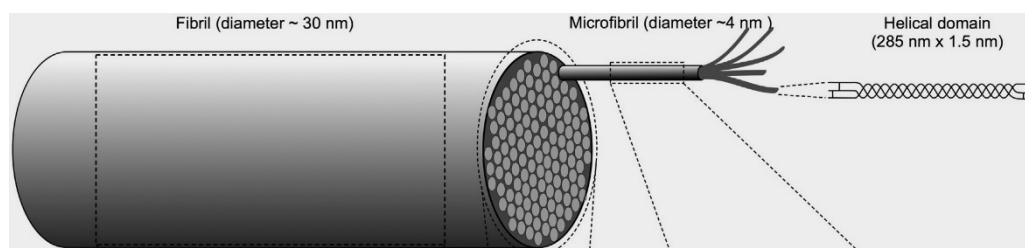


Figure 1. 2 Schematic representation of a collagen fibril (left), a microfibril (middle) and collagen molecule (right). This image denotes the relative hierarchy of structures, specific to corneal and scleral collagen, with the five collagen molecules coiling to form the microfibril, which in turn coils to comprise a full collagen fibril. Adapted from (Meek and Knupp 2015).

Electrostatically, maximal attraction occurs when the molecule ends are staggered. Because the length of the molecule is not integrally related to this stagger, there is a gap between one molecule and the one in front (marked by an asterisk in Figure 1.3) which leads to a regular periodicity along the fibril axis (Figure 1.3A).

In order to view this structure using the electron microscope, stains are used to 'fill in' the gaps between the molecule ends, and a striated pattern arises through negative staining of the tissue. The recurring sequence is seen as 'banding', whereby fibrils appear striped (Figure 1.3B), and the period of such banding is specific to fibrillar collagen. In striated fibrillar corneal collagen, this so-called D-periodicity is 65nm as measured by Meek and Boote (2004).

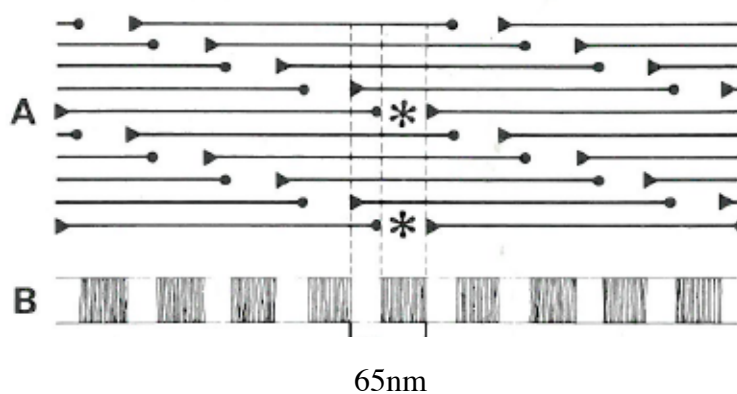


Figure 1.3 Organisation of collagen molecules (A). The electrostatic 'gap' formed between molecule ends is denoted by *. When a stain is introduced to image fibrillar collagen, this results in a 'striped' banding pattern observed with electron microscopy, demonstrated in (B). Adapted from Ghadially (1997)

1.2.2 Collagen genesis

As for any protein, each of 26 known vertebrate collagen types are coded for by a specific gene, and this genetic information is generally held in the nucleus of the fibroblast that produces it. Post-transcription, fibrillar collagen is initially produced as procollagen molecules. Procollagen itself consists of repeating amino acid sequences that form a polypeptide α chain. The terminals of procollagen are N- and C- type propeptides, and upon exit from the fibroblast into the extracellular environment, these propeptides are cleaved by specific procollagen proteases (Njieha et al. 1982). This cleaving allows the natural collagen structure to be released and subsequently 3 collagen molecules wrap around each other to form the triple-helix collagen fibril arrangement as described by Hulmes (2002).

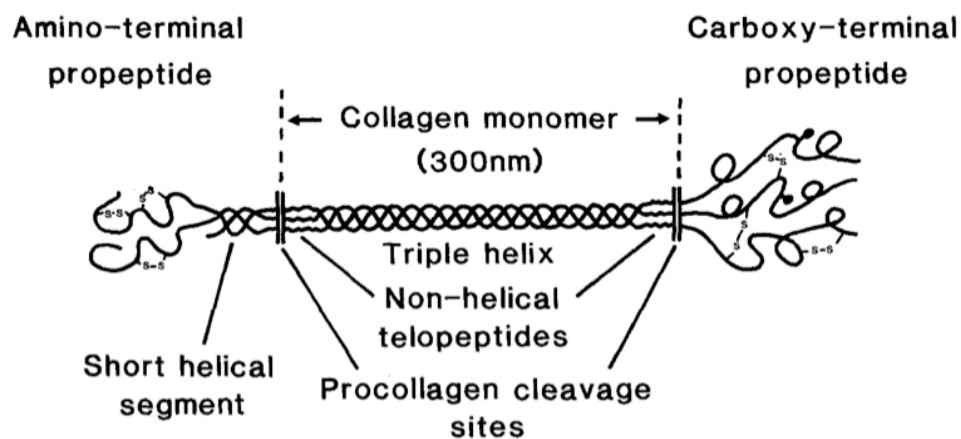


Figure 1. 4 Diagram to show an assembled collagen fibril. Adapted from Robins (1988).

While fibril-forming collagen discussed above forms the bulk of corneal collagen (providing structure and shape), other non-fibril forming collagens facilitate attachment of collagen structures to each other, to neighbouring cells, to basement membranes, or the ECM.

Further still, some collagens produce short chains, and others microfibrils. Such collagen becomes part of a scaffold that may be structural or aid cellular communication. The site of ocular corneal collagens and their genetic locus are set out in table 1.1.

1.2.3 Ocular Collagen types

Table 1.10 Corneal collagen types, their site and their genetic reference.

| Collagen type | Corneal site | | | | | Gene | Human Chromosome | Source |
|---------------|--------------|---|---|---|----|----------------|------------------|--|
| | Ep | B | S | D | En | | | |
| I | | X | X | | | COL1A1, | 17q21.31-q22 | (Bryan Sykes 1990) |
| | | | | | | COL1A2 | 7q21.3-q22 | (C Junien 1982) |
| III | | | X | | | COL3A1 | 2q24.3-q31 | (Solomon et al. 1985) |
| IV | X | X | | | | COL4A1, COL4A2 | 13q33-q34 | (Griffin et al. 1987; Boyd et al. 1986) |
| | | | | | | COL4A3, COL4A4 | 2q35-q37 | (Momota et al. 1998) |
| | | | | | | COL4A5, COL4A6 | Xq22 | (Tryggvason et al. 1993; Zhou et al. 1994) |
| V | | | X | | | COL5A1 | 9q34.3 | (Caridi et al. 1992) |
| | | | | | | COL5A2 | 2q14-q32 | (Huerre-Jeanpierre et al. 1986) |
| | | | | | | COL5A3 | 19p13.2 | (Imamura 2000) |
| VI | | | X | | | COL6A1, COL6A2 | 21q22.3 | (Weil D 1988) |
| | | | | | | COL6A3 | 2q37 | (Weil D 1988) |
| VII | | X | | | | COL7A1 | 3p21 | (Parente et al. 1991) |
| VIII | | | | X | X | COL8A1 | 3q11.1-q13.2 | (Tamura et al. 1991) |
| | | | | | | COL8A2 | 1p34.2-p32.3 | |

Collagen I

The most abundant collagen in the cornea is collagen I, its fibril forming nature providing the biomechanical strength and scaffolding nature of the corneal stroma (Marshall et al 1993). In the cornea, this collagen does not function alone, and Birk et al (1998) demonstrate that type I and V coexist and copolymerise along the same fibril, and this is known as a heterotypic fibril. Such heterotypic fibrils possibly exist as a mechanism to regulate fibril diameter and maintain corneal transparency – because type V is not found alongside type I so readily in other non-transparent tissues such as bone or tendon.

Collagen III

Collagen III is found in the anterior stroma and Bowman's membrane, and is considered prevalent when the cornea is in a growth or a healing state, in both rabbit and human cornea (Cintron et al. 1988).

Collagen IV

Type IV collagen is non-fibril forming, but instead exists in strands that are associated with such striated fibril-forming collagen. Type IV, alongside type VII collagen is thought to stabilise the architecture of fibrillar collagens (including type I) through their formation of anchoring plaques. Collagen IV is found in Bowman's layer, and in Descemet's membrane – both basement membranes of the cornea (Marshall et al 1993).

Collagen V

As previously discussed, type V collagen co-exists with type I collagen (Birk et al. 1988). It is considered that collagen type V is partly responsible for the maintenance of small and regular fibril diameter of type I collagen (Linsenmayer 1993). Impaired collagen V, as found in certain variants of Ehlers-Danlos syndrome (EDS), results in abnormal fibrillogenesis and clinical signs of skin fragility and joint hypermobility.

A comparison of normal and EDS collagen fibril organisation is seen in Figure 1.5 and 1.6. This results from mutations in either the COL5A1 or COL5A2 gene (Richards et al. 1998; Schwarze et al. 2000).

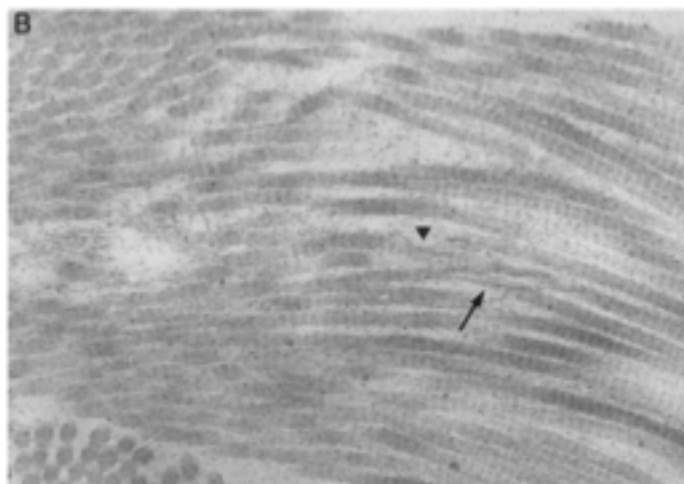


Figure 1. 5 Granulo-filamentous material (arrowhead) seen in longitudinal view in the skin of a patient with EDS, and collagen fibrils that may have unraveled and become disorganised (arrow). Adapted from (Nuytinck et al. 2000).

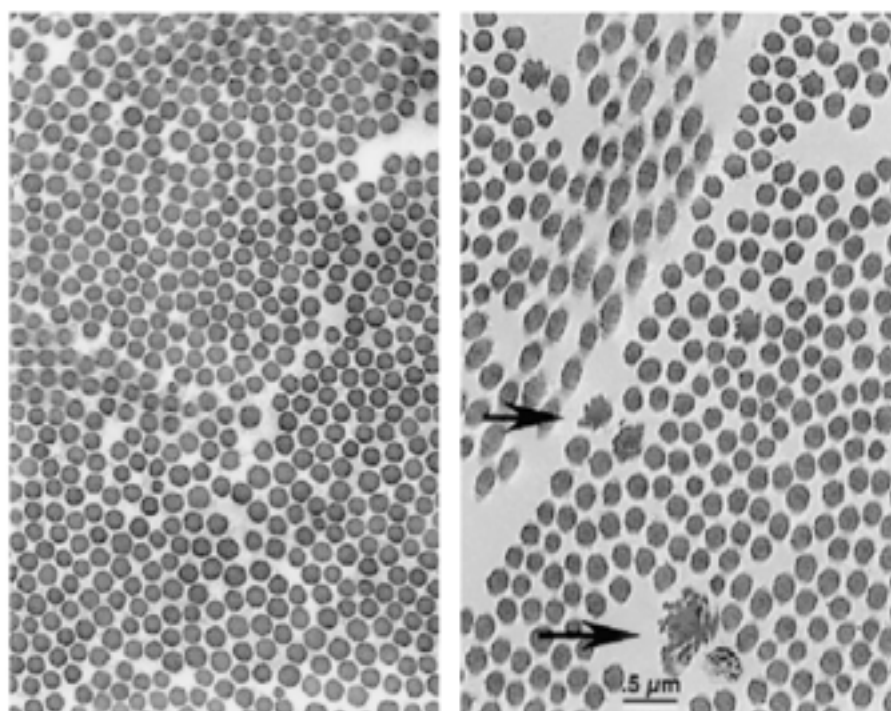


Figure 1. 6 Electron EM photograph to show fibrillar organisation in normal skin (left), and that from a patient with EDS (right). Unusual 'composite' fibrils are seen in abnormal tissue (arrows) Schwarze et al. (2000).

Collagen VI

The $\alpha 1$ and $\alpha 2$ chains of collagen VI are produced along chromosome 21 in humans (Lampe 2005), the chromosome triplicated in Down's syndrome. Collagen VI is a non-fibrillar collagen that has long, flexible strands that carry periodic beading (Bruns 1984), such as that seen in Figure 1.7. The extent of any change to collagen VI in the DS cornea is unknown, but the collagen VI anomalies present in DS skin will be discussed later in this chapter. Little is known about collagen VI in KC, but in a Western blot study by Chwa et al (1996) found that collagen VI does not stain well in ectatic corneae, and as such may be in some way degraded or the genetic expression or secretion of altered in KC.

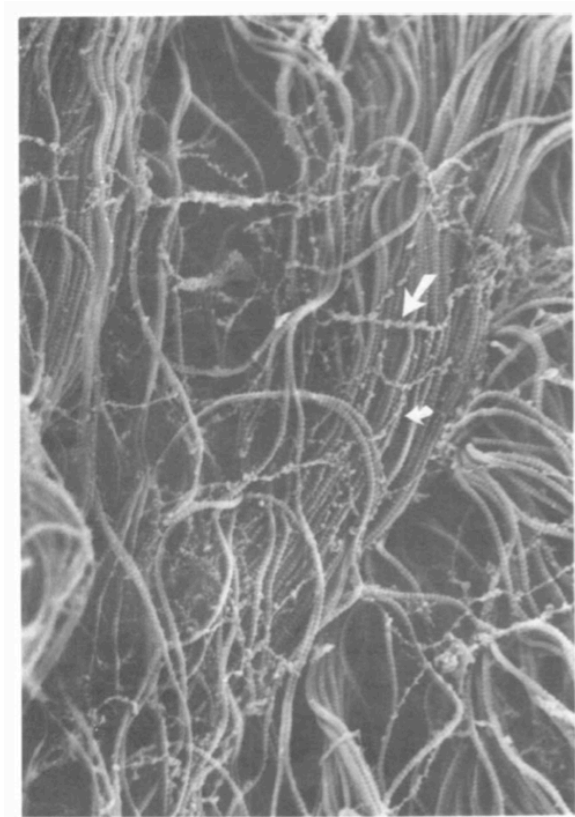


Figure 1. 7 The beaded collagen VI network intertwined among striated collagen fibrils (Burgeson 1988).

Collagen VII

Collagen VII thought to be important as anchoring fibrils in basement membranes. Microscopically, they are found arched or contorted, indicating their flexibility (Keene 1987). The production of collagen VII is thought to be controlled, to some degree, by innervation of the trigeminal ganglion (Baker et al. 1993).

Collagen VIII

Collagen VIII is a non-fibrillar short chain collagen, produced by endothelial cells, keratinocytes, and mast cells. Collagen VIII is prominent in Descemet's membrane, and is sometimes found in association with elastic fibres or microfibrils. It may have a role maintenance of corneal thickness and structural stability (Puk et al. 2009), and a role in permitting the passage of fluids (Jones 2013). The presence of collagen VIII is thought to stabilise the neighbouring endothelial cell phenotype, and thus have a facilitating role in angiogenesis - both in developmental tissue and in wound healing (Shuttleworth 1997).

1.3 Proteoglycans

Proteoglycans (PGs) are small molecules that act as spacers between collagen fibrils in the corneal stroma. They have a protein core and a glycosaminoglycan (GAG) or oligosaccharide side chain, or ‘tail’ (Michelacci 2003). Corneal proteoglycans have been long since considered important in different key properties, including transparency (Chakravarti et al 1998), hydration (Almubrad et al. 2010), and the genesis of collagen itself (Rada et al. 1993). Both the normal ageing process, and abnormal corneal scar tissue are associated with altered levels of proteoglycans (Malik et al. 1992).

In PGs, since the GAG side chain is so large compared to the core protein, the GAG property dominates the PG, and this is reflected in the grouping of PGs according to their associated GAG chain. The two main types found in the cornea are:

- **Keratan sulphate (KS)** – corneal KS is thought to impact primarily upon collagen fibril structure (Chakravarti 1998)
- **Chondroitin sulphate/dermatan sulphate (CS/DS)** – as well as involvement with fibrillary structure, corneal CS/DS is thought to impact upon cell signalling and the inflammatory cascade (Kawashima et al. 2002; Sugahara et al. 2003)

KS PGs have core proteins: keratocan, lumican, or mimecan.

CS/DS PGs have the core proteins: decorin or biglycan.

GAGs produce such significant hydration because they are highly negatively charged and attract an abundance of water molecules with respect to their own weight. Specific GAG content and thus controlled hydrostatic pressure allows for the precise ‘spacing’ property of corneal GAGs, regulating collagen fibril organisation and permitting the maintenance of collagen transparency. The degree

of spacing is controlled by both the availability of free GAG terminals to bind to water and the PG core protein chemistry. PGs, as a whole therefore, can be considered to modulate collagen fibril formation.

Sulphation of GAGs is the addition of a sulphonic acid to the polysaccharide GAG chain. In the cornea, the sulphation of GAGs varies depending upon GAG type, tissue type, and species. The degree of sulphation of the GAG is considered to impact upon corneal hydration, interfibrillar spacing, fibril organisation and thus corneal transparency, and is altered in particular disease states.

Hyaluronan is a particular GAG of note, because it is not found attached to a core protein, and yet is found in abundance in the body, being more prevalent in those tissues requiring hydration and load distribution. Produced by most human cells, hyaluronan acts as the body's natural lubricant, and found in abundance in the synovial fluid surrounding joints (Fraser and Laurent 1997). Hyaluronan was first isolated from purified bovine vitreous humour, and named after hyaloid, meaning vitreous (Hascall and Esko 2009). Hyaluronan is found in the cornea, considered important in the proliferation of fibroblasts and in modulating the healing response and the resultant collagen produced, in order to minimise scar tissue (Price et al. 2007). The metabolism of hyaluronan may differ in DS (Raio et al. 2005; Karousou et al. 2013; Bohlandt 2000).

1.3.1 Keratan sulphate proteoglycan

Keratan sulphate (KS) is of particular interest in the study of KC because of its abundance in the normal cornea and its correlation with fibril organisation and corneal transparency (Borcherding et al. 1975). Corneal KS is altered in various disease states, an effect of fibroblastic repair response during wound healing that causes a down-regulation in the production of normal KS (Funderburgh 2000).

The study of KS and component-specific antibodies show that, while the core protein of KS remains unchanged in KC corneae, the formation of KS GAG is altered in some way in the keratoconus cornea (Funderburgh et al. 1989). KS antigen staining was reduced by 48% in KC corneae from control tissue. The authors suggest that reduced staining of KS PG could occur for several reasons:

- I. Decreased KS sulphation
- II. Shorter KS chains
- III. Decrease in the number of KS chains per molecule

KS core protein (keratocan) mRNA is overexpressed in the corneal stroma of those corneae of eyes with keratoconus, as measured by immunohistochemical staining and PCR (Wentz-Hunter et al. 2001). Results from the same study indicate that there was actually less KS in the severe keratoconus with significant scarring, results that resonate with others (Sawaguchi et al. 1998; Wollensak and Buddecke 1990). Meek et al (2003) examined a keratocan-null mouse model, calculating both larger fibril diameters alongside reduced regularity of interfibrillar spacing from small angle X-ray scatter data. The authors hypothesise that keratocan-null mice exhibit less KS and thus reduced governance of fibrillar diameter and organisation.

Lumican, another KS core protein, was first discovered as a product of corneal keratocytes, and more recently found to be expressed by the corneal epithelium during wound healing (Saika 2000). Lumican is found in abundance in both the developing chick eye and heart valves (Chakravarti et al 1998). In lumican knock-out (null) mice studies, the absence of lumican results in a 25% loss in the total KS present in the cornea, and the mice have bilateral corneal opacities and skin laxity and fragility (Chakravarti et al 1998). A further study by the same authors indicated that the posterior cornea appeared particularly susceptible to the adverse effects of the lumican-knockout gene, corresponding to collagen fibrils of a more variable diameter, creating greater light scatter and a reduction in transparency as seen in figure 1.8 (Chakravarti et al. 2000). X-ray diffraction patterns obtained from the lumican-null mice reveal that the spatial arrangement of their stromal collagen is in ‘disarray’, with diffractive evidence that a much greater range of collagen fibril diameters existed (Quantock et al. 2001).

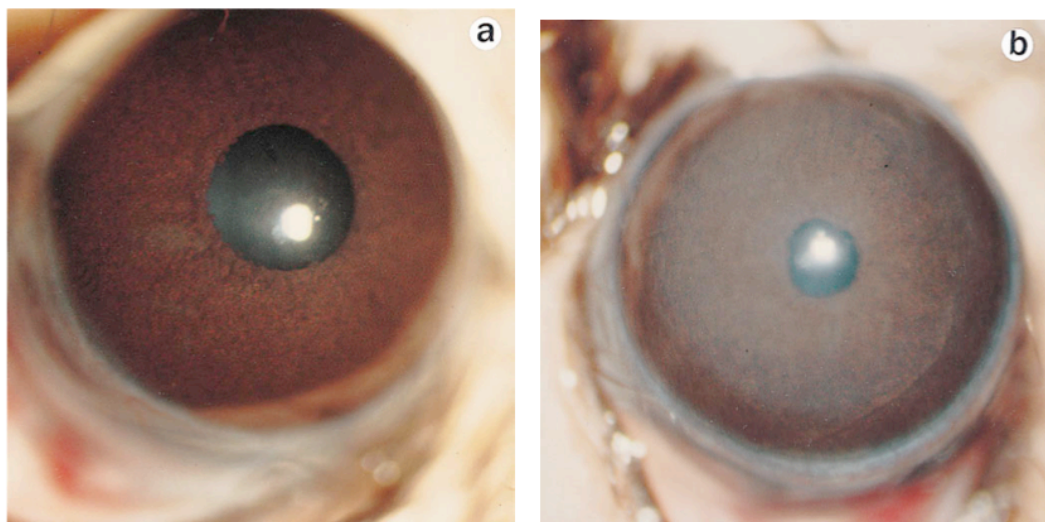


Figure 1.8 Wild type mouse, (a), and lumican null mouse, tm1Sc, (b). Chakravarti (1998) Note the ring of clear cornea found in the far periphery of the cornea in (b).

1.3.2 Chondroitin sulphate / Dermatan sulphate proteoglycan

Decorin and Biglycan are two core proteins of CS/DS and are implicated in corneal transparency and tissue strength, but also in the regulation of inflammation and cell signalling (Tiedemann 2005). Both CS and DS are thought to have an important role in forming or organising collagen, particularly during embryological development (Zhang et al. 2009).

In humans, a mutation in the Decorin gene resulted in autosomal dominant expression of an inherited corneal opacity in a family studied by Bredrup et al. (2005). Unlike humans, Decorin-null mice displayed normal corneae but weakened connective tissue (skin and tendon) (Danielson 1997). Ultrastructural analyses of the mouse tissue revealed coarser fibrils that were irregular in size and shape. The mice exhibited less collagen-bound proteoglycans, considered to be the cause of the biomechanical weakening. While the relationship between transparency and biomechanical strength is uncertain, a connective tissue weakness in PG abnormality lends weight to the lamellar ‘slippage’ theory suggested by Meek et al. (2005).

Decorin and biglycan are found in the normal basement corneal epithelium, Bowman’s layer, and the corneal stroma. Both core proteins and their PG counterparts are upregulated in diseased cornea and dermatan sulphate side chain exhibits abnormally increased sulphation (Funderburgh et al. 1998; Sawaguchi et al. 1998). Funderburgh suggested that a fibrotic ECM produces the abnormal PG and core protein formation, in work later supported by Tiedemann (2005). Such a suggestion is consistent with the now well documented inflammatory cascade and aberrant healing response that the keratoconic cornea are thought to undergo (McMonnies 2015).

Sawaguchi et al (1991) found an accumulation of abnormally thick CS/DS PGs in keratoconic corneal tissue when compared to that from a healthy cornea (figure 1.9).

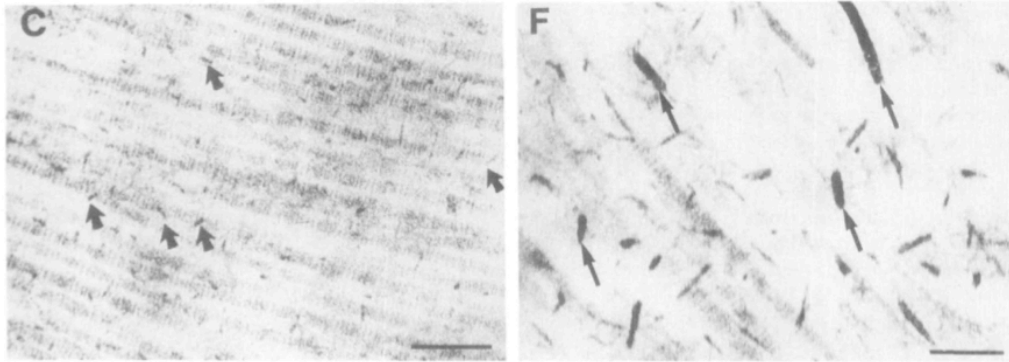


Figure 1.9 Control cornea (C) and keratoconus cornea (F) and staining with cuproinic blue following pretreatment with chondroitinase ABC. The small arrows in (C) point to medium-sized filaments expected in healthy tissue and the large arrows in (F) point to the abnormally thick filaments in the KC cornea. (Sawaguchi et al. 1991).

It is not yet known if the change in composition of CS/DS in some keratoconic corneae could be an underlying cause of biomechanical instability, or simply an effect of the inflammatory nature of the disease.

1.4 Corneal lamellae

It is thought that a variation in fibril organisation and fibril thickness throughout the corneal structure is important to the biomechanical integrity in the human cornea (Boote et al. 2003). Fibrils are arranged into flattened bundles, and these *lamellae* run parallel to the face of the cornea, providing 90% of its thickness (Thomas 1955). Whilst lamellae occur in many orientations in order to cover the whole cornea, there appear to be two preferred directions across the cornea: one running superiorly to inferiorly, and the other naso-temporally (Meek et al. 1987; Aghamohammadzadeh et al. 2004). This orthogonal arrangement is thought to provide tensile strength and anchorage to the cornea as the pulling force of the extra-ocular muscles attach locally to move the globe vertically and horizontally. This orthogonal nature is highlighted in figure 1.10, which also shows a construct of the peripheral collagen in a circumferential arrangement.

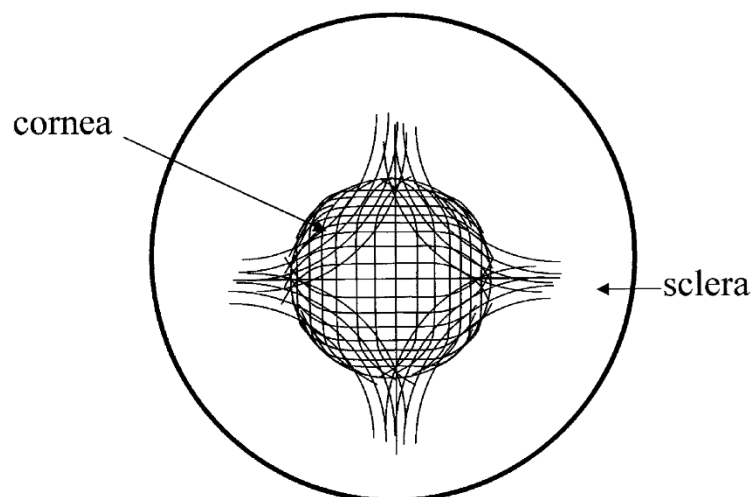


Figure 1.10 Anchoring lamellae that appear to have their origins outside of the peripheral cornea may enter the cornea at a given principle meridian, curve within the cornea and exit at a neighboring principle meridian (Aghamohammadzadeh et al. 2004).

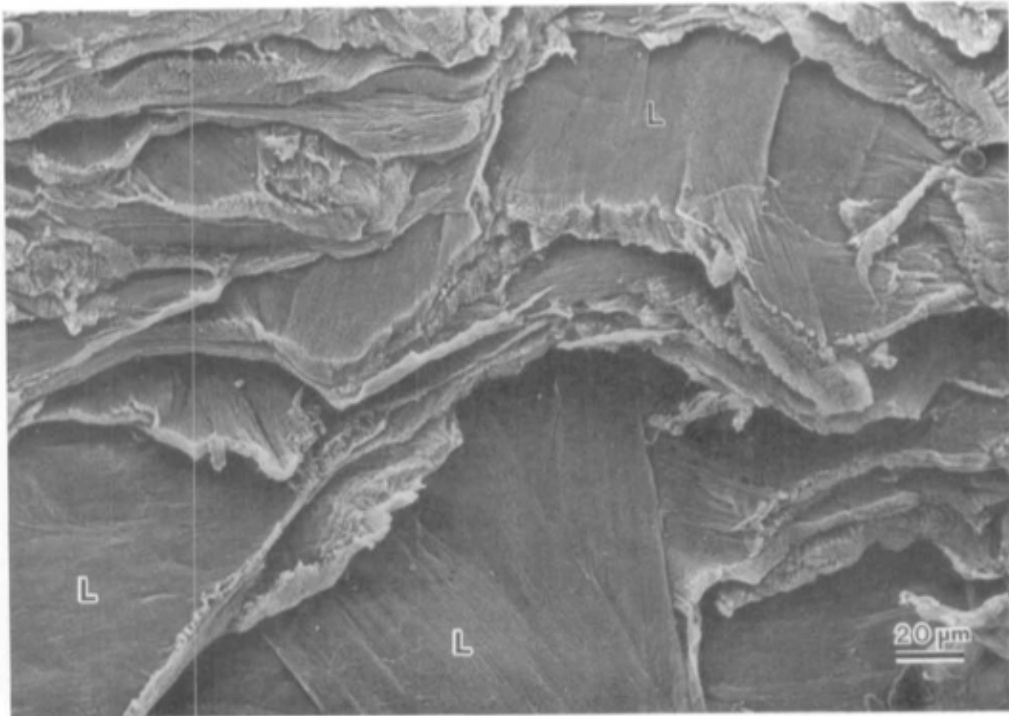


Figure 1. 11 Stacking of lamellae (L) in the deep corneal stroma as viewed with scanning electron microscopy (Komai & Ushiki 1991). While the corneal lamellae are predominantly orthogonal, it is apparent from images such as these that a great deal of angular overlapping is present.

1.5 Collagen in keratoconus

The knowledge gained into the changes in collagen of the keratoconic cornea is from those corneae deemed severe enough to require a corneal transplant, and for which the removed tissue has been gifted to research. The organisation of central collagen lamellae within the cone is profoundly altered from the normal orthogonal alignment into oblique directions (Meek et al. 2005). This demonstrates large-scale changes within the architecture of collagen bundles around the site of the ‘cone’, but in addition, the authors found that the arrangement of peripheral lamellae is also affected. ‘Anchoring’ lamellae that lie in a diamond pattern are thought to strap the cornea in place in the corneal periphery, and the formation of this typical diamond pattern is also disrupted in keratoconus. This finding was mirrored using second harmonic imaging of the peripheral lamellae (Morishige et al. 2007).

Redistribution of collagen in the periphery as well as the central cornea implies some sort of unravelling of normal corneal architecture that appears to be at odds with the ‘focal’ defect as postulated by Roberts & Dupps (2014). Since the cornea is composed of many collagen fibrils weaving together, it is thought that the ends of the fibrils are like ‘sutures’ and integral to holding the structure together. Another concept in the aetiology of keratoconus is that the bonding between neighbouring fibrils may act as a ‘interfibrillar glue’, and a generalised degradation of such bonds may allow fibril bundles to split and unravel, or slip across each other, and a redistribution of tissue would result, just like a small tear in fabric under tension in a garment (Meek et al. 2005). Since there is a great deal of regularity and interweaving in the normal anterior stroma, this hypothesis carries much weight in explaining the clinical anterior changes seen, but does not explain why the posterior cornea shows preferential damage in early disease, even with an intact anterior curvature (de Sanctis et al. 2013). It seems reasonable that in early to moderate keratoconus, collagen is redistributed rather than ‘lost’, and that this redistribution is perhaps governed by the changing/altered strain in the tissue. “Biomechanical coupling” causing flattening peripheral to the cone may compensate for the increased curvature of the cone in the central cornea. This is supported by clinical evidence, such that both corneal surface area and corneal volume in keratoconus are both conserved in keratoconic corneae (Smolek and Klyce 2000).

Vogt’s striae are a hallmark of keratoconus, were considered ‘folds’ of tissue seen in the stroma of patients with keratoconus. These are now considered to be lamellae under stress (Hollingsworth and Efron 2005), and are much more prevalent when viewed using the confocal microscope than compared to what is clinically appreciable using a clinical slit lamp biomicroscope (Mocan et al. 2008). It is thought that their presence is proportional to the direction and magnitude of the biomechanical tension that exists in the keratoconic eye, and that the striae radiate outwards from the cone apex (Hollingsworth et al. 2005).

Ordinarily, Bowman’s layer rigidity and resistance to swelling suggest that it may be integral to the maintenance of normal corneal curvature and architecture, and key

aspect of the refractive stability of the eye (Holtzman et al. 1996; Müller et al. 2001). Reporting of alterations in Bowman's layer in KC appear to conflict. From a biomechanical view, the behaviour of anterior stromal lamellae intersecting with and just posterior to Bowman's layer is of interest. Morishige and colleagues noted a decrease in the number of short lamellae intersecting into the layer, suggesting a degradation of lamellae at this level, and a loss of interlamellar weaving in the anterior stroma (Morishige et al. 2007). This complements the findings of Meek et al (2005) who suggest that the failure of regulation of insertion of mechanically important lamellae causes a redistribution and slippage of the fibrils. Horne et al. (2003) discovered electron-dense formations in the Bowman's layer where lamellae may terminate, hypothesising that a loss of these areas in KC may result in reduced adhesion and facilitate collagen slippage. More recently, this research group proposed that corneal thinning in KC results from splitting of the anterior lamellae at this region due to the stress induced by ocular forces Mathew et al. (2015).

In advanced keratoconus, breaks in the collagenous Descemet's membrane result in a large influx of water and an acute swelling of the cornea (Figure 1.12).

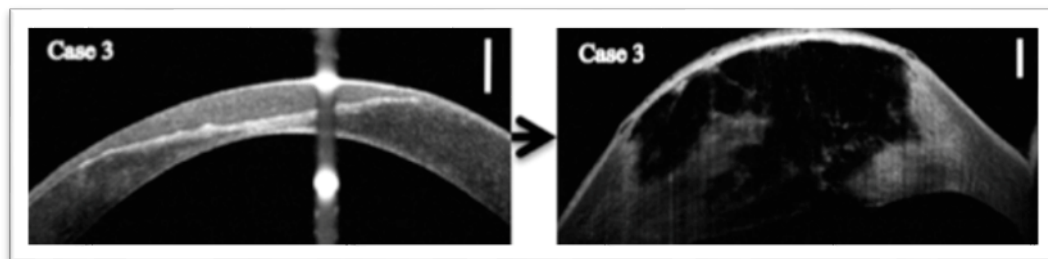


Figure 1. 12 A cornea before, and with, corneal hydrops, as viewed with optical coherence tomography (Fuentes et al. 2015).

There is evidence to suggest that the late finding of corneal hydrops is more common in Down's syndrome (DS). Six of 22 patients (27%) with hydrops in a general corneal referral population had DS (Grewal et al. 1999). In another study comprising 8 patients with DS who had keratoconus in at least one eye, 5 of the 16 eyes (31%) were blind due to acute keratoconus (hydrops) (Cullen 1963; Butler and Cullen 1963). This is significantly higher than the prevalence of hydrops in a general KC population at 2.8% (Tuft et al. 1994). Conversely, in a study of 147

hydrops cases in contact lens wearers, just two had DS. Since the learning disability inherent in DS makes successful contact lens fitting and wearing more difficult, it is likely that keratoconics with DS are under-represented in this particular contact lens sample. A minority of patients with corneal hydrops can identify a potential traumatic trigger such as an incidence of prolonged eye rubbing or a traumatic contact lens insertion. It is both the author's experience and denoted in the scientific literature that most patients are unable to identify a cause (Grewal 1999). While it is postulated that people with DS may induce hydrops by rubbing (Grewal et al 1999), it is entirely possible that the DS cornea is simply more prone to developing the latter stages of the disease, for biomechanical, metabolic or biochemical reasons.

1.6 Corneal Biomechanics

Corneal biomechanics is the study of the response to applied stress or strain on either side of the corneal tissue. Corneal biomechanics are intrinsically related to the content and distribution of collagen fibrils and their surrounding ground substance. Since the corneal stroma comprises the majority of corneal thickness, it is the most modelled aspect of the cornea. The influence of Descemet's membrane (posteriorly) and Bowman's layer (anteriorly) carry conflicting reports in scientific literature (Seiler et al. 1992; Jue and Maurice 1986; Weed et al. 2006; Hollman et al. 2002; Yoo et al. 2011; Danielsen 2004).

1.6.1 Elasticity

The cornea exhibits elastic behaviour after a force is applied, because it can stretch, bend, and return to its original shape. This allows for corneal flexibility when the eye is rubbed, and for corneal curvature to be regained upon removal of the force. Inflation testing and extensometry testing (Vellara and Patel 2015) are two primary methods of ex vivo biomechanical research. Young's modulus, a characterisation of the elasticity of a material, relates stress (force per unit cross sectional area) to the resultant strain (relative linear deformation) (Edmund 1988). Elasticity, that is, the ability to deform the cornea, was found to be greater in keratoconic strips than those from known healthy corneae.

The limbal and peripheral corneal structure and shape mitigates the shape change in the central cornea due to intra-ocular pressure (IOP) increase, but in general the cornea becomes stiffer as IOP is raised in an experimental setting (Boyce et al. 2008). IOP, therefore, is a confounding variable in the measurement of whole-eye corneal biomechanics, such as that measured with whole-eye inflation testing, or in vivo systems as discussed below. Another confounding variable in the study of corneal biomechanics is central corneal thickness (CCT), whereby a thicker cornea,

in general, results in an artificially higher applanation tonometer reading (Doughty and Zaman 2000), although when studied experimentally with an intercameral tonometer, this is shown not to be a systematic error and therefore cannot be directly accounted for by a simple regression clinically (Feltgen 2001). Such work demonstrates the complex interplay between IOP, corneal thickness and corneal biomechanics.

While not yet fully understood, Descemet's membrane appears to hold important elastic properties, holding the greatest elastic modulus of any part of the cornea measured by atomic force microscopy, despite being only 10 microns thick (Last et al. 2012). Maurice postulates that Descemet's membrane may preferentially take up corneal strain in rabbits, although the same is not seen in humans (Jue and Maurice 1986). Descemet's membrane increases in thickness with age in normal eyes (Thomasy et al. 2014). It is conceivable that this increase could go some way to explain the relative stabilisation of the keratoconic cornea often seen in the fourth decade of life as noted by Rabinowitz (1998).

1.6.2 Viscoelasticity

The relationship between the distension of the cornea and the force applied is non-linear, and therefore there are other attributes to the cornea's biomechanics besides elasticity (Kotecha 2007). Elasticity that occurs within the cornea is considered a function of its collagenous and proteoglycan structure. Viscosity is the flow of a material when an external shear force is applied, and represents the fluidity of the cellular, extracellular and extrafibrillar environment that inherently accompanies the collagenous base. Viscoelasticity is the combined effect of the elastic and viscous attributes, and provides the cornea with a strong structure that is able to both absorb some force and dissipate the energy, whilst returning to its original shape upon removal of that force. Hysteresis is a measure of the energy absorption by the material during the loading/unloading stress/strain cycle in a viscoelastic material (Ewing 1889) and is represented by Figure 1.13.

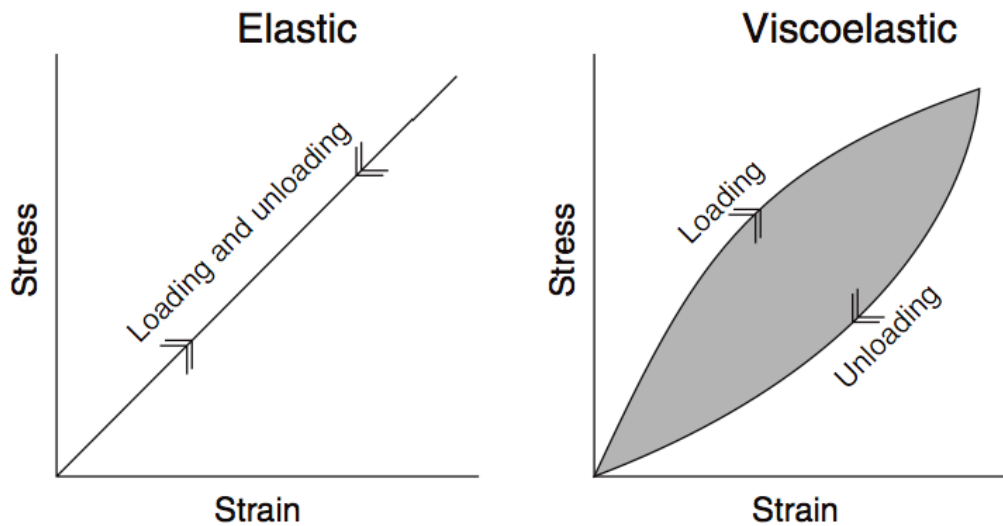


Figure 1.13 Stress-strain graphs of elastic materials (left) and viscoelastic materials (right). The stress-strain curve is noted to follow a different path in the viscoelastic material, demonstrating the property of hysteresis (Vellara and Patel 2015).

1.6.3 *In vivo corneal biomechanics*

The drive toward manufacturing an instrument to measure the ‘true’ IOP of the eye by way of accounting for corneal biomechanical properties, has provided a mechanism for corneal researchers to study corneal biomechanics in the ectatic cornea. Two commercially available systems are currently available.

The Ocular Response Analyzer (ORA) (Reichert Ophthalmic instruments, Depew, NY, USA) deploys a collimated air pulse to the ocular surface, whilst measuring a reflected infra-red signal throughout indentation of the central cornea, and its recovery. The metered air-puff is said to ‘shut off’ upon detection of the first appplanation. The cornea passes through two appplanations: The inward appplanation of the cornea (as it is flattening) and the outward appplanation (as it begins to regain its natural convex shape). Peaks of maximal signal are produced as the cornea passes through each appplanation event (figure 1.14), corresponding to the flattened surface acting as a mirror to reflect the incident beam. At every other point through, the beam is only partially reflected from the convex or concave surface and hence the signal on the receiver is reduced.

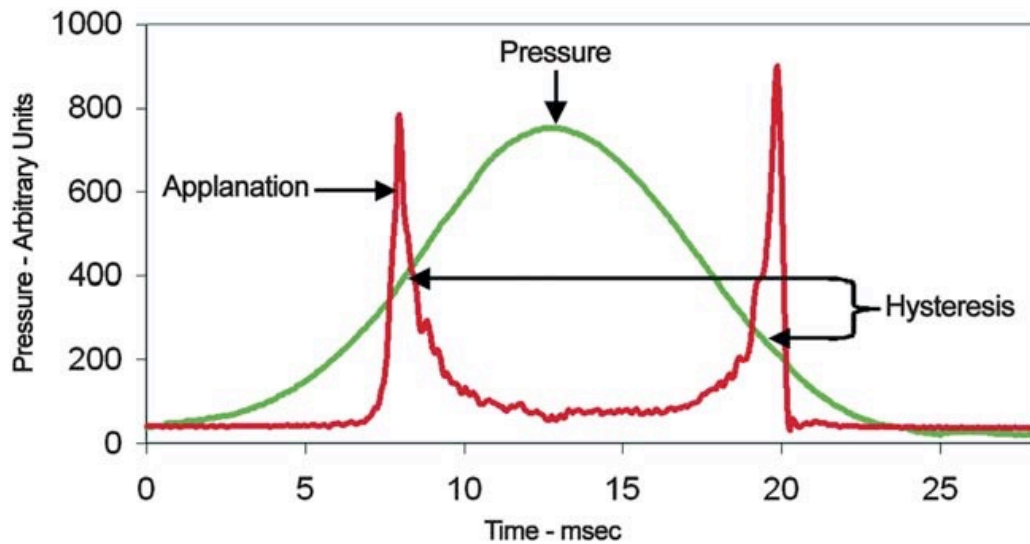


Figure 1. 14 Graph to illustrate a pressure gradient (green) and a two-peak applanation signal (red) from the Ocular Response Analyzer. Reproduced from (Luce 2005) with permission.

Two collimated IOP readings are supplied for each measurement, one on the inward and one on the outward applanation. Theoretically, in a solely elastic material (absorbing no energy), both applanation events would correspond to an equal applied pressure and produce a symmetric graph. The ORA assumes that difference in the two applanation pressure results produced by the cornea represent corneal hysteresis. Similarly, the ORA assumes that the cornea behaves elastically, and as such obeys the Imbert-Fick law stating that the inward pressure force is equal to the underlying IOP. This is demonstrated by figure 1.15.

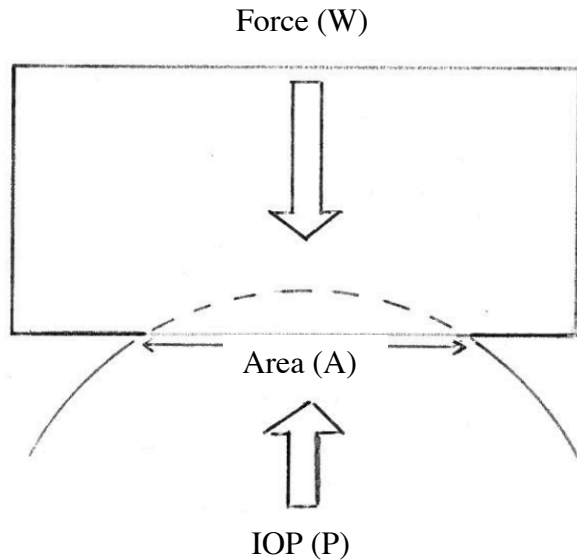


Figure 1. 15 $P=AW$, Representation of Imbert-Fick law, where $P = IOP$, $A =$ applanated area and W is the external force applied (Gloster and Perkins 1963).

The Imbert-Fick law assumes that the cornea is completely dry, infinitely thin, completely flexible and elastic (i.e. assumes no viscoelastic behaviour). Since the cornea is wet, has a thickness and an associated viscoelastic structure, the Imbert-Fick principle has been modified in this context to include the presence of a force of surface tension acting in the same direction as the applanating force, with a force representing corneal resistance acting against the applanation force. The latter two forces were assumed to nullify each other, when the applanated area was 3.06mm² (Goldmann 1956). Contact tonometry including such Goldmann tonometry may be considered a static form of tonometry, in that the forces acting upon the cornea are not changing with respect to time once the applanation has been made. Non-contact tonometry methods such as the ORA have a *dynamic* force, changing with respect to time, rather than the stable force acting in contact tonometry. Since the cornea is carrying a velocity and relative acceleration in one of two directions, an accelerating force is at play which is more difficult to characterise in biophysical terms. It may be for this reason that non-contact devices are calibrated to Goldman applanation tonometry measures in order to achieve clinically useful results. In the case of the ORA, the accelerating force will act in the same direction as the incident air-puff force in the inward applanation, whilst acting in the opposite direction to the air-puff during the outward measurement. This does not appear to be considered with

respect to hysteresis in the introductory material by the developers (Luce 2005), or, to the best of the authors knowledge, subsequently.

This ORA system makes several further assumptions:

- The cornea is sufficiently moist and smooth to reflect the incident light appropriately
- The corneal apex is located in the centre of the cornea
- There is negligible latency effect of the shutting-off of the air-puff after the first applanation
- There is a standard corneal curvature
- There is a standard applanation area

The primary limitation of the use of the ORA in corneal ectasia studies is that the output measures rely on the reflection from the corneal surface. Since the keratoconic eye has an increasing irregular ocular surface often compounded by tear film irregularities and dry eye, poor signals may be as a result of deflection of the beam from an irregular ocular surface rather than biomechanical reason. Further, since most often the apex of the cone is decentred from the geometrical centre, the alignment of the beam is likely to be governed by a measure of reflections that do not in fact represent the geometrical centre or indeed the apex. The resulting air pulse may therefore be delivered to an unknown location, and this may further compound the optical measures. The resulting signals and derived measures may therefore be a representation of the irregularity of the cornea rather than the biomechanical properties in the study of ectasia, and may be unduly influenced by dry eye.

The Oculus CorVis ST (Wetzlar, Germany, figure 1.16), delivers an air pulse to the cornea, providing two applanations as the ORA above. Unlike the ORA, the air pulse is standardised to deliver the same force to a cornea at each measurement. The CorVis utilises a Scheimpflug camera that takes 140 images during the deformation, tracing the form of the anterior and posterior corneal surfaces dynamically throughout the impact of the force and the recovery from it, as seen in Figure 1.17.



Figure 1.16 Oculus Corvis ST (Wetzlar, Germany. From Ambrósio et al. 2013)

Primarily, the CorVis measures the attributes of corneal deformation, which is an effect of corneal biomechanics. Such attributes are shown in Figure 1.19. It also measures standard IOP, using the timing of first applanation with respect to the metered air puff (as for any standard non-contact tonometer). The CorVis appears to demonstrate that keratoconic corneae deform more than healthy controls, even when matched for confounding variables such as IOP and corneal thickness (Figure 1.18). This corresponds to Edmund (1988) who found that ocular rigidity and steady-state Young's modulus of elasticity were both reduced in keratoconus. Both concepts are fitting with the clinical view of the cornea in KC having lost its structural resistance and resulting deformed appearance, however is not yet clear how the attributes of air pulse deformation correspond to ectatic deformation.

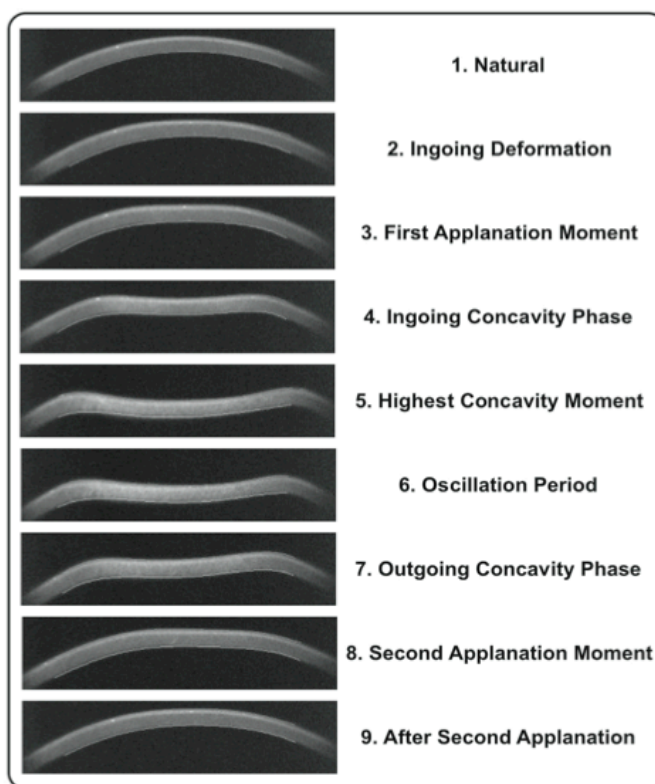


Figure 1. 17 The phases of corneal applanation with air-puff tonometry (Ambrósio et al. 2013). There are two applanations: one on the ingoing movement, and one on the outgoing movement. Note the oscillation period that occurs only after the cornea has reached maximum concavity.

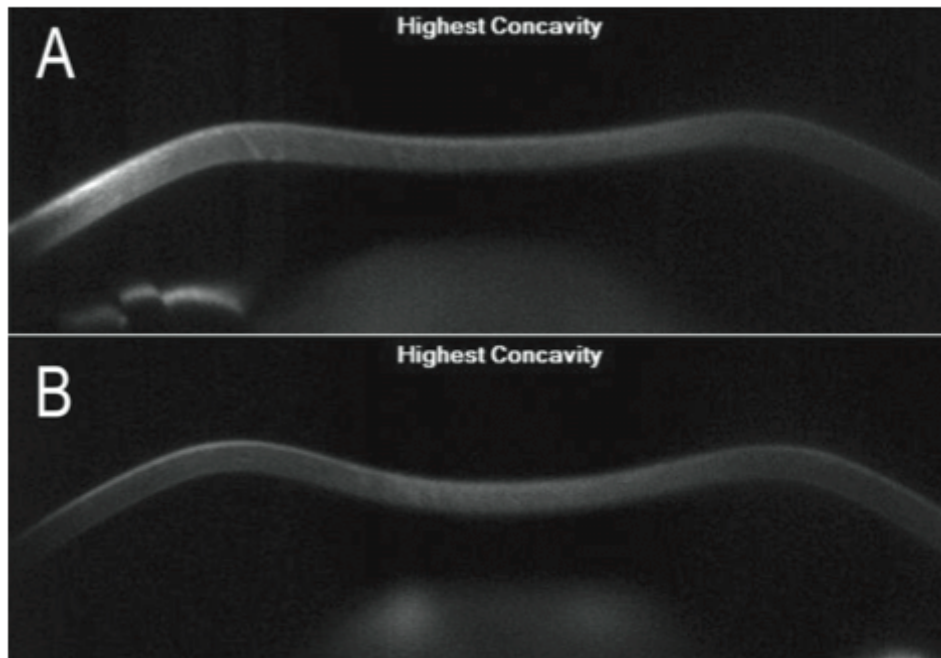


Figure 1.18 Deformation of a healthy cornea (A) and a keratoconic cornea (B) matched for IOP and CCT. Note the increased deformation and concavity seen in the keratoconic eye, whereas the healthy eye is relatively less affected by the air-puff (from Ambrósio et al. 2013).

The deformation parameters measured by the CorVis are confounded by the factors that intrinsically confound corneal biomechanical measurements in general. Asaoka et al (2015) found that the CorVis parameters (such as deformation amplitude, DA) were very strongly (negatively) correlated with IOP measurement (which is derived from the first applanation) and that IOP affected several other parameters including the time of, and velocity at, the first applanation ('A1T' and ' V_{IN} ', positively and negatively, respectively) and the radius of curvature at the highest deformation (positively).

The effect of corneal thickness was investigated by Huseynova et al (2014). An increased CCT increases the time of the first applanation, for a given IOP range. Increasing CCT correlates with increasing radius of curvature at the highest deformation.

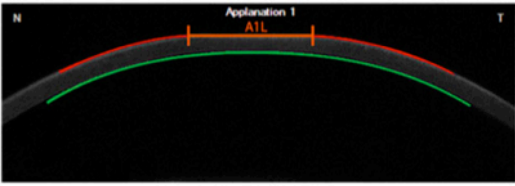
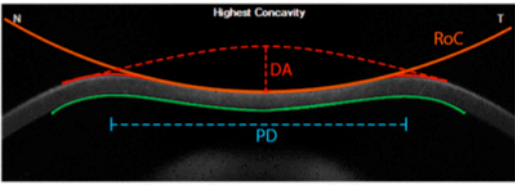
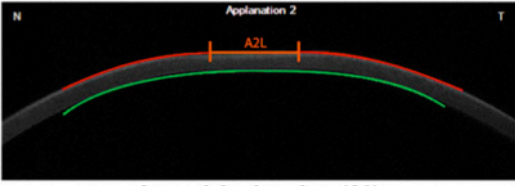
| | Parameter and Abbreviation | Description |
|---|---------------------------------|--|
|  <p>First Applanation (A1)</p> | First applanation time (A1T) | Time taken for cornea to reach first applanation |
| | First applanation length (A1L) | Total length of flattened portion at first applanation |
| | Velocity inwards (V_{IN}) | Velocity of inward motion at first applanation |
|  <p>Highest Concavity (HC)</p> | Deformation amplitude (DA) | Displacement from original position at highest concavity |
| | Radius of curvature (RoC) | Radius of curvature at highest concavity |
| | Peak Distance (PD) | Distance between the peaks at highest concavity |
|  <p>Second Applanation (A2)</p> | Second applanation time (A2T) | Time taken for cornea to reach second applanation |
| | Second applanation length (A2L) | Total length of flattened portion at second applanation |
| | Velocity outwards (V_{OUT}) | Velocity of outward motion at second applanation |

Figure 1. 19 Software-derived characteristics of the CorVis. From Vellara and Patel (2015)

Non-commercial systems for analysing corneal biomechanics are also available. Swept-source OCT provides dynamic capture of corneal deformation with an air-puff, and although in its infancy, is considered to be a promising mode of analysing the behaviour of a cornea under stress and may be useful to measure the response to treatment (Maczynska et al. 2016). Recent investigations of in-vivo biomechanical properties in KC examine *Brillouin shift*, an indirect measure of elastic modulus, indicating changes in biomechanical integrity across the sample, with particularly low modulus in the zone of the cone (Scarcelli et al. 2014). Since Brillouin microscopy can be carried out in vivo, and is undergoing much investigation at present, including the use of adaptive optics interferometry to measure the elastic background signal (Lepert et al. 2016). The possibility of measuring live biomechanical properties across the cornea may shed light on whether the presumed biomechanical weakness in keratoconus is indeed focal as suggested by Roberts et al (2014) or generalised across the whole cornea (Andreassen et al. 1980).

1.7 Topography

Keratoconus causes alterations in the corneal profile, both to the front and to the back surfaces. It is now possible to gather data from both corneal surfaces, but in the early history of corneal ophthalmology, reflections from the front surface of the cornea were used to detect irregular corneae, by observation of the asymmetry of the reflected concentric mires. The simplest of systems involves having the patient fixate at the centre of a series concentric rings, coincident with a viewing space (Placido 1880). Hofstetter used a similar technique (termed keratoscopy), to estimate a population prevalence of irregular cornea of 0.6%. (Hofstetter 1959). Videokeratoscopy, or topography, is simply a digitisation of this technique. Whilst early videokeratoscopy produced a photo of reflected mires, modern topography provides data analytics about the corneal surfaces images. Strictly, corneal topography refers only to equipment imaging the front surface of the cornea, whilst corneal tomography refers to equipment measuring *both* front and back surfaces. It follows that only tomography devices can derive information of corneal thickness. In the remainder of this thesis, for simplicity and to follow clinical convention, the term, ‘topography’, will be used for both.

Accurate topography imaging is considered vastly superior in diagnosing early KC than the reliance of slit lamp observations alone, where tell-tale slit-lamp signs have not yet developed. The drive toward accurate topography and automated detection of keratoconus was propelled by the expanse of refractive surgery, for which incipient KC is a significant contraindication – a high sensitivity to KC is vital. Since refractive surgery is primarily a private venture and a significant source of income for such refractive surgeons, a high degree of specificity became very important, such as not to unnecessarily decline surgical income. The proliferation of diagnostic indices in the literature since the mid-1990s have been primarily designed for this remit, but grading and classification systems are of great use to eye care researchers worldwide. Several methods of using topography to identify keratoconus are discussed below:

1.7.1 Classification by topographical pattern recognition

A typical KC cone lies inferiorly (Krachmer et al. 1984), and so imaging reveals relatively steeper cornea below the midline of the corneal centre (Figure 1.21), whilst the superior cornea is imaged as relatively flat. Such characteristic steepening is typically easily identified by an experienced clinician ‘by eye’.

Protrusion of the cone is denoted by forward movement, or elevation of the front surface topography (Figure 1.20), whilst posterior bowing of the cornea is only seen using tomography imaging of elevation of the back corneal surface. There is evidence to suggest that very early ectatic changes occur at the posterior corneal surface prior to the anterior (Schlegel et al. 2008; Tomidokoro 2000; de Sanctis et al. 2013) and thus an increased posterior elevation will alert an experienced clinician even in the presence of a normal front surface topography. In tomographic imaging systems, the differential of anterior and posterior elevation data provides the relative thickness profile of the cornea. When the thinnest point of the cornea is displaced with respect to the corneal centre (Figure 1.22), and particularly when this change is coincident with the area of steepest curvature and elevation, a high likelihood of keratoconus is reached in the mind of the skilled observer.

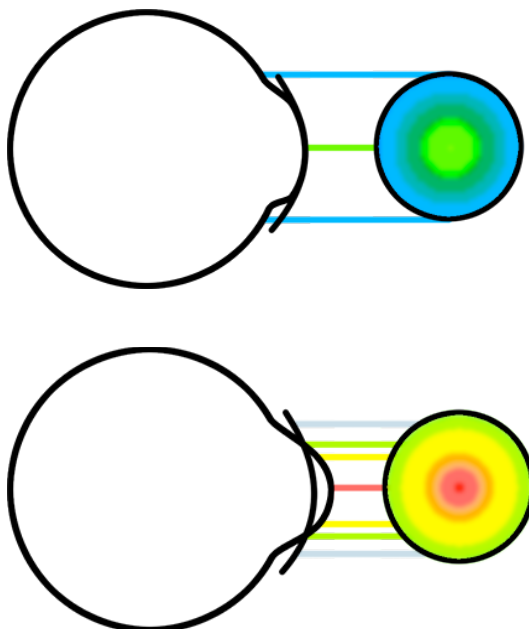


Figure 1. 20 A reference sphere aligned with the profile of a normal cornea, and the resulting topographic pattern (top). The bottom shows a relatively steep cornea and resulting ‘hot spot’ where the corneal elevation is above that of the reference plane

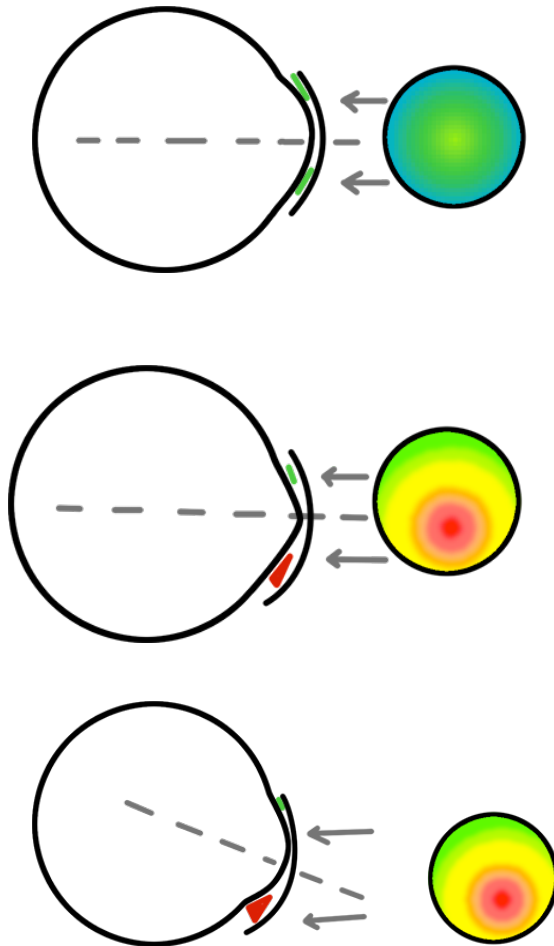


Figure 1. 21 A reference shape relative to a normal cornea (top), an inferior keratoconus cone with an en-face reference shape that highlights the relative steepness of the inferior cornea to the reference shape and the relative flatness of the superior cornea (middle). The last image depicts a healthy eye looking inferiorly and the en-face reference shape inappropriately displaying inferior steepening (bottom), artificially creating the impression of KC, as can be the case when fixation is poor.

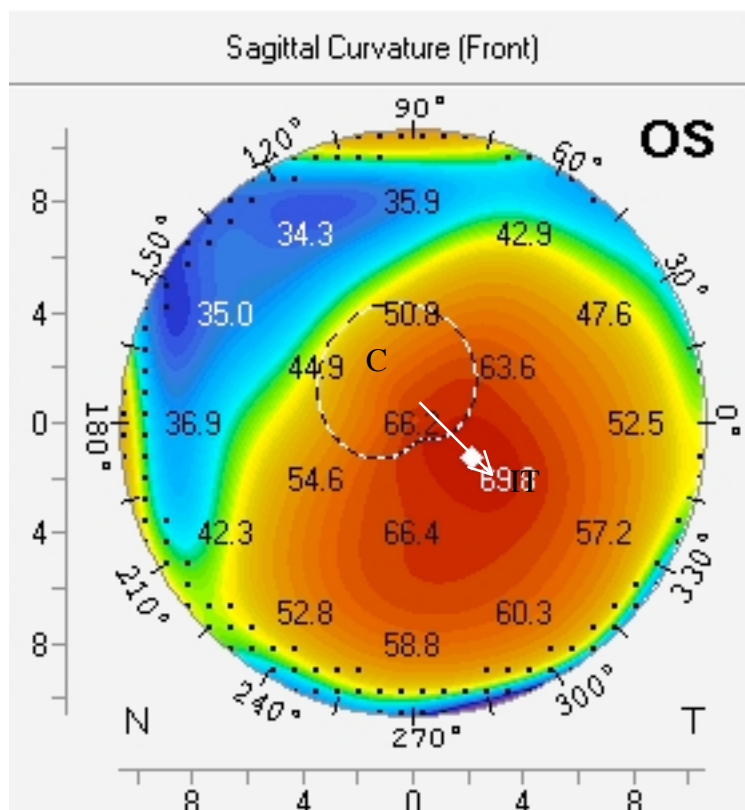
1.7.2 *Classification by monitoring for changes in corneal tomography within individuals*

When a topographical pattern as discussed above appears in conjunction with reduced vision, alongside slit lamp signs of KC, and in the absence of other explanatory pathology, a diagnosis of KC can be made. However, in very early keratoconus, before vision is affected, keratoconus is diagnosed after an increase in conical shape parameters from the measured baseline. A clinically significant change in K_{\max} in early keratoconus is 0.95D (Flynn et al. 2015).

1.7.3 *Classification with respect to a ‘fellow eye’ dataset*

Data from clinically unaffected fellow eyes of keratoconus subjects (a ‘fellow-eye’ dataset) may be used as a benchmark over which indices should raise suspicion for KC in a healthy population (Lema et al. 2009). This does have the limitation of assuming that the clinically affected eye started out as a symmetrical version of the fellow eye (Bühren et al. 2007). Such pre-clinical keratoconus is termed, ‘*forme fruste*’ KC (FFKC), and was identified by Amsler (1946, who noted that although patients presented with ‘unilateral’ KC, early changes were present in the fellow eye, despite this not yet being symptomatic, possibly representing an ‘aborted’ form of the disease. Lema and colleagues looked at topographic, corneal wavefront and pachymetric indices, to study the ability of a ‘fellow-eye dataset’ to significantly differ from healthy eyes, or those of genetic relatives. Of the topographical indices studied, only the ‘location of the steepest point’ (location of K_{\max}) was significantly useful at 80% specificity. All wavefront indicators were useful in differentiating from controls, and all but coma were sensitive enough to differentiate from relatives (Lema et al. 2009). Pachymetric data, however, was found to be the most useful. The distance from the maximum point of posterior elevation from the central cornea was the most predictive of keratoconus. Since healthy corneae with DS are known

to be, on average, steeper (Vincent et al. 2005), thinner (Haugen et al. 2001) and DS eyes carry abnormally high whole-eye aberrations (McCullough et al. 2013), Lema's approach is not transferrable to DS eyes without prior validation.



1.7.4 Topographically-derived single indices

Corneal Power

Since keratoconus is characterised by a steep cornea, measuring the steepest aspect of the central cornea is considered a fundamental measure of the course of the disease. The steepest corneal measurement is known as K_{\max} . This is derived from topographical data with the use of computer software and can detect the maximum corneal steepness regardless of the location on the cornea. In the case of the Oculus Pentacam, the location of the K_{\max} is provided as polar co-ordinates with respect to the central reference (line of sight).

As per a traditional keratometer, modern topography systems are calibrated to provide simulated K readings, from the central 3 mm of the cornea. This builds on the original work by Klyce (1984) and Maguire et al. (1987) who investigated the mathematical derivatives of the elliptical reflection of a circular projection onto an astigmatic eye, to provide a map of corneal power across the central cornea. K1 represents the flat K reading and K2 the steeper. Average corneal power value (ACP) is the mean of the maximum and the minimum corneal powers as per (simulated) keratometry. Since significant astigmatism can exist in keratoconus, this measure takes into account flatter regions also. Using corneal steepness alone (K2), Rabinowitz found that a K-value greater than 47.2D is indicative of possible keratoconus, and classed corneae over 48.7D as clinically keratoconic (Rabinowitz and Rasheed 1999). Others have used average K values of from just 45.57D to identify keratoconus suspects (Smolek and Klyce 1997).

However, it is not accurate to classify neighbouring grades by average central corneal curvature alone. Average corneal curvature, maximum corneal curvature, and simulated steepest keratometry readings did not differ significantly between the *forme fruste* eyes and those of controls (Lema et al. 2009). This demonstrates that the elimination of early keratoconus cannot be ensured through keratometric values alone, but rather that more sophisticated, or multiple measures must be taken.

Regular astigmatism

Regular corneal astigmatism is the difference between K1 and K2 using keratometry, or simulated K's. In refraction, regular astigmatism is simply the cylindrical component of the spectacle prescription. In a typically developing population, Serdarogullari and colleagues determined that patients with refractive astigmatism over 2D should be screened for early keratoconus (Serdarogullari et al. 2013). However, Krachmer warns against using high astigmatism to categorise patients into KC group, stating that this artificially increases the observed prevalence unnecessarily (Krachmer et al. 1984).

Corneal irregularity and aberration

Topographic indices representing irregularity or aberration highlight corneal asymmetry and the measure of the deviation of the cornea from an optically regular shape. Since the typical keratoconic cone lies inferior to the corneal horizontal, producing relative inferior steepening it is possible to quantify the difference between the average corneal power across the inferior cornea relative to that of the superior cornea. The difference is known as I-S and is usually a positive value. When used alone, in the TMS-1 system (Tomey Corporation, Nagoya, Japan), methods by Rabinowitz show an I-S >1.4D is indicative of keratoconus (Rabinowitz and Rasheed 1999).

Keratoconus Index (KI) is provided by the Oculus Pentacam, expressing the ratio between mean sagittal radius values in the upper and the lower segment. The Pentacam itself highlights that which it considers >2.5 standard deviations (S.D.) from the healthy mean as a yellow flag and >3 S.D. from the mean as a red flag. Independently, Kanellopoulos found that a KI value larger than 1.07 correlated with early clinical KC, and that a measure of 1.04 should raise suspicion of KC. (Kanellopoulos and Asimellis 2013). This is in line with data from Goebels who recommends thresholds for the classification of KC using the KI specifically (Goebels et al. 2015).

Corneal irregularity is also measured by the corneal aberrations, whereby the shape of the anterior, or posterior cornea is mathematically compared to Zernike polynomial shapes. While ophthalmic sphere and cylinder correction represent low-order aberrations, high order aberrations such as horizontal coma, vertical coma and spherical aberration represent a large proportion of the vision loss experienced by keratoconic patients. The healthy cornea has minimal aberrations and so aberrations can be used as an early pre-clinical indicator for KC. Alió found that coma-like aberrations are most significantly different in a keratoconic group with respect to normal (Alió and Shabayek 2006). Coma is produced when decentered optics exist within an optical system – in the keratoconic case, a decentering of the powerful corneal apex. The defocus spherical aberration is induced by the protrusion of the cone and the relative shape change in the centre of the cornea in comparison to the periphery. The authors found no correlation between corneal thickness and corneal HOA in the 40 eyes studied - the two factors may be independent. The authors suggest a modification to the well-accepted Amsler-Krumeich classification to include total coma quantification, but do not discuss the impact of vertical or horizontal coma components separately.

Schwiegerling and Greivenkamp propose an aberration classification system that utilises Zernike polynomial decompensation of corneal height data to classify between normal and keratoconic eyes, using a factor called $-Z_3$, measured in μm (root mean squared aberration, RMS) representing the ‘bump’ shape of the cone that remains when the spherical and cylindrical values of the ocular system have been removed. This is, in effect, a measure of spherical aberration. The dataset consisted of 15 keratoconic eyes (3 mild, 10 moderate and 2 severe KC), using myopic eyes as controls. When used on the test group, normal eyes and keratoconus eyes were separated without overlap, with the closest keratoconic eye being 3 S.D. away from the cut-off value $-Z_3 = 0.00233\mu\text{m}$ (Schwiegerling and Greivenkamp 1996). Lema and colleagues found that corneal wavefront indices exhibited the best performance for discriminating between controls and the fellow eye of ‘unilateral’ keratoconus patients (Lema et al. 2009). In particular, total HOA RMS and vertical coma RMS

were the most statistically different parameters and the best clinical detector of KC, over corneal curvature and corneal thickness.

The Pentacam provides two particular irregularity indices of note:

- (i) *The index of surface variance (ISV)* denotes the value of curvature variation from the mean curvature across the anterior cornea.
- (ii) *The index of vertical asymmetry (IVA)* denotes the value of curvature symmetry between the upper and lower hemisphere of the anterior cornea.

Elevation

Mean posterior elevation is a value derived from the Oculus Pentacam and quantifies in microns the average distance that the posterior cornea sits above the reference plane. Values above $29\mu\text{m}$ are considered suspect for KC while above $35\mu\text{m}$ is considered indicative of KC. While the sensitivity (97.3%) and specificity (96.9%) are high for KC, the values are lower for the identification of sub-clinical KC (sensitivity 68.0% and specificity 90.8%) (de Sanctis et al. 2008). Thus, this index may be more useful for establishing a diagnosis than for screening.

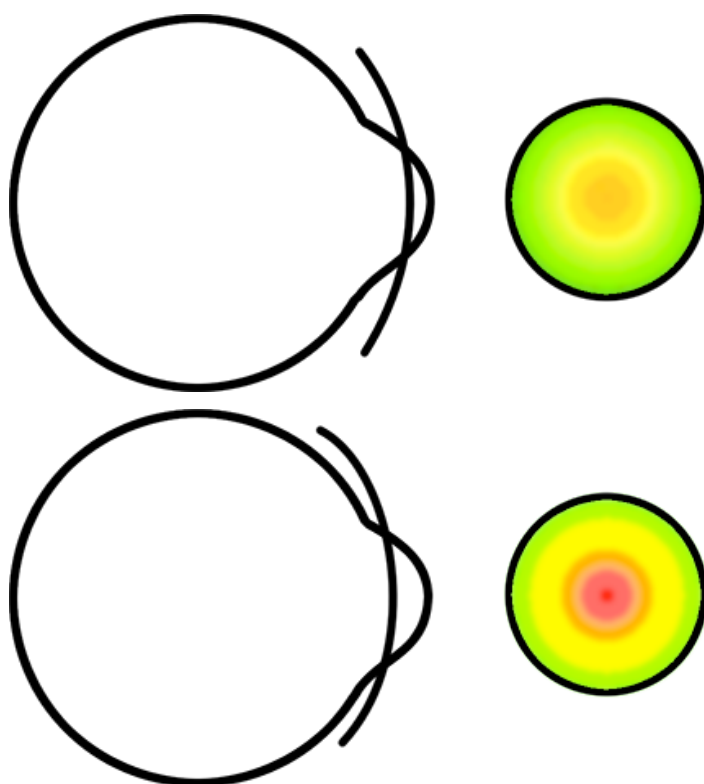


Figure 1.23 Derivation of elevation height data with respect to the reference plane. The lower image depicts an increased height (elevation) from the reference plane, compared to the upper.

The Pentacam provides two particular elevation-derived indices of note:

- (i) *The index of height decentration (IHD)* is a value denoting the decentration of height data in the vertical direction.
- (ii) *The index of height asymmetry (IHA)* denotes the value of height data symmetry between the upper and lower hemisphere of the anterior cornea.

1.7.5 Combined indices

Combined indices are typically found within software built-in to corneal topographers and tomographers. Primarily used to screen refractive surgery candidates prior to corneal refractive surgery, they were initially designed to objectively quantify the likelihood of keratoconus for each patient. The same output, however, is used to either stratify, or chart the progression of keratoconic patients in clinical research.

Belin-Ambrosio Enhanced Ectasia display

This display is additional software available for Oculus Pentacam, providing a corneal tomography view whereby a more sensitive reference shape is used in order to increase the ability for the software to detect an early protruding cone presence (Belin and Khachikian 2007). Figure 1.24 shows how the new reference shape ('enhanced BFS') is fitted to the peripheral corneal shape rather than as an average of the cornea as a whole. Without this software, the automatically-chosen reference shape can be influenced by the portion of abnormal cornea, and mask subtle defects.

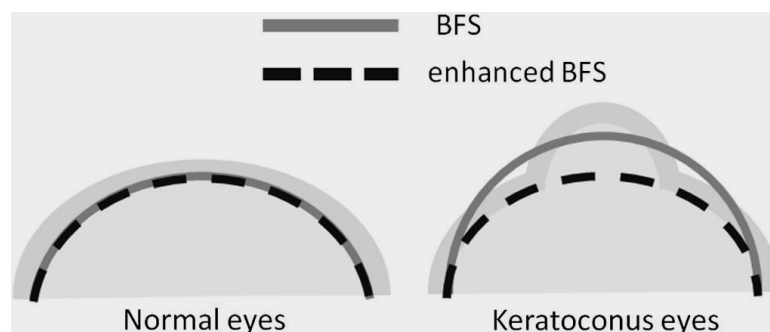


Figure 1. 24 Depiction of Belin Ambrosio enhanced ectasia display. Adapted from (Ishii et al. 2012)with permission. The superimposition of the standard 'BFS' (best fit sphere) demonstrates how the residual elevation would be masked. In this case the enhanced BFS would highlight the focal defect more readily.

1.8 Shape classification

The most comprehensive classification of cone morphology is proposed by Rabinowitz (1996). This topographically-based classification system was originally conceived for the TMS-1 topographer, but the application is relevant across systems. The colours representing the steepest areas of the cornea were used to analyse the shape of the central curvature.

Table 1. 11 Classification of topography from Rabinowitz et al. (1996)

| Category | Acronym | Description |
|----------|---------|--|
| A | R | Round |
| B | O | Oval |
| C | SS | Superior steepening |
| D | IS | Inferior steepening |
| E | I | Irregular |
| F | SB | Symmetric bowtie |
| G | SB/SRAX | Symmetric bowtie with skewed radial axes |
| H | AB/IS | Asymmetric bowtie with inferior steepening |
| I | AB/SS | Asymmetric bowtie with superior steepening |
| J | AB/SRAX | Asymmetric bowtie with skewed radial axes |

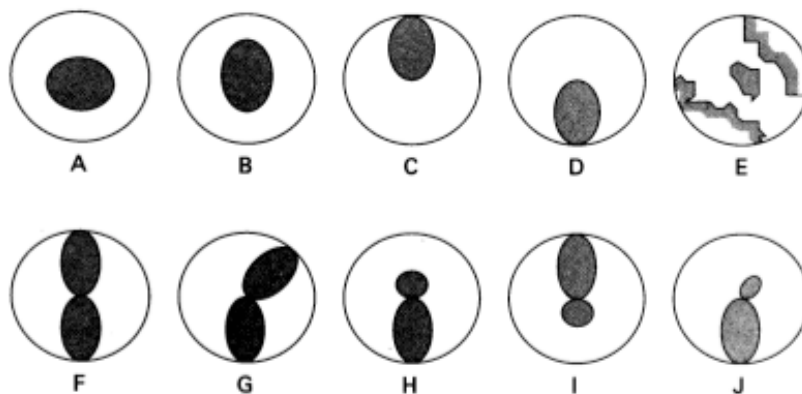


Figure 1. 25 Classification of topography from Rabinowitz et al. (1996)

1.8.1 Scheimpflug profiling

A qualitative profile of the cornea can be extracted from the Oculus Pentacam. This provides several useful points of understanding in the assessment of a keratoconic cornea:

- The distension of the cornea with respect to the anterior chamber. The protrusion of the cone is accounted for by the ‘sag’ value of a scleral contact lens.
- The transparency of the cornea is shown directly, and scar tissue is highlighted by a whitened appearance.
- The profile of the cone becomes clear, in terms of its centrality and symmetry about the centre of the imaged plane.

1.8.2 Corneal apex location

The corneal apex is defined as the maximum curvature or height [elevation] of the front surface (Demirbas and Pflugfelder 1998). This is in contrast to the Oculus Pentacam’s notation of ‘apex’, which is simply an arbitrary point coinciding with the line of sight. This point is used as the reference for all (x,y) locations. When using the Oculus Pentacam, the true apical locations can be derived from either the co-ordinates of K_{max} or the co-ordinates of the point of maximum elevation. In a normal eye, the apex of curvature or elevation may be generally considered to be coincident with the line of sight, although, in reality, it is laterally displaced by a small angle, kappa, which is typically of no clinical relevance (Artal et al. 2006). The formation of a cone in KC will naturally alter the original apex, and may displace it from a location that is typically just infero-temporal to the pupil centre (as in figure 1.22).

Demirbas (1998) used data from typically developing individuals and categorised the corneal apex location into 5 categories from both axial curvature and elevation data: central 1mm zone, and into the following quadrants: inferotemporal, inferonasal, superotemporal and superonasal. Using elevation indices, the apices of most cones are displaced inferotemporally, whilst using axial curvature, most apices were displaced in the central and inferior vertical zone (Fig 2.1). Demirbas suggests that an elevation-based approach is likely to produce an apical location that is more reliable for use when fitting contact lenses or planning corneal surgery.

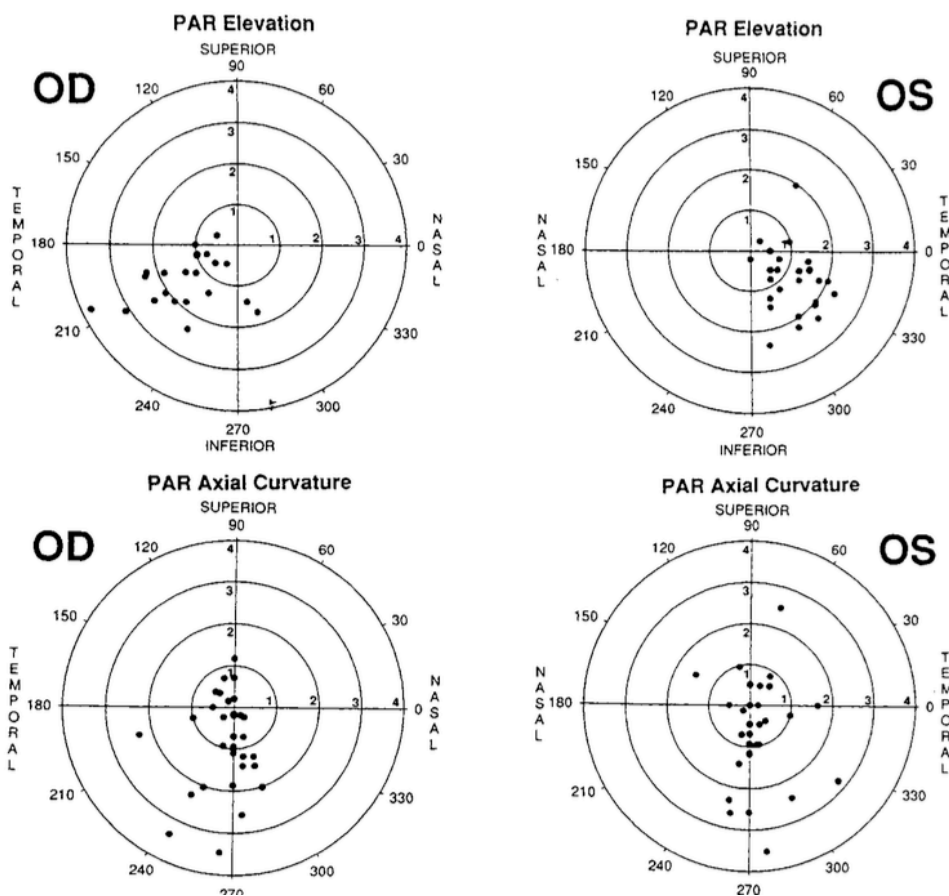


Figure 1.26 Location of the corneal apex using an elevation based method (above) and an axial curvature method (below). Note the symmetry between the eyes and the predominance of the cone in the inferotemporal quadrant of each eye. adapted from Demirbas and Pflugfelder (1998)

1.8.3 Corneal shape: Eccentricity and Asphericity

Eccentricity of a conical section is a measure of the deviation from a circular shape. This is an important measure since the corneal shape cannot be defined by the central cornea alone (Bibby 1976). In corneal terms, eccentricity ϵ is considered as the degree of flattening toward the peripheral cornea. The normal cornea is inherently aspheric, that is, steeper centrally and flattening toward the periphery – this controls spherical aberration in the normal eye (Holladay 1997). The mean eccentricity value of a healthy cornea in a typically developing individual is 0.41 ± 0.11 and is defined as the square root of the difference of the average values of the radius of curvature of the sagittal and the mean central radius (Benes et al. 2013).

Asphericity, Q , is a useful derivative of eccentricity $Q = -\epsilon^2$. Values >0 denote an oblate cornea, i.e. relatively steep in the periphery – such as that found after myopic LASIK (Hersh et al. 2003). In the normal eye, a prolate cornea is typically present, steeper in the centre relative to the periphery and Q having a value between 0 and -1 (a flattening ellipse). Mean Q values for a typically developing adult population are -0.19 ± 0.1 for a 6mm diameter of central cornea (Read et al. 2006), -0.29 ± 0.09 for a 8mm diameter (Piñero et al. 2010), and -0.36 ± 0.1 for a 10mm diameter (Read et al. 2006). Shape factor, p , indicating the rate at which the peripheral cornea departs from the central curvature, is given by the equation $p = 1 - \epsilon^2$, or $p = 1 + Q$ (Guillon et al. 1986).

It is notable that keratoconic cornea have a greater negative asphericity of both the front and back of the cornea than healthy eyes (Piñero et al. 2010). This indicates a more significant prolate shape and represents the relative protrusion of the central cornea. An average early keratoconic cornea has an asphericity of -0.65 ± 0.27 over an 8mm diameter. Despite this difference, asphericity is not a useful diagnostic indicator of early disease (Piñero et al. 2010).

1.8.4 Pachymetric profiling

Since there is a large variation in corneal thickness in any population, relative pachymetric indices are more useful at characterising pathological thinning than an absolute cut-off value.

In a keratoconic eye, focal thinning exists around the location of the cone. Such focal thinning is in contrast to the generalised thinning that exists in a normal thinned cornea (Fig 5.4). Relative thinning results in a relative increase in thickness when moving away from the cone, or the thinnest point. This is the basis of Percentage Thickness Increase (PIT) graphs provided by the Corneal Thickness Spatial Profile (CTSP) programme of the Oculus Pentacam.

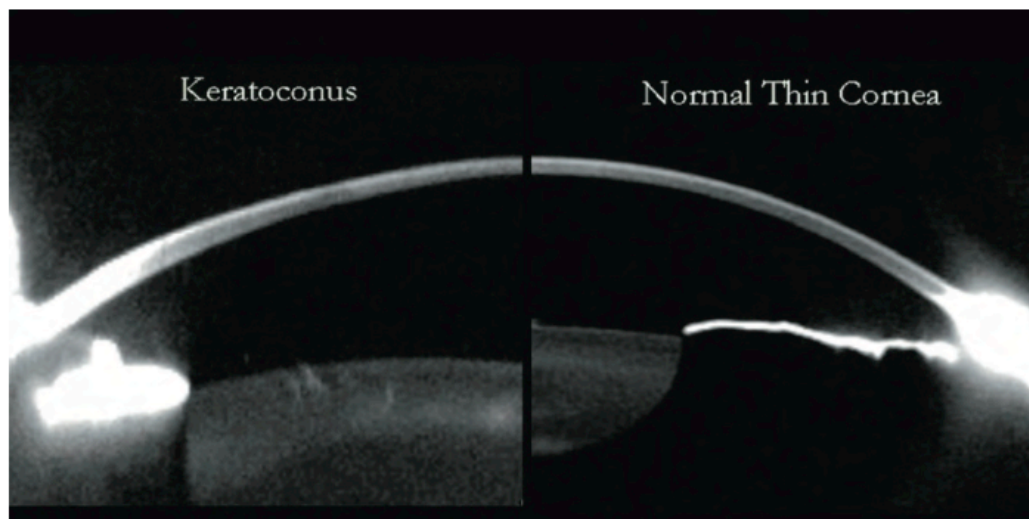


Figure 1. 27 Difference in thickness profile of a pathologically and physiologically thin cornea (Halstenberg, Oculus Pentacam)

Corneal thickness at the thinnest point is determined and the average thickness values of the points within 22 imaginary circles (centred around the thinnest point) at 0.4mm intervals, are determined.

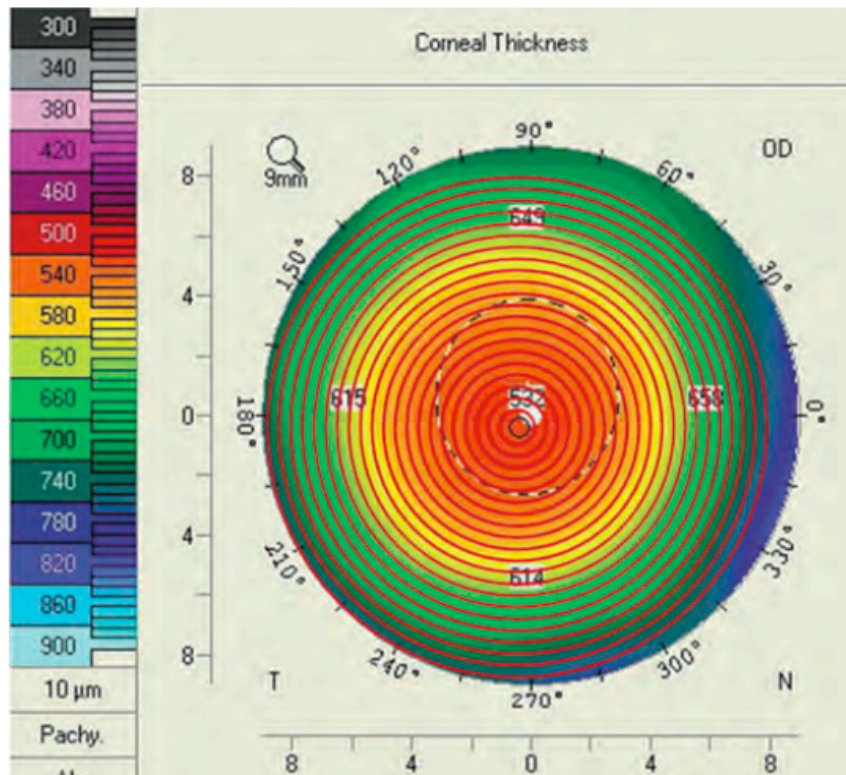


Figure 1.28 CTSP display from Oculus Pentacam.

PIT (for a given ring)

$$= \frac{\text{mean corneal thickness in the ring} - \text{minimum corneal thickness}}{\text{minimum corneal thickness}}$$

PIT is then plotted graphically on the lower display, allowing a relative thickness increase profile to be built up. A normal (Fig 5.6a) and ectatic (Fig 5.6b) cornea provides a visual depiction of the standard deviation within which the healthy cornea is expected to lie, and how the pathological cornea falls outside this expected range. With increasing distance from the thinnest point, the difference between normal and pathological spatial profiling values increases (Ambrósio et al. 2006).

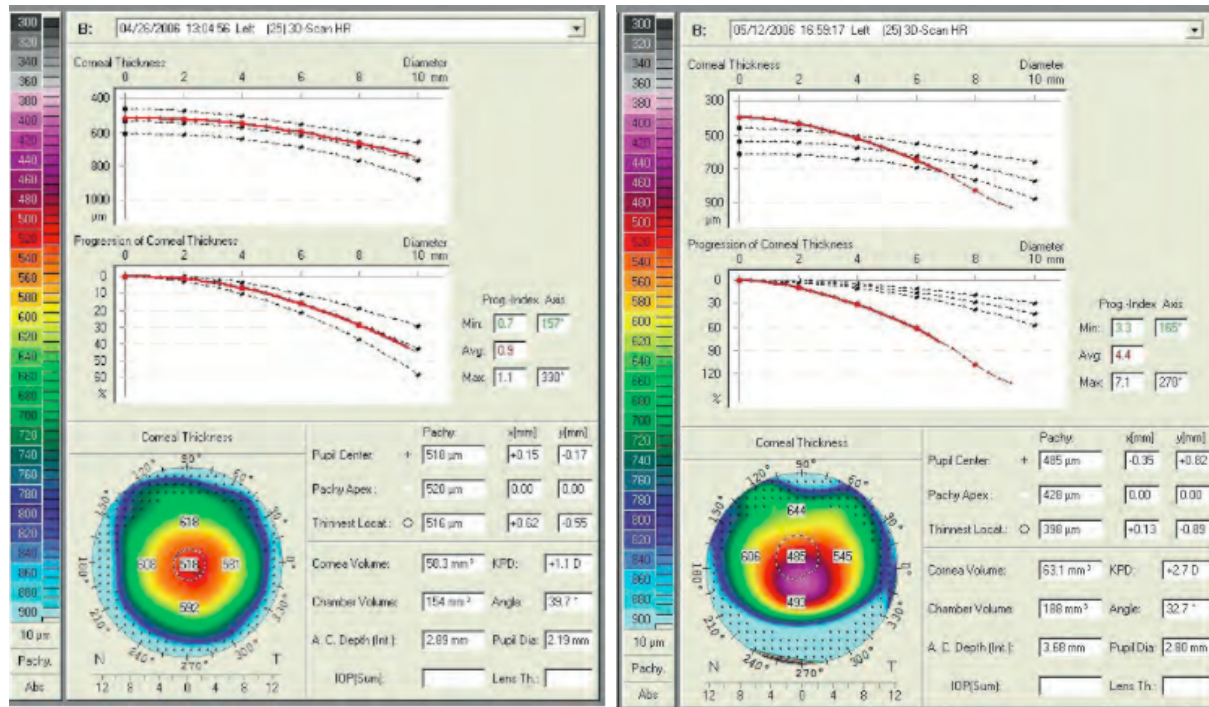


Figure 1.29 (left) and 5.6b (right) to show the Pentacam display of a healthy cornea CTSP and an ectatic CTSP respectively. The actual corneal thickness values are plotted in the superior plot, whilst the PTI is plotted inferiorly. Corneal thickness maps are provided for reference.

1.9 Grading scales for KC

Amsler was the first to suggest a grading scale for KC, categorising the disease into 4 stages (Amsler 1938). This utilised the skewing of axes seen by placido disc viewing, and Amsler noted the increasing irregularity with advancing disease distorting the keratometer mires, and noted the increasing disparity in shape between the central and peripheral corneal areas. Fundamentally, Amsler pointed out that keratoconus could be identified by the asymmetry of the principle refracting meridians of the cornea.

Amsler's work has been supplemented by further understanding and imaging of the corneal power across the corneal surface, and by corneal thinning with disease progression. The Amsler-Krumeich scale (from Alio and Shabayek 2006) is a modern expression of the original scale:

Stage I

- Eccentric steepening
- Myopia and/or induced astigmatism
- Mean K readings <48D

Stage II

- Myopia and/or induced astigmatism from 5D to 8D
- Mean central K readings <53D
- Absence of corneal scarring
- Minimum corneal thickness >400 μ m

Stage III

- Myopia and/or induced astigmatism from 8D to 10D
- Mean central K readings >53D
- Absence of scarring
- Minimum corneal thickness 300-400 μ m

Stage IV

- Refraction not measurable
- Mean central K readings >55D
- Central corneal scarring
- Central corneal thickness <200 μ m

This scale has significant limitations. This scale is extremely prescriptive with dioptric power, refraction and corneal astigmatism and there is frequently cases in which a subject will overlap on at least two of the 'stages'. For example, a cornea that is 350 μ m with corneal scarring would fit into both III and IV, whilst an early keratoconic with hyperopia, low astigmatism and a relatively flat cornea might not reach the specification for grade I. In a population of people who have naturally thinner, steeper cornea, such as that seen in DS (Haugen 2001), healthy corneae could easily be attributed to group I if eccentric steepening was not clearly defined by corneal topography analysis in another manner.

1.10 Cross-linking of collagen and elastic fibres

A vital aspect of molecular-binding is the generalised intermolecular crosslinking across collagen and elastin within tissues. Crosslinking is a developmental necessity in order to provide structure and biomechanical strength to various organs and systems (Bailey et al. 1998). The degree of cross-linking of tissue is important. Under-crosslinking of tissue results in reduced tensile strength whereas over-crosslinking results in poor elasticity and brittle tissue (Buehler 2006).

Bonds between collagen fibrils are covalent in nature and allow formation of larger, stronger collagen fibrils. These occur from the onset of life, through to corneal maturity - with additional crosslinks adding throughout adulthood. Cross-links result in collagen that is tougher, less elastic, less soluble, and less prone to enzymatic degradation (Malik et al. 1992). Two contrasting types of crosslinking are relevant to the predominant collagen type I found in the corneal stroma – enzymatic and non-enzymatic crosslinks (Raiskup and Spoerl 2013).

- I. *Oxidation* – enzymatically controlled cross-linking that occurs during development and maturation.
- II. *Glycation* – the spontaneous addition of sugar (usually glucose) to a protein, is a key part of the ageing process.

Developmental crosslinks in young tissue occur due to aldehyde formation and are reliant upon the production of oxygen by the enzyme lysyl oxidase in the presence of copper (Bailey et al 1998;Bykhovskaya et al. 2012).

In older tissue, crosslinking is aldehyde-based but non-enzymatic, instead is facilitated by the presence of glucose. Such *glycation* is shown to increase with age (Malik et al. 1992), and so collagen fibrils grow in volume over a lifetime as new

collagen molecules are covalently bonded to the fibrils with time (Daxer et al. 1998). As the diameter of collagen fibril increases, the flexibility decreases (Bailey et al. 1998). Indeed, in vivo study of older corneae indicate that they are firmer, less elastic, and show less viscoelastic absorption of force than younger corneae, whilst corneal thickness remains constant (Schwarze et al. 2000; Ohmoto 2009).

Diabetic patients are more susceptible to glycation due to their increased blood glucose availability (Nuytinck et al. 2000; Jerums et al. 2003). While the addition of cross-links to vascular tissue is thought to increased blood pressure and the susceptibility for adverse cardiac events (Wenstrup et al. 2004; Kass et al. 2001), natural cross-linking of the cornea appears to show no great clinical disadvantage. Interestingly, diabetes may be protective against keratoconus, possibly due to the additional glycation and crosslinks in the cornea (Seiler et al. 2000) (KUO et al. 2006).

The gene coding for lysyl oxidase, *LOX*, is located on chromosome 5 (5q23.2), and variants in this gene are thought to account, in some part, for susceptibility to keratoconus in some individuals (Sethi et al. 2012). A deficiency in LOX activity is seen in certain connective tissue disorders, Ehlers-Danlos syndrome type V and cutis laxa (Byers et al. 1980). Since LOX is considered a cross-linking agent, a search has taken place for an abnormal variant of the LOX gene in keratoconics, which could theoretically cause a decrease in normal cross-linking of corneal collagen, biomechanically weakening the cornea and leading to a susceptibility to keratoconus (Bykhovskaya et al. 2012).

In human epithelial cells from the apical area, LOX expression is reduced in keratoconic eyes versus healthy controls (Pahuja et al. 2016). LOX activity across fibroblast culture medium produced from keratoconus samples was found to be decreased 2.5 fold compared to that of controls (Dudakova et al. 2012). Further work from Dudakova and colleagues, suggests that a LOX abnormality may be the common basis for both the biomechanical instability seen in keratoconus and that of mitral valve prolapse, particularly because both are common in Down's syndrome (Dudakova and Jirsova 2013). Other papers disagree about the involvement of LOX

in KC (De Bonis et al. 2011). However, lysyl oxidase, or the LOX gene does not yet appear to have been explored specifically in DS.

1.10.1 Induced Corneal Cross-Linking (CXL)

CXL was developed in order to strengthen the cornea and reduce its propensity to deform in shape (Spoerl et al. 1998). Since the biomechanical integrity of a biomaterial is determined at least in part by the extent of the crosslinking within the structure, the introduction of further crosslinks between the collagen fibrils should make the stroma stiffer and more robust, thus preventing degradation (Spoerl et al. 2009) or fibrillar slippage.

In standard protocol, after removal of the corneal epithelium, the stroma is irradiated with UV light (370nm) in the presence of Riboflavin and dextran solution (Wollensak et al. 2003). This is usually carried out in a surgical theatre as a day case procedure under local anaesthetic, with the patients taking intensive topical medication and returning several days postoperatively to ensure healing of the corneal epithelium.

1.11 The healing cornea

As can be seen from Figure 1.30, the normal corneal epithelium is typically at least 5 layers thick, and as such is able to fend off much mild biological and chemical trauma. Epithelial stem cells are produced in the limbal crypts (Dua et al. 2005), migrate centrally to form the basal layer, move anteriorly, and are naturally sloughed off at the ocular surface.

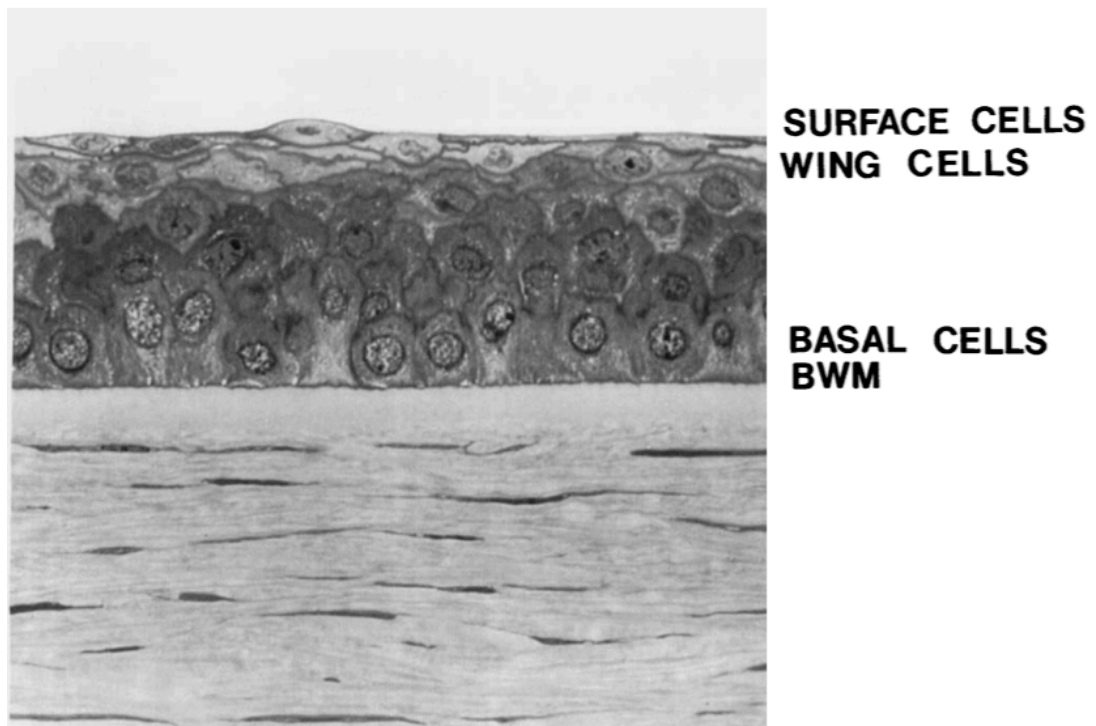


Figure 1. 30 Healthy corneal epithelial cells progress from the basal layer upwards to wing and surface cells. Bowman's layer underlies the epithelium and is morphologically distinct from the underlying corneal stroma (Beuerman and Pedroza 1996), with permission.

In repair, it is the deeper cells basal cells of the epithelium which are most important, undergoing mitosis and quickly replacing the protective superficial cells that have been lost to ocular damage (Thomas 1955). When epithelial trauma occurs, basal cells are thought to release inflammatory cytokine interleukin 1 (IL-1) into the underlying collagen layers (Wilson et al. 2001).

Keratocytes are differentiated fibroblast cells that are specific to the corneal stroma. They produce the components for the production and the maintenance of collagen. They appear mitotically quiescent in absence of pathology, and very slowly maintain the slow turnover of corneal collagen (Davison and Galbavy 1986). Keratocytes are derived from neural crest cells and are thought to possess stem cell-like qualities in terms of their ability to repair and regenerate tissue (West-Mays and Dwivedi 2006). When the corneal epithelium is damaged, IL-1 infiltrates the stroma and binds to the IL-1 receptors on the keratocyte cell. This initiates the keratocytes either to apoptose or assume a repair phenotype (Fini and Stramer 2005). Depending upon the extent of the damage, keratocytes proliferate and migrate resulting in the upregulation of the expression of collagenases and metalloproteinases to degrade damaged tissue. Upon injury, some keratocytes transform into ‘myofibroblasts’, cells that control the deposition and organisation of ECM specific to corneal wounds (Jester et al. 1999). These specialized cells are phenotypically different to keratocytes, and produce altered secretions including altered glycosaminoglycans for the resulting new collagen to be laid down to the extracellular matrix (ECM) (Funderburgh et al. 2003). A significant product of myofibroblasts is actin (akin to that of smooth muscle cells) that contracts, sealing the open wound (Majno et al. 1971). See figure 1.31.

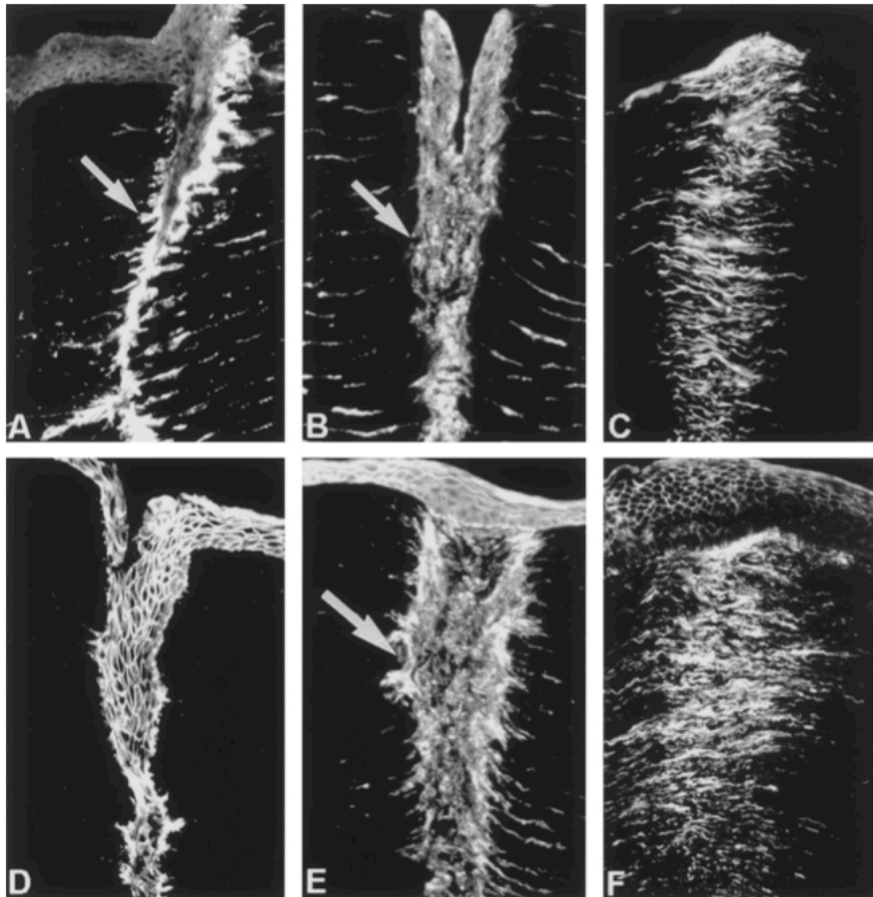


Figure 1.31 The healing of an incisional corneal wound at 3 days (A&D), 14 days (B&E) and 30 days (C&F). A-C show staining of presumed keratocytes with anti-fibronectin antibodies whilst D-F show presumed staining of epithelial ingrowth by phalloidin (Garana et al. 1992). It appears that the wound is contracting and sealing, a function of cellular activity, both by the epithelium and the keratocytes.

Jester (1999) describes that, in general, the healing of controlled, incisional wounds involve:

- (i) Early sliding of the corneal epithelium over the wound margins
- (ii) Formation of an epithelial plug
- (iii) Epithelial plug is replaced by fibroblastic cells
- (iv) Formation of scar tissue

Such healing is modulated by the activation of the corneal inflammatory pathway, and the studies of the pathophysiology in KC reveal interesting parallels that are worthy of note.

1.12 Cellular changes in Keratoconus

On the very first page of his 1854 book, Nottingham remarks that [keratoconus], “...in most instances, of inflammatory action, although this antecedent may not, at all times, be observed.” Despite this, for many years, scientific keratoconus literature has typically introduced the disease as a “non-inflammatory corneal ectasia” and research has focussed upon the biophysical and structural changes present. Recently, more attention has been placed upon investigating the inflammatory aspects that may contribute to the disease state, and their impact upon the existing biochemical changes that are well established in KC.

1.12.1 Tear film

The tear film exhibits inflammatory markers that are altered significantly between eyes that do and do not have keratoconus (Lema and Duran 2005; Lema et al. 2009; You et al. 2013). Further, Lema et al (2009) compared the levels of proinflammatory markers in patients affected by asymmetric disease divided into 3 groups: (i) the clinically keratoconic eye (ii) the fellow sub-clinical keratoconic eye and (iii) that of control subjects with no overt evidence of keratoconus. The authors found that tear composition is altered in KC (relative to control patients) even in the eye with subclinical disease.

1.12.2 Corneal Epithelium

In keratoconus, there is a loss of epithelial basement membrane integrity and altered protein expression (Nielsen 2003). The morphology of epithelial cells appears disturbed in keratoconus, cells appear longer and elongated, even when contact lens wear is accounted for (Tsubota et al. 1995). Reflective deposits are noted in the basal epithelial cells under *in vivo* confocal microscopy, thought to be the microscopic component of the haemosiderin, the accumulation of iron in the cornea that leads to Fleischer’s ring seen under slit lamp biomicroscopy (Uçakhan et al. 2006; Efron and Hollingsworth 2008). In severe inflammatory ocular surface disease, reflectivity of the epithelial cells is also affected (Alsuhaibani et al. 2006),

and the nuclei of epithelial cells appear altered (Efron and Hollingsworth 2008), see figure 1.32. When viewed using histological light microscopy, the overlying epithelium is noted to thicken beside areas of breakage in the underlying collagenous layers in KC (Sykakis et al. 2012). This is also noted in some corneae using in-vivo imaging with Scheimplug imaging (Figure 1.33). Taken together, this altered epithelial cell behaviour is a likely indicator that the cells are under metabolic stress in KC, and suggestive that epithelial cells may be phenotypic of healing properties.

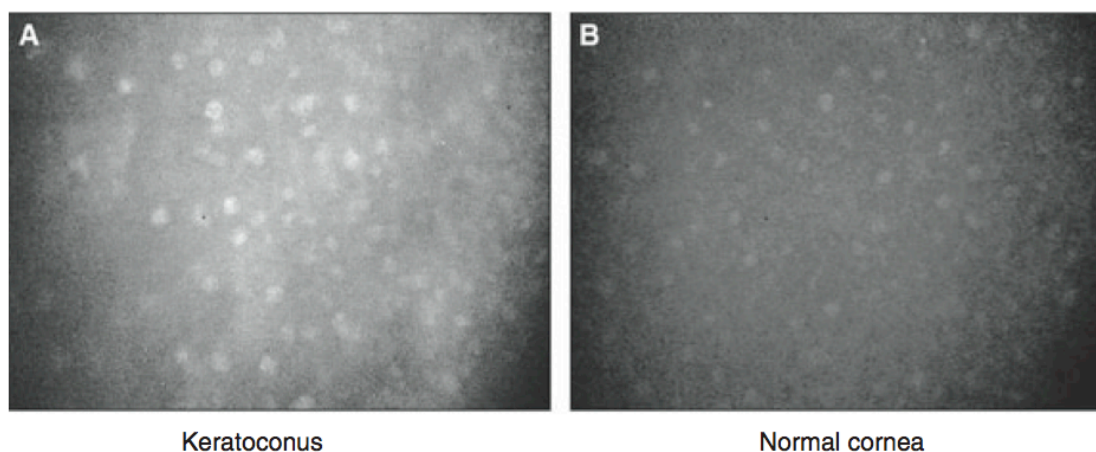


Figure 1. 32 Reflective epithelial nuclei in KC and those in the normal eye (Efron and Hollingsworth 2008), changes which may indicate that the epithelium is under metabolic stress.

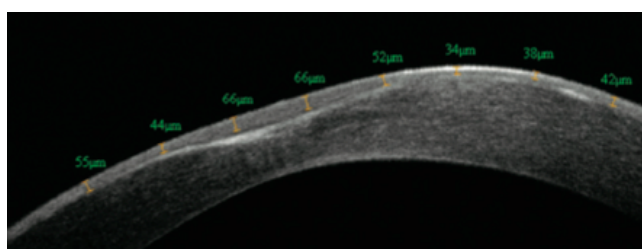


Figure 1. 33 An image adapted from (Rocha et al. 2013) displaying hypertrophic epithelium overlying a particularly thinned area of corneal stroma in a keratoconic eye. Such cellular volume changes make a dramatic difference to the curvature of the anterior cornea and mitigate the detrimental refractive impact of keratoconus.

1.12.3 Bowman's Layer

In KC, Bowman's layer is noted to develop breaks and growing evidence to suggest these breaks become infiltrated by keratocytes and epithelial cells surrounding the area (see Figure 1.34) (Sherwin et al. 2002; Sykakis et al. 2012). Importantly, alterations in the collagen expression found in Bowman's layer appear to be specific to the pathogenesis of keratoconus, and are not as a direct result of the scarring alone (Tuori et al. 1997). Sykakis et al (2012) also found apoptotic cells in the area of Bowman's layer breaks. In a healthy cornea, the barrier between epithelium and stroma is maintained such that inflammatory mediators do not reach the stromal keratocytes (Wilson et al. 1996). Figures 1.34 and 1.35 may show some evidence to the contrary in KC, albeit in a small sample sizes.

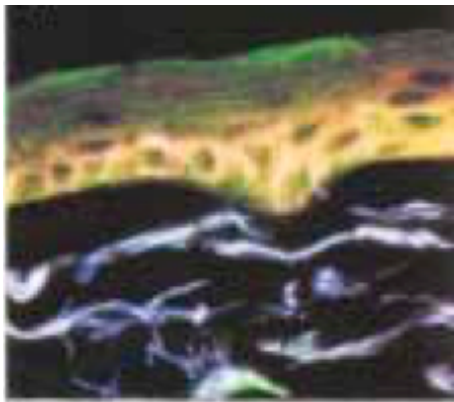


Figure 1. 34 Examination of Bowman's layer in KC reveals that epithelial cells (yellow) invaginate into Bowman's layer in a keratoconic sample (Sherwin et al. 2002).

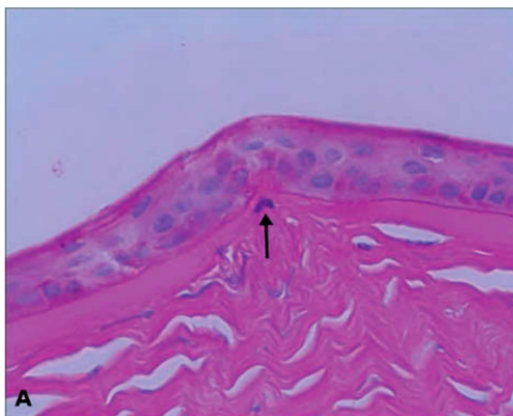


Figure 1. 35 Histological examination of a keratoconic cornea shows a break in Bowman's layer and the invasion of a keratocyte cell into it (arrow denoting the nucleus) (Sykakis et al. 2012).

Corneal stroma

Interleukin-1 has been discussed as the initial mediator of inflammation and healing in damaged corneal stroma, through the IL-1 binding sites on keratocytes, causing apoptosis or cell transformation. Four times more IL-1 receptors are found in keratoconic keratocytes cultures than in controls (Fabre et al. 1991). Since IL-1 increases the synthesis of collagenase, and subsequent reduction in collagen bulk, Fabre and colleagues postulate that a genetic abnormality in the number of binding sites in keratocytes could predispose to KC.

Corneal endothelium

In the absence of hydrops, the corneal endothelium remains largely unchanged in keratoconus (Del Viva et al. 2015; Rabinowitz 1998), although precautions are taken during CXL so that the endothelium is not irradiated, and hence a minimum corneal thickness is required prior to most treatments (Kymionis et al. 2012).

Cellular changes seen in KC are in many ways akin to an inflammatory and healing process. It is hypothesised that an abnormality in such a system could be implicated in the pathogenesis of keratoconus, allowing a chronic sub-clinical inflammatory process that in some way contributes to degradation of collagenous tissue.

1.13 Atopy

Atopy is a term originally coined by Professor ED Perry in 1923, stemming from the Greek word, “ατοπια”, meaning out-of-place, or “strange disease” that was an inheritable “abnormal hypersensitiveness” to an infection, or what we now term an *allergen* (Coca and Cooke 1923). The world allergy organisation (WAO) define atopy as “a personal or familial tendency usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary allergens, usually proteins” (Johansson et al. 2004). Scientifically, atopy is characterised by high levels of immunoglobulin E (IgE) antibodies in the blood or on mucous membranes. Clinically, atopy results in the tendency to develop classic allergic diseases such as asthma, eczema and hay fever. Keratoconus itself has long been associated with atopy (Copeman 1965). Indeed, clinicians who fit contact lenses to patients with keratoconus consistently report that keratoconics tend to have particularly red, itchy eyes, and tend to suffer from at least two of the atopic triad mentioned above.

1.13.1 Inflammation

Atopy itself is considered part of a wider allergic disease, immune-mediated hypersensitivity. Whilst the immune system that typically guards the body from infection, in hypersensitivity it instead reacts to an antigen that is not otherwise dangerous. This results in the unnecessary activation of what can be a serious inflammatory cascade.

In the normal physiological sense, regulated inflammation exists to eliminate invading organisms, and has two main roles:

1. To recruit high levels of immune cells to the potentially infected area
2. To facilitate the destruction and subsequent healing of damaged tissue

Atopy, therefore, creates an undue pathological process that serves no benefit to the host and produces local tissue irritation and destruction.

There are two phases for an allergic reaction to occur:

1. Sensitisation. The allergen enters the immune system and is presented to the T-helper lymphocyte cells (Th), triggering the release of inflammatory cytokines. Cytokines activate B-lymphocytes to produce antigen-specific antibodies – in the case of allergy, these are from the IgE family. Not only are these released in excessive quantities, they are also bound very tightly to the cell membranes of mast cells in tissues, and basophils around the body (Fukagawa et al. 1994).
2. Re-exposure. Upon the second or subsequent re-presentation to the immune system, the allergen binds to the specific IgE-antibodies on the surface of immune cells (MacLean and Eidelman 2001). This causes the cells to degranulate, and burst their contents into the extracellular matrix (Figure 1.31). It is the contents of the granules within these inflammatory cells that initiate the inflammatory cascade and result in the signs and symptoms associated with inflammation. The relevant factors are described in table 1.3.

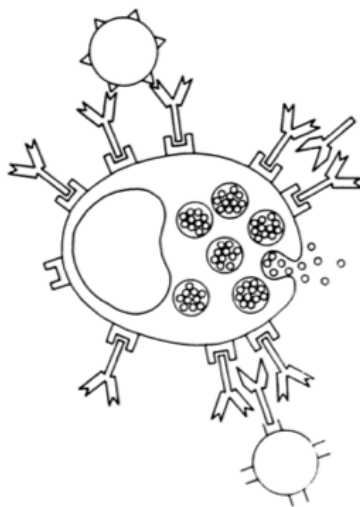


Figure 1.36 IgE antibody receptor on the cell surface and the subsequent degranulation from vesicles containing abundant levels of histamine (Stump et al. 1988)

Table 1. 12 A series of inflammatory mediators involved in the inflammatory cascade.

| <i>Inflammatory mediator</i> | <i>Purpose</i> |
|---|---|
| Histamine | A hormone that causes vasodilation, increased vaso-permeability, mucous secretion, itch |
| Tryptase | An endoproteinase specific to mast cells (Fukagawa et al. 1994), an enzyme that breaks down collagen and amino acids. |
| Prostaglandins and heparin | Hormones causing vasodilation and preventing clot formation |
| TNF- α (tumour necrosis factor) | Promotes neutrophil migration to tissue site and apoptosis of neighbouring cells |
| TGF- β (transforming growth factor) | Inflammatory cytokine |
| Interleukins | Inflammatory cytokines - signalling molecules produced by T helper cells to mediate inflammation IL-1, IL-4, IL-9, IL-13. IL-4 and IL-13 directly promote the production of IgE |
| MMP (matrix metalloproteinase) | Endoproteinases that break down extracellular matrix. MMPs can induce apoptosis of neighbouring cells through the release of ligands (such as FAS), and activation of inflammatory cytokines. |

The factors in table 1.3 comprise part of the inflammatory cascade, responsible for all allergic reactions, from a mild skin rash through to life-threatening anaphylaxis. Like most allergies, ocular allergy can be acute or chronic in presentation – this will depend on the allergen itself, its concentration and duration of exposure. In acute allergic eye disease, the onset is usually within 1 hour of exposure, and causes severe swelling of the conjunctival and dermal tissues. Chronic allergen exposure can lead to low grade, persistent symptoms. While the tissues of the eye do not swell, sustained histamine release causes the blood vessels on the conjunctiva to become dilated, causing a red eye, and itchiness to occur. Persistent high levels of inflammatory cytokines cause hypertonic tears on the ocular surface and continue the

cascade that causes stress to the ocular surface cells and ultimately to epithelial cell death, and possibly to those in the corneal stroma.

1.13.2 *Mast cells*

While the dead epithelial cells of the skin's epidermis provide a physical barrier to infection, the living cells of the mucous membranes are in contact with the air, and so are constantly exposed to antigens; therefore the mediators for protective inflammation must reside locally. Mast cells predominate in locations such as the lungs, the nose, the eyes (and to some extent the skin). Thus, it is these areas that are affected most severely in allergic disease (Figure 1.32). These cells contain histamine (Riley and West 1953) – released upon the activation of the cells and their rapid degranulation to initiate a surge of inflammation in the area. Mast cells are found in greater abundance in those with, than in those without atopic dermatitis (Damsgaard et al. 1997).

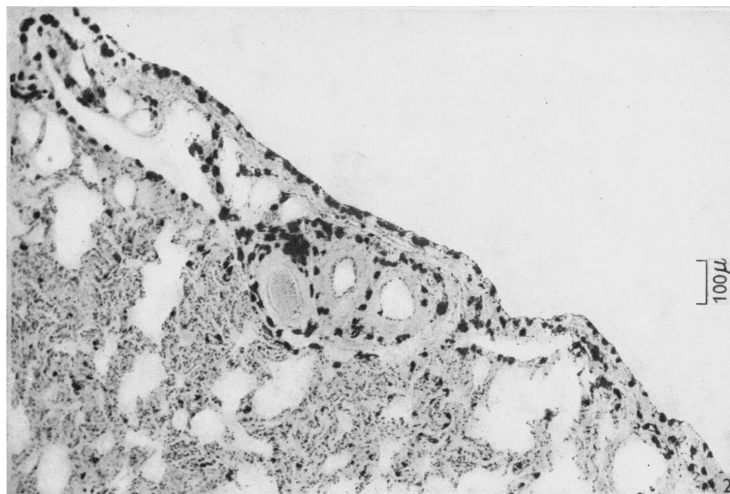


Figure 1. 37 Mast cell in the epithelium of the ox lung – note the abundance of histamine granules present at the surface of the tissue (Riley and West 1953).

1.13.3 Inflammation of the eyelid margin – blepharitis

Inflammation of the eyelid margins results in red rimmed, crusty and uncomfortable eyes. Although prevalent in the typical population seen by optometrists (37%) (Lemp and Nichols 2009), blepharitis is particularly problematic in DS often leading to ophthalmological referral. The tendency of significant blepharitis in DS is attributed to congenital nasolacrimal duct obstruction in this group, preventing sound drainage of tears and debris. However it is possible that the microbiome and inflammatory system is altered somewhat in DS and that the inflammatory environment in the tarsus is exacerbated biochemically. Blepharitis in corneal disease has common associations with atopy, allergy and eye rubbing (McMonnies and Boneham 2003). Significant eye rubbing due to blepharitis-derived ocular irritation could, in theory, lead to keratoconus according to some evidence (Krachmer 2004).

1.13.4 Prevalence of atopy

The lifetime prevalence of individual atopic components varies from 6.9% (allergic asthma) to 25.7% (atopic dermatitis), but varies considerably with a variety of factors including sex, age and geographical location (Mortz et al. 2001; Warner 1999). Despite the inherent variation, the prevalence of atopy appears to be increasing over time in developed countries. An Eastern Australian study based in Wagga Wagga studied the increase in atopic conditions between 1992 and 1997 finding an increase in asthma of 8.1% (to 38.6%) and hayfever of 6.7% (to 45.4%) (Downs et al. 2001). In Leipzig (East Germany), the prevalence of hay fever increased by 2.8% (to 5.1%) and diagnosed atopy increased by 7.4% (to 26.7%) between 1991-92 and 1995-96 (Mutius et al. 1998). It is commonly thought that this increase is due to cleaner living environments and less exposure to infection whilst young. The hygiene hypothesis (Schaub et al. 2006) states that exposure to pathogens early in life aids the regulation of the allergy responses later in life, and the absence of suitable exposure results in atopy. Th2 cytokines (which predispose

to allergy) predominate in infancy, are slowly replaced by Th1 cytokines that are allergen-specific and do not cause allergy to innocuous substances. It is only through 'exercise of infection' that the maturation of Th1 cytokines can occur (Holt 2000), and it is thought that children who are brought up in a very clean environment are deprived of the viruses and the bacteria usually available to assist in the development of a healthy immune system. This is supported by the inverse correlation of atopic risk and family size (Strachan 2000). Since IgE is thought to be an evolutionary throwback to the need for protection against helminth infection (Yazdanbakhsh et al. 2001), something much less common in developed countries. Others suggest that the diet and gut flora of very young children (Devereux and Seaton 2005; Kalliomäki et al. 2001), or the diet of their mothers during pregnancy (Chatzi et al. 2008) could impact upon risk of atopy, through the mechanism of antioxidant or polyunsaturated fat exposure. The possible impact of psychological stress on the neuroimmunoregulation and hypersensitivity is also proposed (Wright et al. 2005).

1.13.5 Diagnosis of atopy

There is no agreed system for the identification of those with atopy. There is a great variation in the literature of methods used to provide an indication of atopy; and many reports of prevalence do not discuss a justification for the methods employed. Prospective clinical trials often focus on biochemical indicators in blood, whilst others observe clinical signs and their severity.

- (i) The blood serum IgE content is used as a key inflammatory marker measured from the systemic circulation. Specific IgE for the suspected allergen may be quantified, or that of total serum IgE concentration which represent a general atopic response (Paganelli et al. 1998). Alternatively, blood samples may be taken to mix with antigens in a lab setting.

- (ii) The allergen skin test is the most common invasive method used in primary care to measure response to common allergens. Each allergen is dissolved in

an aqueous solution and is injected into, or placed on the superficial skin layers in labelled rows alongside a positive control of histamine and a negative control of a dilutant (Spergel et al. 2002). If the patient reacts positively to the allergen, inflammation occurs locally at the injection site.

(iii) Atopy questionnaires have been used extensively in preliminary research in the investigation of atopic prevalence in a population. Since atopy has clinical features clinically determined by consensus (Edfors-Lubs 1971; MacLean and Eidelman 2001), questionnaires are considered a practical approach for groups when invasive testing is impractical or excessive to clinical need. The current study aims to produce clinical guidelines for primary and secondary care for those with learning difficulties, questionnaires were considered both appropriate and minimally invasive when screening for atopy in this Down's syndrome cohort.

1.13.6 *Grading atopy*

The literature on grading of atopy predominantly focuses on atopic dermatitis. Despite the extensive use of questionnaires, the agreement of physicians on what constitutes atopic dermatitis is poor – and a ‘working party’ was initiated in order to design and test a method for eliciting significant signs of the disease that allowed both good agreement between physicians and also that were of sound clinical use (Williams et al. 1994). This appears to have been the most extensive and validated research on questionnaires for the condition, and its inclusion of general atopic criteria is of use to the current study. Authors first identified all possible diagnostic criteria (31 in total) (Williams et al. 1994), identifying those criteria which carried the largest sensitivity and specificity, and then tested the agreement to maximise the integrity of the questionnaire (Williams et al. 1996). Patients who have history of itchy skin, in combination with 3 or 4 of: itching of the skin creases (flexures), visible flexural eczema, asthma or hayfever are considered *complete atopic*, whereas those with 1 or 2 categories in addition to a history of itch are considered incomplete or *partial atopic* (for dermatitis).

Another system of atopic classification was used by Rahi and colleagues at Moorfields Eye Hospital (Rahi et al. 1977). Patients were asked for a history of hay fever, asthma, allergic dermatitis, urticarial or vernal catarrh. Those with at least one of asthma, allergic dermatitis or hay fever were considered atopic. Pearce (1999), however, suggests that atopy is responsible for less than half of asthma incidence, which would suggest that this population is easy to overestimate.

1.13.7 Genetics of Atopy

The pattern of atopic inheritance does not follow Mendelian rules that would typically be seen in single-gene disease (Romagnani 2000). The mode of inheritance, therefore, is thought to be relatively complex and genetically multifactorial (Coleman et al. 1997). It is highly likely that environmental conditions help to determine atopic status, and so atopy may be considered multifactorial. Furthermore, since atopy is characterised by a variety of clinical traits (phenotypes), each trait is likely to have its own set of coding genes. In the study of asthma, key genes are excellent candidates for their involvement based on the scientific understanding of the inflammatory cascade: Polymorphisms for genes coding for interleukins IL-4 and IL-13 (that directly promotes the production of IgE) have been found on chromosome 5, whilst FCER1B (high affinity IgE receptor) exists on chromosome 11 and has been maternally linked to atopy (Sandford et al. 2000). Polymorphisms in these genes increase the risk for high levels of serum IgE and the asthma phenotype (Kabesch et al. 2006; Hizawa et al. 2001).

1.13.8 Atopy in KC

Several authors have found a significantly higher prevalence of atopy in KC groups than controls. A UK study of 182 patients from Moorfields Eye Hospital found a three-fold increase in atopy with respect to non-atopic controls (Rahi et al. 1977). Serum IgE was raised in the KC group over controls, but particularly in those with co-existing atopy and KC. In a Canadian study of 49 cases of KC, complete atopy was present in 20.4% of 49 cases of KC versus 4.2% of 71 controls (Bawazeer et al 2000). Gasset (1978) found the prevalence of asthma to be 17.9% in the KC group versus just 1% of controls. The DUSKS (Dundee University Scottish Keratoconus Study) found a higher proportion of atopic sufferers in their keratoconic population than expected from the normal population. In their KC group, 41.5% had reports of clinical atopic manifestation: asthma (in 23% of keratoconics, 6% of controls) and

eczema (14% of keratoconics, 16% of controls) and hay fever (30% of keratoconics, 16% of controls) (Weed et al. 2007).

Several studies did not find significant relationships between KC variables and atopy. Harrison et al (1989) did not find an earlier onset of keratoconus diagnosis in individuals with atopy, nor did they have a higher rate of keratoplasty; but the keratoconus in atopics was more likely to be bilateral. Lowell and Carroll (1970) studied 31 patients with keratoconus and 30 without, and found no difference in eczema or allergic rhinitis (hay fever) between the groups. Eosinophil count, skin reactivity to allergen and familial atopy were comparable between the two groups.

1.13.9 Atopy in DS

The literature surrounding atopy and DS is dominated by atopic dermatitis. Several papers suggest that a majority of young people with Down's syndrome have atopic dermatitis (Carter 1976; Scherbenske et al. 1990; Thomas et al. 1994). However, Schepis et al (1997) challenged this accepted belief, and identify that such papers citing a high incidence of atopy in Down's syndrome, they have not published the diagnostic criteria used. Their own work suggests a much lower prevalence, of 3%, when facial dermatitis is excluded as a criterion. It is certainly the case that objective identification of atopic traits is fraught with inaccuracy, and there is no commonly accepted diagnostic system. Mannan (2009) used a skin prick test to elicit the response from children with Down's syndrome compared to that found in the general population. Only 18% of cases were found to have a reaction to just one allergen – approximating that of typically-developing children. The author suggests that it is in fact altered DS anatomy causing symptoms of congestion, especially in the case of allergic rhinitis.

1.14 Eye rubbing

“Vigorous” and “habitual” eye rubbing has long since been associated with KC (Ridley 1961). There is also a strong association with eye-rubbing and atopy and Copeman (1965) suggested that atopy provides the stimulus for eye rubbing. This suggestion is supported by modern understanding that the ocular itch that stimulates eye-rubbing in atopic individuals is considered to be a clinical expression of the histamine released by degranulation of mast cells on the ocular surface (Leonardi 2000; Wahlgren 1999). Weed (2007) found that within a keratoconic population, those with atopy rub their eyes more frequently than those without atopy. Lema et al (2009) found that, in the majority of cases of asymmetric KC, the itchiest eye was the one that had the most advanced keratoconus. Corneal epithelial damage through excessive eye rubbing has been implicated as the causation of keratoconus itself (Krachmer 2004). An intact epithelium is thought to protect the corneal stroma, preventing the major corneal inflammatory cytokine IL-1 from spilling into the post-epithelium layers and causing stromal apoptosis of keratocytes (Wilson et al. 2001). Epithelial damage from vigorous eye rubbing may release inflammatory cytokines down into superficial collagen layers that may adversely affect the anterior stromal keratocytes. Apoptosis in the anterior stroma is becoming accepted as an *in vivo* and *ex vivo* hallmark of keratoconus (Kim et al. 1999). The authors suggest that chronic apoptosis of keratocytes release degradative enzymes into the extracellular matrix (degrading the collagen) and that the loss of keratocytes impedes the generation of new collagen into the stroma.

Case studies of patients who are pathological ‘eye-rubbers’ have been published and suggest a strong link to eye rubbing in cases of unilateral disease (Jafri et al. 2004); however, in studies such as these, a longitudinal analysis is required to confirm this finding by following up these subjects to find out if KC develops in the ‘good’ eye (since KC may be bilateral but just markedly asymmetric). Further, not all such case reports use topography and therefore there is the possibility of sub-clinical keratoconus being present in the fellow eye. Korb et al (1995) measured the

forcefulness of eye rubbing in KC versus non-KC controls (both contact lens wearers and non-contact lens wearers) and confirmed that the keratoconics rubbed more forcefully than the other groups, and also that they rubbed in a typically rotary motion with their knuckles (Korb et al. 1995). The strongest evidence of an association between eye-rubbing and KC is the case-control study by Bawazeer et al (2000), where univariate analysis found that atopy, eye rubbing, and a family history of KC were all associated with keratoconus development, but multivariate analysis showed that only eye rubbing maintained statistical significance and it may therefore be causative of the disease in a typical population.

The CLEK study (Collaborative Longitudinal Evaluation of Keratoconus) found that in a large study group of non-DS individuals 48.2% of patients reported rubbing both eyes vigorously whilst 46.3% of patients reported rubbing neither eye (Zadnik et al. 1998). In this case, grading was made by the patient self-reporting “yes/no/unsure” to each eye separately during the ‘examination’ questions. It has been strongly suggested that self-reporting of ocular rubbing is inaccurate due to under reporting by patients themselves in comparison to the observations of family members (McMonnies and Boneham 2003).

The DUSKS study (Dundee University Scottish Keratoconus Study), eye rubbing was measured in two ways (both self-reporting): a closed answer set to the question, “Do you rub your eyes?” (Great deal/A fair amount/Sometimes/Never) and secondly, an analogue scale whereby participants graded their tendency to eye rub by placing an X along a line (Never, A fair amount, Always). The former analogue method of the DUSKS study revealed no significant difference in the rubbing behaviours between the KC subjects (11% reported never rubbing, 48% reported rubbing ‘a great deal’) and the control subjects (4% reported never rubbing, 39% reported rubbing ‘a great deal’). However, when the visual analogue scale was quantified, there was a statistically significant difference between the two groups. It is not made clear to what degree this is, or if it is clinically significant at all.

The evidence for eye rubbing is mixed. While vigorous eye-rubbing appears to be associated with some cases of KC, it appears unassociated in others. In the authors

experience, it is a widely-held clinical view that two groups of keratoconics exist, those who rub in response to a distinct ocular itch and those who appear to have developed eye rubbing in the absence of eye rubbing.

1.14.1 Down's syndrome and eye rubbing

In the non-DS population, eye rubbing has been implicated both in the progression of keratoconus (Bawazeer 2000), the transition to corneal hydrops, and in the aetiology of keratoconus itself (Koenig and Smith 1993). *Corneal hydrops* certainly appears more common in Down's syndrome than that of the typical population (Grewal et al. 1999). However, the evidence to support a strong association, let alone a causation of eye rubbing and the development of KC Down's syndrome weak, yet is frequently cited in scientific literature as the probable cause of the origins of such a high prevalence of keratoconus in people who have Down's syndrome. Table 1.4 provides an overview of this literature along with the supporting evidence for the genesis of keratoconus in Down's syndrome.

Table 1. 13 An overview of the scientific literature attributing keratoconus in DS to eye-rubbing, relevant extracts, and the supportive citations and experimental data.

| <i>Author</i> | <i>Extract</i> | <i>Supporting evidence</i> |
|---------------------------|--|---|
| Fong et al. (2013) | “This, together with frequent eye rubbing, may predispose Down’s syndrome patients to keratoconus” | This statement is not supported by evidence (neither referenced nor experimental work within the article itself) |
| Stoiber et al. (2003) | “Habitual eye rubbing, which is frequently observed in patients with Down’s syndrome and other forms of mental deficiency, has been postulated as an important factor not only for the development of keratoconus itself but also for the progression to the acute condition of the disease” | Cites Pierse and Eustace (1971); see below. |
| Pierse and Eustace (1971) | “Seven mentally defective and ten mongoloid. Eye rubbing has been blamed...” It is not clear if the beginning of the second sentence refers to DS or non-DS eyes. | Cites Ridley (1961); see below. |
| Ridley (1961) | In a letter of correspondence to the British Journal of Ophthalmology: “It might be thought that the rubbing is coincidental and not causal, but the large proportion of patients giving a history of habitual rubbing before the keratoconus appeared leaves little doubt that eye rubbing causes the cornea to give way and is also responsible for the progress of the condition” | No reference to DS. |
| Ozcan and Ersoz (2007a) | Case study report entitled: “Severe acute corneal hydrops in a patient with Down syndrome and persistent eye rubbing”, reads, “Eye rubbing has been implicated in the pathogenesis of KC and often a feature in Down’s syndrome, as seen in our patient”. | “The patient was observed rubbing his left eye [with hydrops] persistently”. It is not clear if this was prior or following the onset of hydrops, or observed by the clinician, or parent/carer. Cites Stoiber 2003 (dealt with above), and (Ioannidis et al. 2005), a case study of an eye-rubbing child without DS. |

| | | |
|--------------------------------|---|--|
| Wylegala and Tarnawska (2006) | “Keratoconus appears in 5.5% of Down’s syndrome patients... This phenomenon is most likely the result of vigorous eye rubbing”, | Supported by Grewal et al (1999), below. |
| Grewal et al. (1999) | “...Patients with Down's syndrome tend to rub their eyes to relieve itching” | Cites (Koenig and Smith 1993); see below. |
| Koenig and Smith (1993) | This paper provides a case report of a 35 year old (non-DS) patient with pathological eye rubbing secondary to ‘personality disorder and self-mutilating behaviour’, who presented with a severe corneal infection and who subsequently developed keratoconus upon healing of the ulcer. Quite separately to the case presented, Koenig et al discuss eye rubbing in other demographics, including DS, whereby “...the high incidence of keratoconus and hydrops in Down’s syndrome may also be related to rubbing to relieve symptoms of itching associated with chronic blepharitis”. | This statement is not supported by evidence (neither referenced nor experimental work within the article itself) |
| Rabinowitz (1998) | “The frequent occurrence of keratoconus has been attributed to a high incidence of eye rubbing in these two disorders, owing to increased blepharitis in Down syndrome...” | This statement is not supported by evidence (neither referenced nor experimental work within the article itself) |
| McElvanney and Adhikary (1997) | “...and the increased incidence of eye rubbing and self-inflicted trauma” | Cites Haugen (1992) – discussed below. |

A single study explores KC specifically within a DS population, as a subset of an institutionalised learning disabled population (Haugen 1992). Quantification of eye-rubbing was estimated by institution staff, into: ‘normal’, ‘often’, or ‘very often’. Eye rubbing ‘very often’ was reported in 3 out of 6 (50%) cases of KC in DS. Healthy DS without KC were used as controls. This small study also found that eye rubbing occurring ‘very often’ was significantly correlated to keratoconus in individuals with intellectual disability generally. However, since half the patients with DS and KC were found not to rub their eyes abnormally, it is difficult to draw firm conclusions about the causal relationship between eye rubbing and KC in DS. Haugen remarks that it is a common surgical experience during corneal grafting of the DS eye, that the consistency and the mechanical properties appear to differ from those KC eyes that do not have DS.

1.14.2 Corneal hydrops

There is anecdotal evidence to suggest that eye rubbing may be an inciting event to the progression to hydrops in both DS and non-DS eyes (Wylegala and Tarnawska 2006; Ozcan and Ersoz 2007b), and there is evidence to the contrary (Rehany and Rumelt 1995; Aldave et al. 2003). In a larger study, Grewal et al (1999), 21 consecutive presentations of hydrops were recorded. Significant eye rubbing was considered present in 15 out of the 15 non-DS cases, and 5 out of the 6 DS cases. It appears that patients with DS are highly represented in this particular study. However, given the relative frequency of DS in the whole population, and the relative frequency of KC within the DS population, it is possible that hydrops in DS is proportionally represented, but the manner in which currently available data has been collected makes it difficult to analyse. Further, the limitations of current studies mean that it is difficult to establish if having DS predisposes the keratoconic individual to develop hydrops or not. If indeed people with DS are more likely to develop hydrops, this may be due to an inherent biomechanical weakness in the DS eye, altered immunological systems, or it may be due to external factors such as eye rubbing. It is conceivable that patients with intellectual disability and who already have KC may rub their eyes in an attempt to ‘clear’ their vision, and are less able to understand the potential impact of vigorous eye rubbing when they do.

Limitations of current studies to determine (a) if eye rubbing in the keratoconic eye leads to hydrops, or (b), if eye rubbing predisposes the healthy DS eye to KC; are:

- (i) Except for Haugen (1992), no control group (healthy eyes with DS) existed to compare eye rubbing in those who have DS
- (ii) Except for Haugen (1992), eye rubbing was not assessed quantitatively or qualitatively
- (iii) Confounding eye rubbing factors of blepharitis or atopy have not been explored

Despite much anecdotal evidence, eye rubbing in DS appears relatively unexplored *experimentally*, and thus a causal relationship either on the initial development of KC, or the progression to corneal hydrops, is so far impossible to draw. Further, since it is now widely considered that a significant link exists between KC and atopy, and there is mounting evidence to suggest that KC itself is an inflammatory disease, it is therefore vital to examine the inflammatory issues associated with KC in DS and explore the factors that may underpin a possible causative relationship between DS and KC.

1.15 Vision in KC

1.15.1 Visual Acuity

Visual acuity (VA), the ability to which the eye can discern detail, is reduced significantly in keratoconus and especially in low luminance and low contrast levels (Gobbe and Guillon 2005; Applegate et al. 2003). VA worsens with the progression of the disease, and this is attributed to the increasing detrimental effects of the optical defects outlined below.

1.15.2 Contrast sensitivity

Patients with KC suffer a loss of contrast sensitivity, particularly under glare conditions (Jinabhai et al. 2012). The reduction in contrast sensitivity found in keratoconics is in line with an increasing optical aberrations seen in the distended cornea (Okamoto et al. 2007). Further, there is likely to be significant additional reduction in corneal clarity (and thus contrast sensitivity) from scarring that occurs as KC progresses.

1.15.3 Myopia & Astigmatism

The changing shape of the keratoconic cornea results in axial elongation of the globe, a reduction in the radius of curvature of the cornea, and the power of the eye as an optical system increases. The refractive error therefore tends towards myopia and this effect is countered with negatively powered spectacles or contact lenses.

A pathological increase in corneal curvature is rarely equal in all meridians, and results in high degrees of corneal astigmatism. This is represented in optical terms by the directions of the flattest and steepest curvatures (principle meridians), and in early keratoconus, when the principle meridians are orthogonal, vision can be corrected with cylindrical correction in glasses (as per regular physiological astigmatism). Regular astigmatism represents a deviation of curvature along two orthogonal principle meridians; in irregular astigmatism, the two principle meridians are non-perpendicular. In advancing keratoconus, the axes may become increasingly

skewed, with increasing differences in curvature such that correction with spectacles becomes practically and cosmetically unacceptable (Shneor et al. 2013).

1.15.4 High order aberrations

Full spectacle correction is unable to correct the optical defects of the keratoconic eye completely, because the distension of the cornea produces additional pathological aberrations. Progression towards a conical shape produces an area of steepening, the ‘cone’, that is rarely co-located with the natural centre of the cornea. This results in a poorly aligned optical system that, as Melamund et al (2006) reports, carries an “asymmetry of focus due to relative hyperopia and myopia present in the same meridian”. For example, an inferiorly displaced cone produces an area of steepened cornea that is inferior to the line of sight, and an area of relative flattening above it. Wavefronts from spot of light would be refracted more in the inferior portion of the cornea with respect to the superior aspect, and would be seen as a spot inferiorly with a flare radiating upwards as depicted in figure 1.33. This is known as coma, and vertical coma represents the decentration of the corneal power in the vertical plane, whereas lateral positioning of the corneal power is reflected by the horizontal coma component. Coma is the dominant higher order aberration (HOA) in the keratoconic eye, increasing with the severity of the disease (Alió and Shabayek 2006).

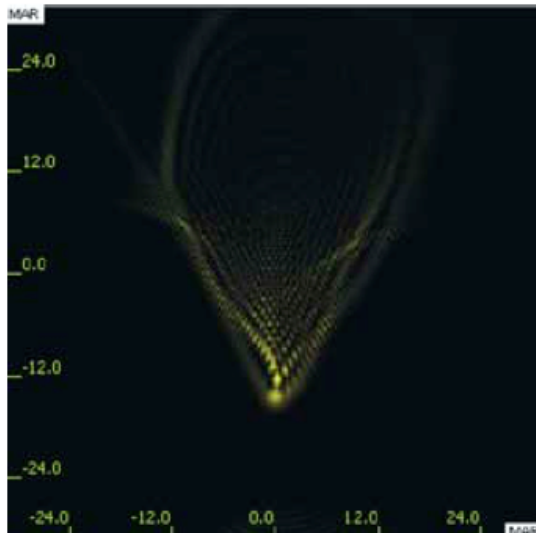


Figure 1.38 A point spread function, demonstrating the often-reported 'flare' perceived by a KC patient, due to significant coma in the eye. Note that in this case, the flare is, rising vertically upwards from a point of relative focus. It is most probable to have arisen from an inferiorly displaced cone. (Jinabhai et al. 2009).

The normal human cornea is an aspheric shape, flattening toward the periphery. This shape compliments the internal ocular surfaces such that peripheral light rays are focussed as closely as possible to those centrally, thereby minimising *spherical aberration* within the eye. As a keratoconic cone develops, the disparity between the central and peripheral powers increase to the extent that the mid-peripheral light rays are increasingly defocussed, producing a detrimental effect on vision (Maeda et al. 2002).

1.16 Vision in DS

1.16.1 Refractive error

Emmetropisation is the tendency toward lower refractive error in infancy. Therefore, a population of babies will have a greater spread of errors than the same group of babies as toddlers, and as children (Brown et al. 1999). The lack of emmetropisation in DS results in much greater magnitudes of refractive error, affecting a greater proportion of the population such that around 60% of children with DS rely on spectacle correction (Cregg et al 2003).

A significant magnitude of corneal astigmatism in the human eye is generally accepted to be $\geq 1.00\text{D}$ in either eye. While most typically developing children ‘grow out’ of their astigmatism, the opposite is observed in DS, whereby an increased prevalence in significant astigmatism is seen in DS relative to peers, and further increasing magnitudes of astigmatism during the early years and throughout adolescence (Woodhouse et al. 1997; Al-Bagdady et al. 2011). These findings are also reported by Haugen (2001) and Little et al (2009). The underlying aetiology of greater astigmatism in DS remains unclear. It is hypothesised that a thinner cornea may permit bending of the cornea from eyelid forces (Haugen 2001) or that reports of ‘slanted palpebral fissures’ give rise to tension necessary to alter the corneal shape and provide large degrees of oblique astigmatism (Read et al. 2007).

A well-held clinical view is that early keratoconus presents with myopic astigmatism (Rabinowitz 1998). This is quite likely to be the case for the general population who are frequently emmetropic, but less likely for those with DS who have a tendency to be highly hyperopic to begin with. In addition, since teenage DS eyes are predisposed to high astigmatism, it is unlikely that the evolution of growing astigmatism and directional change can be relied upon to monitor for the detection of KC in this group, making diagnosis based upon refraction alone very difficult.

1.16.2 Visual acuity and contrast sensitivity

VA in all babies is initially very poor at birth, developing rapidly in the first months and years of life to reach adult levels. From the age of 2, vision in DS is significantly reduced with respect to control subjects (Woodhouse et al. 1996; Tsiaras et al. 1999).

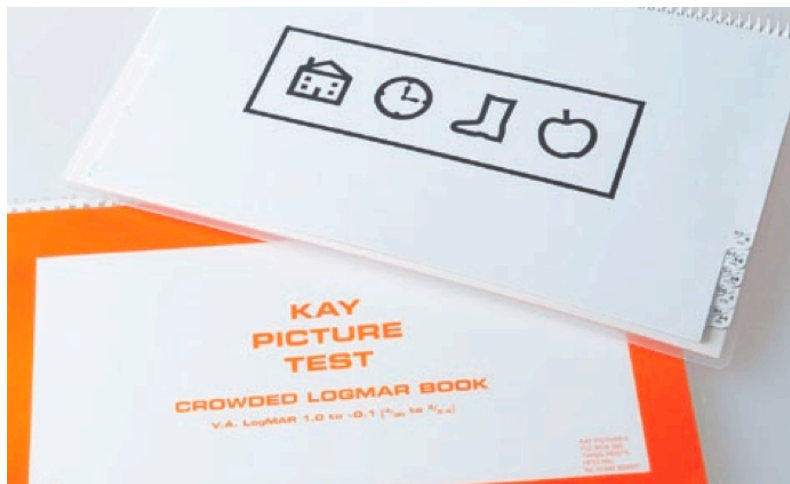


Figure 1. 39 The symbol-matching Kay Picture Test. This recognition acuity test allows the non-verbal patient the opportunity to match a symbol (from the key card on their lap) with a corresponding optotype presented in the distance.

Electrophysiological visual measurement quantifies activity in the primary visual cortex as measured by skin electrodes. The electrical response to patterns of light, diminishing in size, is measured. Electrophysiological testing controls for behavioural variables, and those associated with higher visual, motor and sensory processing systems. Vision in DS subjects as measured by Visually Evoked Potentials (VEPs) for a sine-wave detection stimulus was decreased compared to control subjects' (John et al. 2004), suggesting a disability in vision within the first aspects of neural processing, demonstrating that poor vision in DS is not attributable to motivational or attentional aspects, and that a true deficit in vision exists within optical, retinal or neural factors in DS. This is also demonstrated in the Ts65Dn mouse model of DS (Scott-McKean et al. 2010). Interferometric visual acuity

measurement was used by Little et al to investigate visual acuity in children with DS whilst bypassing the optics of the eye. Since this type of measurement was relatively less affected in DS, the authors conclude that a degradation in optical quality is a major contributor to poor visual performance in this group (Little et al. 2007).

Contrast sensitivity

Sensitivity to contrast is the ability to distinguish between closely matching shades of light and is a useful indicator of ‘real world’ visual function, particularly in low light levels. Patients with Down’s syndrome are known have a reduced sensitivity to contrast (Courage et al. 1997; John et al. 2004), even with full spectacle or contact lenses in place. Since the reduction in contrast sensitivity is in line with the reduction in VA, the deficit in both of these aspects of vision are likely to derive from a common cause. A reduction in contrast sensitivity was also noted in the Ts65Dn Down’s syndrome mouse model with respect to the euploid wild-type mouse (Scott-McKean et al. 2010). Since high contrast VA does not reliably predict low contrast performance, it has been recently recommended to measure both high contrast VA and low contrast VA in patients with DS (Little et al. 2013). This is particularly important for the current study as contrast sensitivity is known to be detrimentally affected in keratoconus (Yang et al. 2014).

1.16.3 Visual Processing

Children with DS appear to have a motion perception deficit that corresponds to an abnormal Visually Evoked Potential (VEP), in common with members of the typically-developing population who have Alzheimer’s disease (Del Viva et al. 2015). Whilst there is a strong body of literature DS and Alzheimer’s disease, suggestive of an abnormal ageing process - it is also of note that the DS brain contains reduced numbers of specific populations of neurons, and abnormalities in dendrites within the cortex (Coyle et al. 1986). It correlates that children with DS may not process the visual world in a typical way, and there remains many aspects of visual processing in DS that are yet to be investigated. An impact of this may be that

young people with DS do not perceive blur in the same way and therefore remain asymptomatic of keratoconus until the moderate or late stages of the condition.

1.16.4 The problem with early detection of KC in DS

In non-DS cases, the diagnosis of keratoconus is usually made after a patient presents to the optometrist with blurred vision. Taken together, prior knowledge of the visual deficits in DS indicate that it would be much more difficult for a patient with DS to:

- (i) Perceive an early vision indicator of KC
- (ii) Articulate a vision change

Further, it would be much more difficult for a clinician to reliably establish whether or not a statistically significant drop in vision has occurred in a patient who has inherently reduced vision, as the confidence interval is greatly increased (KISER et al. 2005).

1.17 Rehabilitation of vision in keratoconus

As described above, increasing irregular astigmatism renders spectacle correction impractical in KC and so it is necessary to rehabilitate vision in another way. Contact lenses rest over the irregular surface and mask many of the irregularities by vaulting over the localised defects and allowing the tear film to fill up the spaces between (Visser et al. 2013). Quality of life is higher in those corrected with contact lenses rather than spectacles (Ortiz-Toquero et al. 2015).

Unfortunately, the pathogenesis of keratoconus can result in changes to the transparency of the corneal stroma, due to the presence of hydrops or corneal scarring and so even a well-fitting contact lens will not improve vision if there is significant scar tissue in the line of sight. Within a 8.5 year period, around 3 in 10 patients will progress to require corneal transplant/graft in order to regain the ocular transparency they will need to see clearly (Weed and McGhee 1998).

Corneal grafting is a major ophthalmic surgery, and there are significant medical risks that accompany the procedure for any patient, as shown in table 1.5. It is of note that the incidence of the infective events appear higher in the studies of those with DS patients, whereas traumatic events appear comparable over the period studied. It is difficult to compare rejection rates since the follow-up periods and the study cohort vary considerably.

Table 1. 14 Surgical risks of corneal grafting for non-DS and DS patients

| <i>Risk</i> | <i>Incidence (non-DS)</i> | <i>Evidence</i> | <i>Incidence (DS)</i> | <i>Evidence</i> |
|-------------|------------------------------|--|----------------------------------|--|
| Infection | 10.5% (8 years) | (Sung et al. 2015) | 14.6% (7 years) 50% (3 years) | (Haugen et al. 2001; Wroblewski et al. 2006) |
| Rejection | 7% (2 years) – 28% (3 years) | (Figueiredo et al. 2015; Bali et al. 2016) | 12.2% (7 years) | (Haugen et al. 2001) |
| Trauma | 2.23% (8 years) | (Tzelikis et al. 2015) | 2.4% (7 years) | (Haugen et al. 2001) |

With such high complication rates, grafting is therefore not entered into lightly, and visual rehabilitation with glasses or contact lenses is usually extensively explored before consideration for surgery of this kind. Furthermore, corneal grafting relies on the supply of healthy donor tissue, which is in short supply in the UK (Gaum et al. 2012).

Additional risks when grafting in Down’s syndrome surround the general anaesthetic needed for the procedure, that puts additional strain on the heart and respiratory system (Kraemer et al. 2010). People with DS are also more prone to infections (Valentini et al. 2015). The ability to comply with post-operative treatment is difficult, but generally patients manage very well after surgery with good support and supervision (Haugen et al. 2001).

It is considered that the early use of corneal crosslinking will reduce the need to graft keratoconus in the longer term (Sandvik et al. 2015). For the reasons outlined above, if crosslinking is successful in this group, it is likely that an early diagnosis of KC in DS would reduce the need for visual rehabilitation with contact lenses, which in the authors experience, is a resource-intensive form of treatment.

1.18 Topography and pachymetry in DS

The difficulties of identifying early keratoconus in DS through subjective measures or prescription analysis have been described earlier. In clinic (non-DS eyes), early keratoconus is ordinarily diagnosed by progressive thinning and steepening of the cornea, in line with slit lamp microscopy signs. Early keratoconus, or sub-clinical *Forme Fruste* keratoconus is diagnosed in the absence of slit lamp signs, and in the absence of symptomatic loss in vision. In such cases, corneal power and thickness maps are used (such as those obtained with the Oculus Pentacam) to classify at-risk eyes, or those presenting with very early clinical keratoconus (Saad and Gatinel 2010; Toprak et al. 2015).

Unfortunately, even the average healthy DS cornea is known to be naturally steeper (Haugen et al. 2001) and thinner (Evereklioglu et al. 2002) than controls, and this presents further problems with the screening of DS patients based on their keratometric power and pachymetry alone. A cut-off set at 2 standard deviations from the mean normal corneal curvature (in non-DS eyes) provides a value of 45.7D (Maeda et al. 1995). Toprak suggests that optimal screening cut-offs for normal corneae are a combination of mean corneal power that lies below 45.2D and central corneal thickness of above 519 μ m. The modified Rabinowitz-McDonnell test, however, is more generous suggesting corneal normality up to 47.2D (Rabinowitz and McDonnell 1989). To the best of the author's knowledge, no literature exists on the exploration of suitable cut-off points in DS eyes.

Table 1.6 summarises the corneal power found in children and young people with DS. From these data, it is clear that screening for KC with the data available from non-DS eyes could produce a significant number of false positives.

Table 1. 15 Table to show existing literature on corneal power in DS vs control subjects.

| <i>Author</i> | <i>Age (years)</i> | <i>Power (D) in DS</i> | <i>Power (D) in controls</i> | <i>method</i> |
|----------------------|--------------------|------------------------|------------------------------|---------------|
| Haugen et al (2001) | 14-26 | 46.39±1.95 | 43.41±1.40 | topography |
| Vincent et al (2005) | 10 months-18 years | 46.66±1.64 | 42.60±1.87 | topography |
| Ji (2006) | 4-16 | 44.5±1.8 | 41.5±1.6 | topography |
| Little et al (2009) | 9-16 | 45.62±2.13 | 43.10±1.37 | keratometer |

Vincent et al (2005) builds on the earlier work by Haugen (2001) to suggest that a significant proportion of eyes with DS carry abnormalities of corneal shape even in the absence of clinical disease; yet the reason for this altered corneal shape in healthy DS eyes remains elusive.

Two hypotheses relevant to the unusual DS corneal shape are:

- I. The abnormal topography in DS is part of an incomplete KC phenotype; or an aborted disease process. An underlying structural abnormality predisposes these eyes to KC.
- II. That DS eyes have steep/irregular corneae as a feature that is unrelated to the risk for, or the development of, keratoconus.

Personal observation to support the second hypothesis is that many DS corneae that have unusual topography are stable for 10+ years and do not develop ectasia in this time. The second hypothesis suggests that the development of keratoconus in DS is not related to the initial shape of the cornea, but to other factors (such as those that are biochemical in nature).

Vincent (2005) discusses possible genetic causes for the increased steepness and suspicious (front surface) topography seen in DS eyes. To the best of the author's knowledge, no research into the specific genetics of KC and DS has been undertaken. Chromosome 21 (triplicated in DS) has however been considered as a candidate chromosome for containing the gene(s) predisposing to KC. Despite this (and analysis of various other chromosomal locations), the mode of inheritance for corneal power and keratoconus is poorly defined (see later).

1.19 Corneal Thickness

Studies into corneal thickness demonstrate a significantly reduced thickness in DS eyes compared to controls. Ultrasound pachymetry revealed corneal thickness of $488\pm 40\mu\text{m}$ (DS) versus $536\pm 21\mu\text{m}$ (Evereklioglu et al. 2002); whilst Scheimpflug imaging revealed $480\pm 40\mu\text{m}$ versus $550\pm 30\mu\text{m}$ (Haugen et al. 2001). Haugen also demonstrated that ultrasound pachymetry carried good agreement with Scheimpflug pachymetry measurement, and found that corneal thickness was not influenced by age or gender. Haugen suggests that the reduced corneal thickness reduces mechanical rigidity of the DS cornea and in turn may be responsible for increased levels of astigmatism and KC in DS eyes. Little (2001) in a study of 29 DS individuals, later found that corneal astigmatism or curvature were not related to the total ocular refraction of the DS eye. This has recently been challenged by (Knowlton et al. 2015), who in a larger study of DS eyes, found that corneal astigmatism was predictive of refractive astigmatism (both in magnitude and in direction). Scheimpflug imaging in Haugen (2001) suggest that the relative corneal thinning is largely at the level of the corneal stroma. It is not clear if the reduced corneal thickness is due to a reduction in the total collagen available, or if the corneal collagen is simply compressed in DS. It is possible that the composition of corneal collagen in DS differs from that expected of a typical non-DS population.

The exploration for the genetics of corneal thickness in non-DS eyes has been relatively successful. Dimasi (2009) suggests that corneal thickness is one of the most highly heritable human traits.

Twin studies are useful in the investigation of heritability: In a UK/Australian study, CT was comparable in sets of monozygotic twins, whilst carried much less similarity in the dizygotic twins (Toh et al. 2005). Since monozygotic twins carry the same genetic makeup, CT appears strongly influenced by genetics and is considered to be of high heritability. The Guangzhou Twin eye study revealed similar findings in Chinese participants (Zheng et al. 2008)

A sample of ethnicity studies (table 1.6) reveal that certain populations appear to have thinner corneae, even when the discrepancies between the methods used have been accounted for (Tam and Rootman 2003; Amano et al. 2006). It is of interest that the Mongolian population have the thinnest corneae, because it was the likening to this population by John Langdon Down that gave the Down's syndrome population the historical term, 'Mongoloid', in the paper, 'Ethnic classification of Idiots' in 1866.

Table 1. 16 A sample of the variation in corneal thickness with ethnicity.

| Author | Method | CT | Ethnicity |
|-----------------------|---------------------------------------|----------|---------------------------|
| Suzuki et al. (2005) | Specular microscopic pachymetry | 518±30µm | Japanese |
| Foster (1998) | Optical (pachymetry) | 505±32µm | Mongolian |
| Landers et al. (2007) | Ultrasound pachymetry | 512±35µm | Aboriginal Australians |
| Landers et al. (2007) | Ultrasound pachymetry | 542±33µm | Caucasian Australians |
| Aghaian et al. (2004) | Ultrasound Pachymetry | 570±32µm | Chinese |
| Aghaian et al. (2004) | Ultrasound Pachymetry | 525±38µm | African |

Studies of known genetic disorders demonstrate a significant departure from the expected values of corneal thickness in certain groups. Ehlers Danlos Syndrome

(EDS type I/II – affecting collagen type V) revealed reduced CT ($436\pm 13\mu\text{m}$) compared to controls ($569\pm 28\mu\text{m}$). The affected corneae were also steeper than controls ($46.30\pm 1.36\text{D}$ and $44.11\pm 1.21\text{D}$ respectively). Patients with osteogenesis imperfecta (affecting collagen type I) have significantly reduced CT ($460\pm 25\mu\text{m}$) versus controls ($544\pm 21\mu\text{m}$) when measured with ultrasound pachymetry (Evereklioglu et al. 2002). Collagen I and V appeared to co-exist in the human cornea, and mutations in human (collagen I) and mouse (collagen V) lead to a reduction in the diameter of collagen fibrils, which was considered to be the cause of reduced corneal thickness in EDS and OI (Mietz et al. 1997; Segev et al. 2006).

In humans with posterior open-angle glaucoma, mutations were found in the COL8A2 gene in patients with a corneal thickness below $513\mu\text{m}$ (Desronvil et al. 2010). Despite this, Aldave et al (2007) found no association with these genes in a group of keratoconic patients.

Marfan's syndrome, characterised by a defective fibrillin gene appears to result in a reduced corneal thickness versus controls ($502\pm 42\mu\text{m}$ and $552\pm 24\mu\text{m}$ respectively) alongside a flatter central cornea ($40.8\pm 1.4\text{D}$ and $42.9\pm 1.1\text{D}$ respectively). The authors suggest that this combination may be as a result of globe enlargement, as the morphologic abnormalities of the elastic components of the eye allow stretching of the globe (Sultan et al. 2002). This suggestion would correspond with the significant myopia in this group (Maumenee 1981). Since the converse is seen in DS, with a large prevalence of hyperopia (Akinici et al. 2009) and increased corneal power (Haugen et al. 2001), it is unlikely that the aetiology of the thinner cornea in Marfan's syndrome is comparable to that of the thin cornea seen in DS.

1.20 The genetics of keratoconus

Keratoconus (non-DS) typically manifests in the second or third decade of life and its prevalence is thought to vary between 0.057% and 0.229% depending upon ethnicity (Pearson et al. 2000) and the method of case selection (eg based on slit lamp signs or topography). Keratoconus appears sporadic in the vast majority of cases (Rabinowitz et al. 1999; Wang et al. 2000), however there are occasions when the disease appears to co-exist with other diagnoses. Down's syndrome is a well-known association, and the prevalence estimates are detailed in table 1.8.

Table 1. 17 Table to show prevalence of keratoconus in studies of patients with Down's syndrome. It is notable that the studies with a higher prevalence of keratoconus are those that include older patients.

| Author | Age (years) | Subjects | Prevalence |
|----------------------------|--------------------|-----------------|-------------------|
| Pires De Cunha (1996) | 0-18 | 152 | 0% |
| Roizen et al (1994) | 0-19 | 115 | 0% |
| Wong & Ho (1997) | 0-13 | 140 | 0% |
| Berk et al (2009) | 0-25 | 55 | 0% |
| Liza-Sharmini et al (2006) | 0-17 | 60 | 0% |
| Kim et al (2002) | 0-14 | 123 | 0% |
| Fimiani et al (2007) | 0-18 | 157 | 0% |
| Doyle et al (1998) | 15-22 | 50 | 2% |
| Jaeger (1980) | 15-64 | 75 | 2.7% |
| Cullen & Butler (1963) | 2-53 | 143 | 5.5% |
| Walsh et al (1981) | 5-60 | 88 | 8% |
| Shapiro & France (1985) | 7-36 | 53 | 15% |
| Haugen (1992) | 15-90 | 30 | 20% |
| Hestnes et al (1991) | 21-72 | 30 | 30% |

The common occurrence of keratoconus within a geographical cluster, a family or particular groups with a chromosomal disease has led research into the possible genetic basis of the condition.

1.20.1 Aggregation studies

Aggregation studies observe the clustering of disease within families is studied to establish if it is significantly atypical to that observed in the general population. Segregation Analysis uses the study of offspring to propose the mode of inheritance of the condition - patterns of offspring affected and unaffected by keratoconus are compared statistically to probabilities expressed from binomial distribution analysis. While Wang and colleagues proposed an autosomal recessive mode of inheritance (Wang et al. 2000). Rabinowitz proposes an autosomal dominant mode; whereby high degrees of regular astigmatism, irregular astigmatism or keratoconus fruste are assumed to represent incomplete expression of the keratoconus gene, this trait is found across three generations (Rabinowitz 1998). Many researchers suggest a complex mode of inheritance with involvement of several alleles that is not represented by Mendelian genetics. However, this also raises the possibility that the advent of topography has exposed variations in corneal anatomy that previously may be considered 'normal'.

1.20.2 Geographical populations

Nottingham (1854) was the first to document the apparent prevalence of keratoconus in certain countries. More recent epidemiology studies confirm an increased incidence of and severity in keratoconus across Asian populations with respect to that seen in Whites (Georgiou et al. 2004). A hospital study based in the English Midlands found that not only is there an increased prevalence in the Asian population, but that this group demonstrated an earlier age of onset, more rapid progression, and a 4 fold greater proportion of Asians than Whites who ultimately progress to requiring a corneal graft (Pearson et al. 2000). Keratoconus appears to be very rare in Japan, Russia and Macedonia whilst very common in Iran, India and Israel (Gordon-Shaag et al. 2012). Suggestions of a link with consanguinity, or relatively genetically-isolated populations have been proposed (Woodward 1984).

1.20.3 Genetic syndromes & Linkage analysis

The association of other genetic syndromes with KC is widely accepted. Down's syndrome, Leber's congenital amaurosis, and Ehlers-Danlos syndrome have all been linked with keratoconus (Weed et al. 2007; Robertson 1975; Damji et al. 2003). Further, the associations with various particular connective tissue disorders or syndromes (osteogenesis imperfecta, mitral valve prolapse, floppy eyelid syndrome), are of particular note here but will be discussed at length later in this chapter (Beardsley and Foulks 1982; Beckh et al. 1995; Naderan et al. 2015; Robertson 1975).

Most conditions described above have been attributed to a particular genetic region. Within a selected chromosome, 'linkage studies' allow gene mapping of suspected faulty genes-the closer these genes lie together, the more likely it is that they have been inherited together and the more significant the finding. Many keratoconus linkage studies have taken place and notable genes have been published in recent years include VXS1, SOD1, LOX1-4, COL4A3 and COL4A4 (Stabuc-Silih et al. 2010). An overview of these and other significant genetic investigations in KC are given in table 1.9.

Table 1. 18 Notable candidate genes in the genetic studies of KC

| <i>Gene</i> | <i>Evidence</i> |
|-----------------|---|
| VSX1 | Inconclusive. VSX1 has been reported as a candidate gene for KC in a small proportion of cases in an Indian population (Tanwar et al. 2010) and Iranian population (Saeed-Rad et al. 2011), but this was not confirmed in a Saudi Arabian population (Abu-Amro et al. 2011). Studies have struggled to locate VSX1 within the cornea (Heon 2002) and others could not confirm a reported association (Moschos et al. 2013; Aldave et al. 2006). |
| LOX | Implicated by (Hasanian-Langroudi et al. 2015) as a significant risk factor in an Iranian population, but excluded in an Italian population studied by (De Bonis et al. 2011).. Significantly down-regulated in the ocular surface of KC eyes (Rohit Shetty 2015). |
| SOD1 | Some found a 'possible causative role' (Moschos et al. 2013) and others indicate that whilst the genetic variant is established, the pathologic link has not been remains unknown (De Bonis et al. 2011). |
| COL4A3 & COL4A4 | (Stabuc-Silih et al. 2009) Found no association between mutations in collagen type IV (from COL4A3 and COL4A4 genes) and KC, but require further examination using function assay, since some differences in genotype did exist between the KC and healthy groups. |

| | |
|-----------------|--|
| COL4A1 & COL4A2 | Polymerase chain reaction amplification excluded mutations in $\alpha 1$ and $\alpha 2$ as a cause of KC in 15 Ecuadorian families (Karolak et al. 2011) |
| COL5A1 | Li et al. (2012) found a non-protein coding regions suggested to contribute to corneal thickness in normal eyes and in KC |
| COL6A1 | Linkage analysis excluded a gene locus for KC on the distal region of chromosome 21 in a family with an autosomal dominant keratoconus (Rabinowitz et al. 1992). |
| CRB1 | Leber Congenital Amaurosis is associated with KC and a common genetic mutation was found on chromosome 1 (McMahon et al. 2009) |
| 5q14.1–q21.3 | Additional chromosomes were studied for the family above, suggesting a genetic site 5q14.1–q21.3 would provide candidate genes for KC (Rabinowitz 2005). |
| GPC6 | Burdon (2015) found that the GPC6 gene on chromosome 13 was a plausible candidate gene for KC. |

None of these genes point specifically to a locus for keratoconus present on chromosome 21, the genetic region triplicated in DS. However, it is well accepted that, given the heterogenetic nature of KC, that a combination of activated genes on different chromosomes may result in the phenotype of KC, or that the cause of KC may be an indirect result of a gene located on chromosome 21.

1.21 Down's syndrome

Triplication of the twenty-first chromosome in the developing embryo results in Down's syndrome. Unlike many trisomies, trisomy 21 is 'compatible with life', so often results in the birth of a healthy baby that will need minimum medical intervention in the early days and years after birth. As the life expectancy of someone with DS grows, so too does the necessity to provide optimal vision for the duration of life, and protect against visual impairment from KC.

People with Down's syndrome carry several advantages over those with the standard number of chromosomes, including a reduced incidence of tumours (Sullivan et al. 2007) and a very low risk of atherosclerosis (Vis et al. 2009) both of which, to some degree, are thought to represent an altered immune response. Down's syndrome, however, carries many significant challenges, both in physical and mental health, and is the primary genetic cause of significant intellectual disability worldwide (Roizen and Patterson 2003).

Since industrialisation in developed countries, those with intellectual disability were removed from their families, growing up in institutions or hospitals, without stratification of individual needs or capabilities (Noll 1995). It was an English doctor, John Langdon Down who first noticed the striking physical and behavioural similarities between many residents was famously identified and named after an, in the paper, 'Ethnic classification of Idiots' in 1866. Rados (1948), an American clinician was the first to describe keratoconus in Down's syndrome, after seeing two patients with both bilateral KC and a diagnosis of DS in quick succession.

1.21.1 Classifications of Down's syndrome

Three different classifications of DS are used and these are of genetic importance in research:

Trisomy 21

Meiosis of gametes necessitates the splitting of each of the 23 pairs of homologous chromosomes in the cell into two halves. But an egg, for example might have 22 normal half-chromosomes and one that has not shed its other half – this is known as nondisjunction. Post-fertilisation, the addition of a healthy paternal set results in the embryo now comprising 47 chromosomes, and further mitosis means that every cell in the body of the foetus will then be affected. This cascade of events is known as Trisomy 21, and is the most prevalent type of Down's syndrome.

Mosaicism

The nondisjunction in mosaicism occurs after fertilisation of two normal gametes is complete, during the mitosis that allows the embryonic ball of cells to grow. This results in two different cell lines within one individual, and as different embryonic cells are destined for different tissues, some parts of the body will be affected, and others unaffected. Even within a tissue, a fraction of the cells may be affected (Wiseman et al. 2010). The phenotype is therefore variable, and some individuals will be affected to a greater or lesser degree than others. Mosaicism is uncommon in DS, accounting for 2-4% of those affected by DS as a whole, although it is of note that due to the nature of mosaicism, the clinically diagnosed population may be smaller than what actually exists. Due to the unpredictable nature of the proportion of ocular tissue affected, for the purposes of this research, potential subjects with DS mosaicism have been excluded. Mouse models of DS inherently carry mosaicism to some degree (Reeves 2006).

Robertson Translocation

This occurs when an additional copy of a chromosome 21 exists, but is combined with another chromosome, usually 14, 15 or a fellow 21. Only an unbalanced form of Robertson's translocation will cause a DS phenotype, a balanced form will produce a typically developing child. The extra genetic material can also be carried and passed on unknowingly by individuals with the 'balanced' form and is therefore the only classification of DS that is hereditary. Robertson's translocation accounts for 1 in 10 of those diagnosed with DS (Mikkelsen 2009). For the purpose of this research, Robertsonian translocations have not been excluded, since individuals with the DS phenotype have all relevant genes affected.

1.21.2 Gene Dosage

The classical phenotype of DS is thought to be attributable to a gene-dosage effect. That is, that the additional copies of the genes on chromosome 21 cause an over-expression in the features that the regular genes would usually code for (Antonarakis et al. 2004). However, not all of the genes on chromosome 21 are likely to be dosage-sensitive, and so it is necessary to examine how expression levels of the genes are each affected by the additional copy of the chromosome, and how they interact with each other (Korenberg et al. 1994; Wiseman et al. 2009b).

Although certain traits are present in all individuals with trisomy 21, the degree to which an individual is affected will vary from one person to the next. Finding out which genes, when triplicated, cause the features that are characteristic in DS, is the aim of genetic DS research in general (Wiseman, Alford, Tybulewicz and Fisher 2009a). This is particularly the case for pathology that is extremely common in DS such as Alzheimer's disease or congenital heart defects – in which studying the genetics of the ordinary population would be unfeasible due to the relatively low prevalence. Characterising Alzheimer's disease in DS may one day unlock the secrets to specific conditions found across the world's population as a whole (Korenberg et al. 1994).

If there is a genetic link between DS and KC, it may be direct or indirect in nature. It is possible that the ‘gene dosage’ directly magnifies the effect of the keratoconic genetic code on chromosome 21; but it is also possible that the dosage of trisomy of chromosome 21 modulates a keratoconic gene at another locus (on a different chromosome) (Vincent et al. 2005).

1.21.3 Congenital Heart Defects in DS

The incidence of congenital heart defects (CHD) in newborn babies with DS is strikingly high, with most reporting an incidence of between 40-60%, although structured epidemiological research indicates that the prevalence is likely to be at the lower end (Freeman et al. 1998). The most common defects are those affecting the septa between the hearts chambers, the ventricles and the atria (figure 1.40). (Patent ducts that do not require surgery and close spontaneously in the early part of life are not considered CHD, as they also occur in the general population).

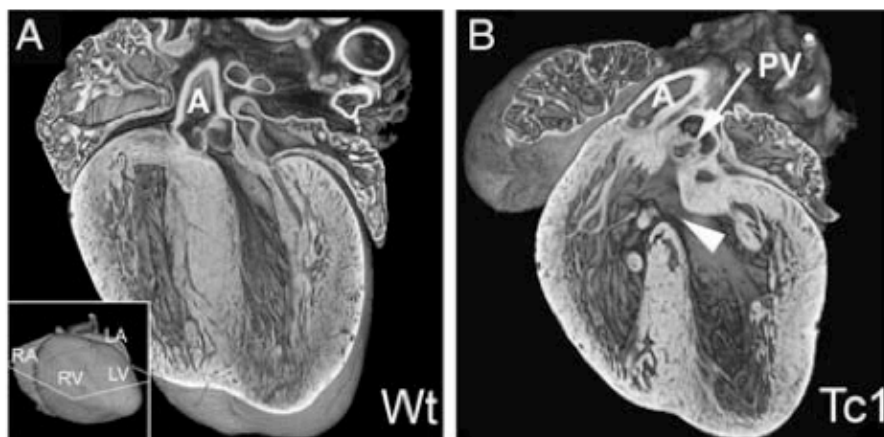


Figure 1.40 Ventricular septal defects in DS (*Tc1*) mice and wild type (*Wt*) (control) mice. Note the aberrant closure of the divisional wall in the centre of the heart (B), and the healthy heart in (A). (Dunlevy et al. 2010).

The cause of such an increased incidence of CHD has been the subject of much research as it is the primary risk factor for early death in DS, and because babies with DS make up 50% of all babies born with CHD (Freeman et al. 1998). The characteristic nature of CHD in DS indicates that genes located on chromosome 21 are normal cardiac valve and septal development (Gittenberger-De Groot et al.

2003), and the characteristic defects in DS suggest that at particular places and times during embryonic development, the additional genetic material in trisomy 21 causes specific disruptions in normal cardiac development (Freeman et al. 1998). The Tc1 mouse model displays a comparable incidence of the specific CHD seen in DS (Dunlevy et al. 2010) (Figure 1.35).

An underlying collagen abnormality is thought to produce such a striking DS phenotype, and led to investigations of the proteins coded for by chromosome 21 and their impact upon cardiac valve development. Collagen VI and other aspects of the ECM in trisomy 21 have been implicated in the formation of CHD (Klewer et al. 1998; Duff et al. 1990).

The co-incidence of ocular abnormalities and heart defects in children with DS has been of interest to several groups and raises the possibility that the conditions may derive from a common genetic weakness. Gardiner (1967) was the first researcher to suggest a possible link between the high myopia that was prevalent in his group, and with the CHD also prevalent in DS, although did not elude to why this might be the case. Cunha followed this up and found a modest association - just 13 out of 89 hyperopics had heart defects versus 27 out of 60 of myopes (DA CUNHA and DE CASTRO MOREIRA 1996). Afifi found that 7 of 9 children with DS and myopia had a heart defect (Afifi et al. 2013). Bromham and colleagues found an association between myopia and CHD, suggesting that visual pathway damage may be a common cause (Bromham et al. 2002). However, in a recent paper with DS subjects, of whom 51 had CHD, Ljubic found that myopia did not correlate (Ljubic et al. 2015). As previously discussed, collagen type VI is found in corneal and scleral tissue as well as in the developing heart, and is just one possible source of common pathology if overproduced by the trisomy of chromosome 21.

Congenital Heart Defects in KC and Evidence of KC as a connective tissue disorder

Mitral valve prolapse (MVP) is the non-closure of bicuspid cardiac valve and is seen in around 0.7% of the young non-DS population, and 2.4% of adults (Seguela et al. 2011). Several reported incidences of MVP in the DS population exist between 14%

and 50% (Barnett et al. 1988; Goldhaber et al. 1986). MVP can be the result of congenital malformations of the valve, connective tissue disorders, from degenerative heart damage or from a combination of these. Regardless of aetiology, most cases are accounted for by a genetic cause (autosomal and X-linked inheritance) (Guy and Hill 2012). For several decades, there has been much interest in the co-incidence of KC and DS, with mixed findings: some finding that MVP appears more commonly in KC than in those without KC (Beardsley and Foulks 1982; Sharif et al. 1992) and others indicating either no correlation (Street et al. 1991) or no clinical consequence (Moodaley et al. 1992). In a study examining the presence of keratoconus in 36 patients diagnosed with MVP, Lichter found a high prevalence of asymptomatic, unilateral KC in 22% of patients (compared to one patient, 4%, in the control group). Lichter used a front-surface topographer and automated detection system (Lichter et al. 2000). Since the presence of KC was not necessarily defined by the presence of slit lamp signs (undisclosed) nor the progression of the condition, it is not possible to be sure that the findings are not merely forme fruste KC or another abnormal topography. If the results do represent true keratoconus, then the findings certainly do appear significant. The possible co-incidence of heart problems and eye problems (MVP in KC and CHD/myopia in DS) is certainly intriguing and requires further research into connective tissue ultrastructure. Dudakova suggests that a decreased availability of cross-linking enzyme, lysyl oxidase, may be a common aetiology that could account for abnormal collagen metabolism in both MVP and KC (Dudakova and Jirsova 2013).

Non-cardiac connective tissue findings in DS

Joint hypermobility and poor muscle tone is very prevalent in DS. In the non-DS population, joint hypermobility is found 5 times more commonly in KC versus non-KC eyes (Woodward and Morris 1990) – leading the authors to suggest that keratoconus is a localised manifestation of a mild connective tissue disorder. Further evidence for their hypothesis is that floppy eye lid syndrome and sleep apnoea are each associated with keratoconus (Ezra et al. 2010). Floppy eyelids are defined as those with a loss of rigidity allowing the lid to be folded over itself with ease, thought to be the result of the upregulation of elastolytic (MMP) enzymes causing

elastic fibre degradation in the tarsal tissue. Reduced presence in histological staining of elastin in the floppy eyelid syndrome is seen in figure 1.41 (Schlotzer-Schrehardt et al. 2005).

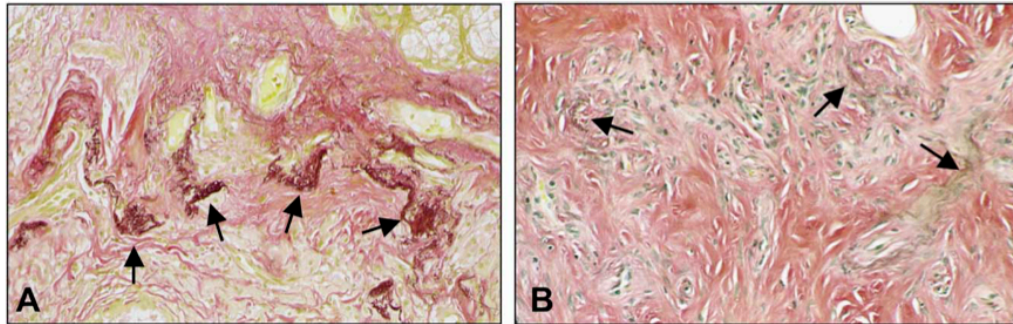


Figure 1. 41 The collagenous tarsal stroma in a healthy eye (A) and an eye with floppy eyelid syndrome (B). Elastic fibres (elastin) appear dark brown when stained, and are marked with arrows. There is notably less elastin present in the floppy eyelid (B). (Schlotzer-Schrehardt et al. 2005)

Evidence of a connective tissue disorder that affects ocular tissues is provided by ultrastructural studies that examine abnormality at the level of the proteoglycan core protein (Chakravarti et al. 1998). A lumican knock-out mouse exhibits skin weakening alongside reduced corneal transparency. This appears to result from abnormal collagen fibril genesis (figure 1.42). These results are interesting, since keratoconus has long been considered a loss of corneal biomechanical integrity with is possibly associated systemic collagen defects such as Ehlers-Danlos syndrome and Cutis Laxa, some of which also exhibit skin laxity in humans (Cameron 1993; Woodward and Morris 1990). Since, in humans, the corneal transparency in keratoconus is generally much better retained than evident in these mice, it is possible that this mouse is showing an exaggerated systemic phenotype of some collagen disorder, which may relate to KC in some way.

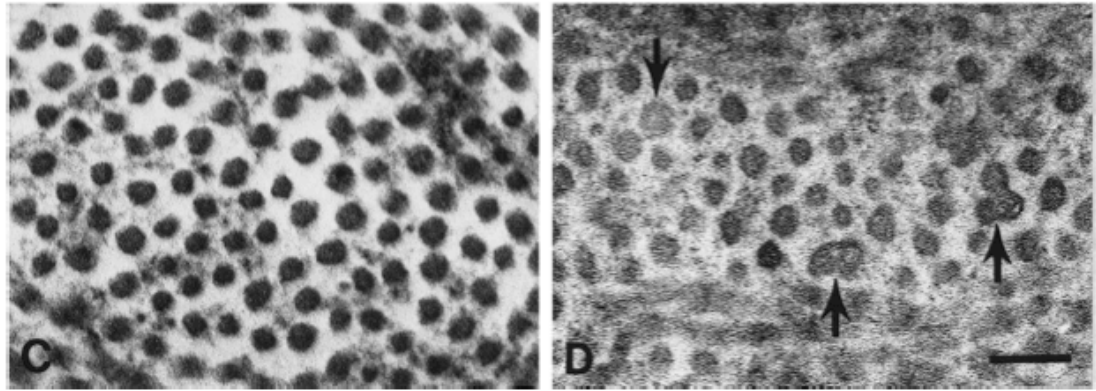


Figure 1.42 Fibril organisation in the posterior cornea of wild type mouse (C) and lumican-null mouse (D). Arrows point to larger, and abnormally shaped fibrils seen in the lumican-null mouse, that are likely the cause of the reduced transparency. Chakravarti et al (2000).

At the ultrastructural level, healthy DS skin also shows abnormalities. While collagen VI is abundant in non-DS endocardial tissue differentiation, occurring at 5-8 weeks gestation, an abnormally large deposition of collagen VI was noted in the developing heart of all trisomy 21 human embryos, all of whom had heart defects (Gittenberger-De Groot et al. 2003). The authors report that the pattern of collagen VI distribution was considered to be in some way be related to the shear stress forces in the normally developing heart, as another load bearing tissue.

Often the first clinical indication of DS is during routine ultrasonography, in which the unusual presence of a relatively transparent nuchal skin fold is imaged in the developing foetus. Ultrastructurally, this is due to an abnormally high concentration GAGs associated with an unusual abundance of collagen VI (Kaisenberg 1998; Brand-Saberi et al. 1994). Figure 5.8 shows the schematic representation of DS (left) and control (right) nuchal skin, with several distinct differences that are represented in figure 1.43 (Brand-Saberi et al. 1994):

- Nuchal skin is significantly thicker in DS
- Collagen I network was less dense in DS, fibrils were more widely spaced
- Collagen VI network was unusually dense throughout the thickness of the dermis
- Collagen VI was randomly orientated in DS, and in parallel with the dermis in controls
- Hyaluronan was abundantly present in DS, whilst scarcely present in controls

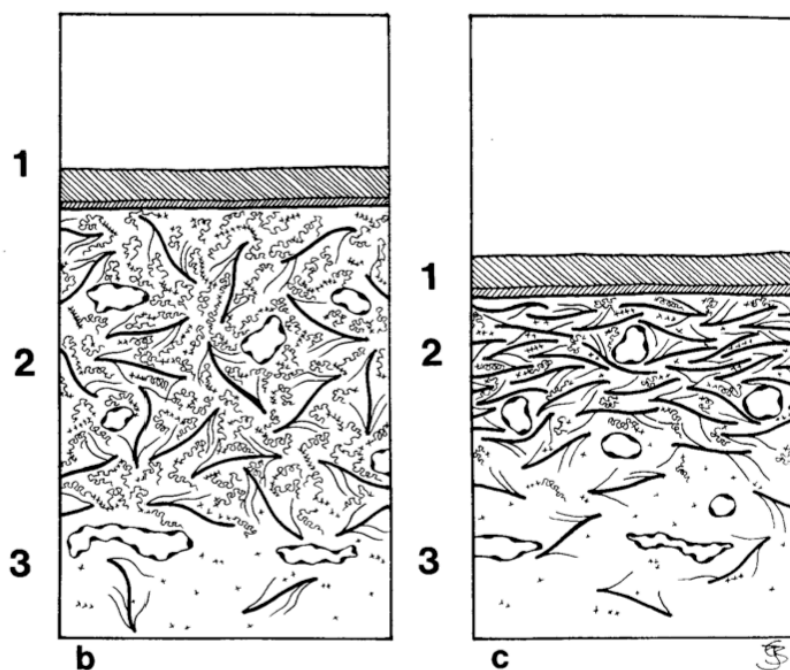


Figure 1.43 Schematic representation of histological sample of the fetal nuchal skin in (b) DS and (c) non-DS. DS tissue (b) harbours an abundance of collagen VI and of hyaluronic acid, with widely space collagen I. Adapted from Brand-Saberi et al. (1994).

Since collagen VI $\alpha 3$ is produced on chromosome 2, it is considered that trisomy 21 could distort the ratio of available ($\alpha 1$: $\alpha 2$: $\alpha 3$) from 2:2:2 (non-DS) to 3:3:2 (DS); thereby allowing for composites consisting exclusively of $\alpha 1$ and $\alpha 2$, and the potential to significantly alter biomechanical dynamics of DS connective tissue (Weil D 1988; Duff et al. 1990).

Hyaluronan, or hyaluronic acid (HA) is highly hydrophilic, and binds strongly to collagen VI (Kielty 1992). (Raio et al. 2004) propose that an increase in type VI collagen by the COL6A1 gene may contribute to an alteration in hyaluronan metabolism resulting in an accumulation of low molecular weight hyaluronan accumulation in DS tissue (Raio et al. 2004; Raio et al. 2005). Karousou et al (2013) later found an increased expression of ‘hyaluronan synthase-2’ an enzymatic precursor to hyaluronan is closely related to the increased expression of the COL6A2 gene found in DS. Conversely, Barlow et al (2001) provide evidence against the disturbance of collagen VI in their 8 human subjects with mosaic DS, some of whom

did not possess triplication of the chromosomal area responsible for the production of collagen VI, despite having CHD.

Currently, there are no studies of the collagen and proteoglycans in a DS mouse model, but these would be useful to investigate if abnormal collagen or PG content may alter the corneal biomechanics in DS.

1.22 Summary and aims of thesis

KC appears much more prevalent in DS than non-DS eyes, yet the identification of early ectatic disease in this group is difficult. Fundamentally, these DS eyes are known to be of an altered shape, yet it is not known if this altered shape leads to biomechanical weakness and the development of KC. Further, it is not known whether the KC seen in DS is of a phenotype similar to that seen in the non-DS population, or if the ectasia is a completely different disease in this group (as seen in a genetically aberrant mouse model).

Eye-rubbing is frequently postulated as the aetiology of KC in DS, yet this traumatic and subsequently inflammatory (biochemical) hypothesis is at odds with the understanding that the DS eye exhibits altered shape and thickness, and the possible biomechanical implications. Existing literature suggests that subtle but fundamental collagen changes that could affect corneal biomechanics are present in DS, yet there appears to have been no ultrastructural investigation of the DS cornea. In particular, no *in vivo* corneal biomechanics have yet been assessed in DS.

This thesis will examine the clinical correlates of KC in DS from a primary care and a topographical perspective. The study will also examine corneal biomechanics in human DS eyes, and probe the ultrastructure in a DS mouse model. It assess if the ectasia in DS shares a common phenotype with non-DS KC in terms of the cone morphology and finally evaluate if KC in DS is a suitable model for the study of keratoconus as a whole.

Chapter 2

Clinical correlates of keratoconus in Down's syndrome

2.1 Introduction

Since the introduction of CXL, there is a necessity to diagnose and refer KC as early as possible in areas where it is available. The early identification of KC in DS is difficult since this group are typically much less able to perceive or articulate early vision loss and so KC in this group may go unnoticed for a number of years. Successful early CXL in DS would stabilise the ectatic cornea before much structural damage has occurred - in particular, before corneal scarring or excessive curvature changes are established, and before the patient becomes dependent on contact lenses or requires a corneal graft. At the time of writing UK CXL guidelines necessitate at least 375 μ m of residual corneal thickness at the thinnest point. Since people with DS typically begin with a healthy cornea of just 475 μ m, this leaves much less tissue as 'buffer' for the period before treatment. Therefore, individuals with DS may need targeted screening and monitoring guidelines.

One aspect of considering whether screening for KC in DS is feasible, is the establishment of the clinical risk factors for KC in a DS group and associations with other aspects of general health, to see if a potential screening population can be refined. In primary care optometry, it is important for a clinician to know the optometric risk factors for KC in DS, in order to aid case finding for onward referral. This is particularly difficult in DS because the cornea is already steeper and the baseline refraction already more astigmatic than that of a non-DS population (Woodhouse et al. 2007). Finally, examining the attributes of KC and healthy eyes within a DS population may be of use in considering underlying aetiology of the disease.

Extensive literature exists indicating that eye rubbing is causative of the high prevalence of keratoconus in a population with Down's syndrome; however, in all but one case this appears unexplored experimentally (Fong et al. 2013; Wylegala and Tarnawska 2006; Stoiber et al. 2003; Haugen 1992). Further, the relevance of

possibly significant confounding variables of atopy and blepharitis have not been explored (Daneshpazhooch et al. 2007; Jaeger 1980). The current study aims to explore the correlates of eye rubbing in KC in DS.

2.2 Methods

2.2.1 *Participants*

Participants were recruited from an ongoing study of 210 children and young people with DS at Cardiff University. The first of these participants were initially systematically recruited through the Cytogenetics Department at University Hospital of Wales, Cardiff, upon the post-natal diagnosis of DS. Therefore, there are extensive optometric records for these patients and some previous extended research data available. Other participants were initially undergoing a regular examination at Cardiff University when they opted to join the cohort. Further participants heard about the KC study online or through the UK KC foundation newsletter and were enrolled into the cohort; all these had KC. Therefore, the Cardiff University ‘cohort’ is the base sample, and the ‘current study’ denotes selected patients from this group who opted to participate in the ‘Keratoconus and Down’s syndrome’ research.

2.2.2 *Ethics*

Ethical Approval was gained from NHS Research Ethics Service and all aspects of the study are aligned with the Declaration of Helsinki (Appendix D).

2.2.3 *Consent*

Participants were provided with study information sheet (appendix A) and consent form (appendix B) prior to the date of visit, and upon arriving both the participant and their parent/carer were talked through the consent form and given the opportunity to ask any questions before taking part. When the young person was able to, they too signed the consent form. The young people with DS taking part in the

study were given lots of time to ask questions about the research, and were reminded that they could change their mind about participating at any time.

2.2.4 Inclusion & Exclusion criteria

Any participants with DS between the ages of 6 and 40 were welcomed to the study. Participants with mosaicism were excluded. Those eyes with a history of injury, infection or surgery were excluded.

2.2.5 Diagnosis of keratoconus

Diagnosis of keratoconus was confirmed with Scheimpflug tomography (Oculus Pentacam, Weltzar, Germany) in all cases, and confirmed by the progression of corneal thinning and steepening over subsequent visits. This was independently confirmed by a keratoconus-specialist optometrist or ophthalmologist. All subjects underwent an extensive optometric examination to include visual acuity, measurement of contrast sensitivity, binocularity, retinoscopy, slit lamp examination and retinal examination.

2.2.6 Atopy Questionnaire

Questionnaires were used to elicit the atopic history of individuals within the study (appendix C). Classification from the 'UK working party's diagnostic criteria for atopic dermatitis' was used to group existing skin conditions into partial atopic dermatitis, complete atopic dermatitis, or none (Williams et al. 1994). This was combined with data of a history of asthma, and hay fever or other allergies to determine the atopic status of the patient – again defined as partial atopic, complete atopic, or none. The final questionnaire included open questions surrounding allergy in order to capture immune responses to allergens not listed (for example penicillin allergy that would be representative of a significant IgE response). Dry skin is particularly common in Down's syndrome, and care was taken not to overestimate

the prevalence of atopic dermatitis by asking questions about acne and dry skin in order to aid parents and carers to differentiate from the more classic allergic responses (such as inflammation on the skin creases – flexures), and provide a more accurate response. The presence of, or a history of, dry skin in the absence of any other atopic sign was therefore not graded as atopic dermatitis.

Presence of eczema alongside a history of all 3 of: dry skin, flexural irritation and either asthma or hay fever was deemed as complete atopic dermatitis. Eczema alongside one or two of dry skin, flexural irritation or asthma/hay fever was classed as incomplete atopic dermatitis, in line with the UK Working Party's classification.

It is well documented that underdeveloped canaliculi and nasolacrimal ducts cause watery and sticky eyes in Down's syndrome in the absence of an allergic trigger (Mannan et al. 2009), so those symptoms alone were not considered indicative of atopy. For similar reasons, suspect hay fever in the absence of formal medical diagnosis or habitual seasonal treatment was therefore not included as a positive result.

2.2.7 Eye rubbing questionnaire

Patients often under-report intensity of eye rubbing (McMonnies and Boneham 2003). In order to gain a more objective reflection of eye rubbing tendency, parents or carers were asked to fill in a questionnaire estimating the frequency of eye rubbing to either eye on an average day. Since a discussion of eye rubbing may well initiate the behaviour in the young person, written information about this aspect of the study was given to parents/carers out of sight from the young person, and questions regarding it were asked out of earshot. Parents were given the questionnaire in advance of the appointment to bring completed on the day, or invited to return it after the eye examination, by post or email. Eye rubbing tendency by observation was divided into one of the following categories in table 2.1. Those whose parents or carers noticed that they rubbed their eyes only when tired (and therefore comparable to typically developing children) and not during the course of the day, were classed as non eye-rubbers.

| | Eye rubbing tendency |
|---|----------------------|
| Not noticed at all | |
| little: once or twice daily | |
| moderate: three to five times daily | |
| regularly: six to ten times | |
| frequently: eleven to twenty times daily | |
| excessively: more than twenty times daily | |

Figure 2.1 Extract from atopy questionnaire from the current study

For graded analysis, the eye rubbing categories were retrospectively reduced to three: “Not noticed at all” with “Little”, “Moderate” with “Regularly” and finally, “Frequently” with “Excessively”. For analyses that required a binary split between significant and non-significant eye rubbing, the following categories were grouped together: “Not noticed at all” and “little”, “moderate” were grouped as ‘non-significant’. “Regularly”, “frequently” and “excessively” were grouped as ‘significant’. Whilst the level of choice available to the parent/carer demonstrated the necessity for a specific answer, the grouping provided the power to the statistical analysis.

2.2.8 History Taking

The participant’s own ocular health was noted to include spectacles, amblyopia and strabismus, history of contact lens use, injuries/infections, ocular surgery or other ophthalmic diagnoses.

The presence or absence of a CHD was noted, and the type where known. Further, a medication list was taken and the young person and their parent/carer were asked specifically about anti-inflammatory medication use, in line with the atopic history taking.

2.2.9 Optometric examination procedure

- i. Retinoscopy – to assess refractive error and to evaluate the clarity of reflex
- ii. Monocular visual acuity measurement using Kay Picture test when possible
- iii. Monocular contrast sensitivity measurement (Cardiff Contrast test)
- iv. Binocular alignment assessment using Hirschberg test, and when possible cover test
- v. Scheimpflug Imaging using Oculus Pentacam (Chapter 3)
- vi. Slit lamp examination

Retinoscopy

Mohindra retinoscopy (Mohindra 1977) was attempted on all participants so that a subjective refraction could be attempted immediately after if possible. The refractive error was neutralised for both eyes and the resulting prescription recorded in sphero-cylindrical format, alongside the quality of the reflection (reflex) seen. In order to reflect possible disease states in KC, the reflex was categorised as either normal or abnormal (split/swirling). Both the magnitude and the direction of the astigmatism were conserved by means of astigmatic decompensation (Thibos et al. 1997). This ‘breaks down’ a single vector component into two constituent parts, 45° apart. The constituent parts are represented along 180° (C_0) and 45° (C_{45}), where $C_0 = C \cos(2\alpha)$ and $C_{45} = C \sin(2\alpha)$ (Fig 2.2)

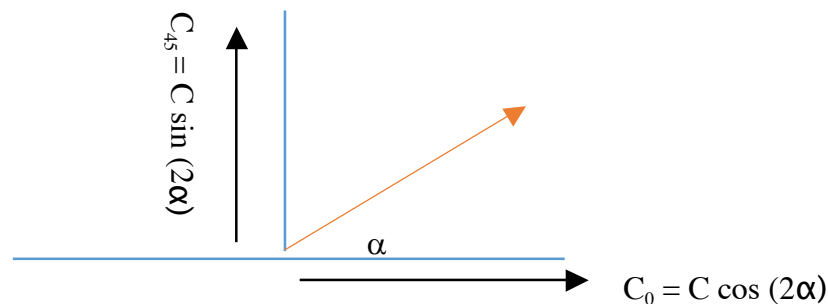


Figure 2.2 Astigmatic decompensation of cylindrical value into its C_0 and C_{45} components.

Slit lamp biomicroscopy

Slit lamp examination was attempted on all participants, and participation maximised through having a parent/carer attract attention with a small toy behind the ears of the examiner. Data were collected on:

- i. Blepharitis – anterior and posterior
- ii. Corneal clarity, with scarring graded as per the Gestalt scale
- iii. The presence/absence of corneal thinning, Munson’s sign, Fleisher’s ring, corneal nerves.
- iv. Any anomalies detected in the anterior chamber.

Corneal scarring

Gestalt grading was used in the current study, mirroring others (McMahon et al. 2006; Szczotka-Flynn et al. 2008), to identify two important clinical parameters: (i) the proximity of the scar to the line of sight (LOS) and (ii) the density of the scar. Graded in 0.5 steps, the scale is shown in table 2.1. Corneal scarring >G2.0 was deemed clinically significant and incorporated into the analysis. The advantage of this scale over the Amsler-Krumeich is that it reflects the variable clinical presentation of corneal scarring (its location, size and density) rather than simply the presence or absence of scarring.

Table 2.6 Gestalt grading scale for assessing corneal scarring

| <i>Grade</i> | <i>Features</i> |
|--------------|--|
| 1.0 | Trace and not on LOS, <1.5 mm total size |
| 2.0 | Easily noticeable and approaching LOS, 1.5–2.5 mm total size |
| 3.0 | Dense but translucent and impinging on LOS, total size 2.5 mm or greater |
| 4.0 | Opaque and on LOS, size 2.5 mm or greater |

2.2.10 *Grading keratoconus in DS*

The potential for misclassification in DS eyes using the modified Amsler-Krumeich grading scale (Alió and Shabayek 2006) is large since this scale was derived using assumptions based on refractive error, CCT and dioptric power of non-DS eyes, when in fact the baseline parameters of healthy DS eyes are significantly different to those of DS eyes. By using the modified Amsler-Krumeich scale, it follows that stratifying DS eyes by corneal power, CCT and regular astigmatism could over-grade KC in DS, while requiring myopia for the classification of early KC would under-grade the disease in DS (since high hyperopia is so prevalent in DS).

In order to prevent misclassification of KC in DS eyes, it was decided to grade the KC in such a way that the resulting grade represented the clinical severity and clinical implication of the disease on the subject in question, and primarily the intervention required to optimally rehabilitate the patient's vision. Since VA, corneal power and corneal thickness are altered in DS (and since it is not known how each interact specifically with KC), the diagnosis of KC and the grade had to be made without those parameters and as such these aspects were removed from a potential grading scale. Instead the grading from 0 to 5 was based upon retinoscopy, slit lamp examination and severity of the topographical pattern and the actual or likely path to visual rehabilitation. Since KC is often asymmetric, each eye was evaluated separately. Since longitudinal optometric data were already in place for each participant, the VA prior to KC was known for those in the suspect/early group. Therefore, the 'optimal' vision level to rehabilitate towards was known by the examiner. The 'study grading scale is outlined in table 2.2.

Table 2.7 New keratoconus grading scale for the current study

| Study Grade | Status | Clinical data used | Visual Rehabilitation |
|-------------|----------------|---|---|
| 0 | Healthy | Clear ret reflex, no dioptric asymmetry on topography (other than that explained by regular astigmatism) | Nil – glasses / contact lenses for standard refractive error only |
| 1 | Suspect / FFKC | Abnormal ret reflex and/or abnormal topography | Nil – glasses / contact lenses for standard refractive error only |
| 2 | Mild KC | Abnormal ret reflex and abnormal topography with or without SL signs. Spectacle correction and BCVA. | Glasses to incorporate astigmatic correction attributable to KC |
| 3 | Moderate KC | Abnormal ret reflex and abnormal topography with or without SL signs. Spectacle correction and or Contact Lens correction and BCVA. | Vision correctable to optimal level* with contact lens |
| 4 | Severe KC | Abnormal ret reflex and abnormal topography with SL signs. Spectacle correction and or Contact Lens correction and BCVA. | Vision corrected to sub-optimal level with contact lens |
| 5 | End stage KC | Abnormal ret reflex and abnormal topography with severe SL signs. Spectacle correction and or Contact Lens correction and BCVA. | Visual rehabilitation not possible / hydrops |

*Optimal level was defined as the usual visual acuity for a patient with DS – 6/12

This new study grading scale is a clinical tool designed to stratify patients based on need and therefore grade necessary clinical intervention/outcome to rehabilitate vision. This will allow classification of patients in a way that is independent of refractive error, numerical topographical characteristics, CCT and should be transferrable across equipment, and both primary and secondary care.

2.2.11 Statistical analysis of data

Statistical analysis of the correlation between keratoconus and each variable was carried out using the Chi-squared test (with Yates' correction where appropriate) in SPSS (Version 23.0, IBM, Chicago, Illinois, USA).

2.3 Results

2.3.1 Subjects

There were 45 subjects enrolled in the current study, all of whom had DS. One subject had significant autism and was unable to tolerate one eye being occluded; they were removed from the analysis. Therefore 44 DS participants (11 with keratoconus) completed this aspect of the study. Two eyes were excluded, one due to a history of retinal detachment (this subject had KC) and one healed corneal ulcer (this subject had no KC). This left 86 eyes of 44 subjects (21 KC eyes, 8 suspect eyes, 57 healthy eyes). There was a higher percentage of male subjects (61%) than female subjects (39%). Participants ranged from 7.5 to 27.8 years (19.5 ± 8.3 , mean \pm S.D.), covering the high-risk age range in which keratoconus typically develops.

2.3.2 Correlations of eye-rubbing and atopy

Data from eye-rubbing and atopy questionnaires were analysed using Chi-squared statistics in order to measure the independence of the two categorical probability distributions presented, by comparing difference between the expected frequency with the observed frequency. The significance of the association between the nominal variables is presented in table 2.3.

Table 2. 8 A summary of the relationships explored using the Chi-squared test for statistical significance

| Feature exhibited | Correlated with | Significance (p value) |
|-------------------------|-------------------------|------------------------|
| Significant eye rubbing | KC | 0.81 |
| Atopic dermatitis | KC | 0.88 |
| Hay Fever | KC | 0.80 |
| Allergy | KC | 0.64 |
| Significant atopy | KC | 0.73 |
| Significant atopy | Significant eye rubbing | 0.05 |

Table 2.3 demonstrates that in the current study, KC is not significantly correlated with atopic dermatitis, hay fever, allergy, nor atopic status as a whole. The association between atopic status and the extent of eye rubbing was examined, and the association approaches significance, so it is likely that eye-rubbing was dependent upon atopy in this group, results that are in agreement with non-DS research (Copeman 1965).

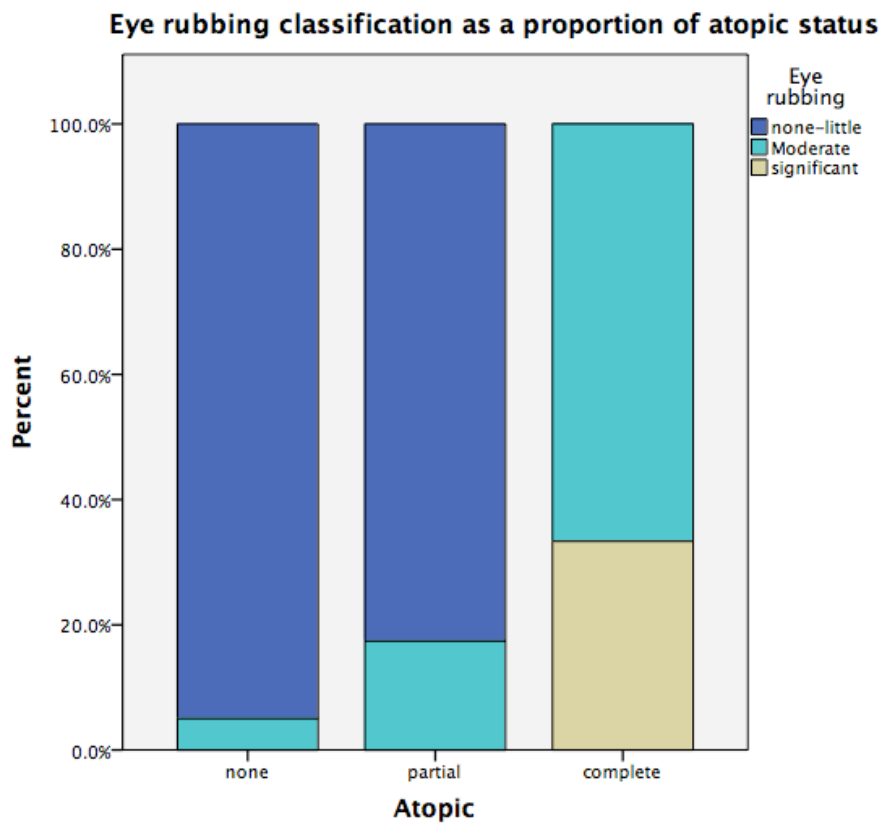


Figure 2.3 Eye rubbing classification as a proportion of atopic status. This illustrates that patients with atopy exhibited more eye rubbing in the atopic groups. Significant eye-rubbers were only found in the complete atopic group.

While associated with atopy, eye rubbing, however, was not associated with keratoconus. In addition, no ‘frequent’ or ‘excessive’ eye rubbers were found within the keratoconic group, as shown in figure 2.4 and figure 2.5.

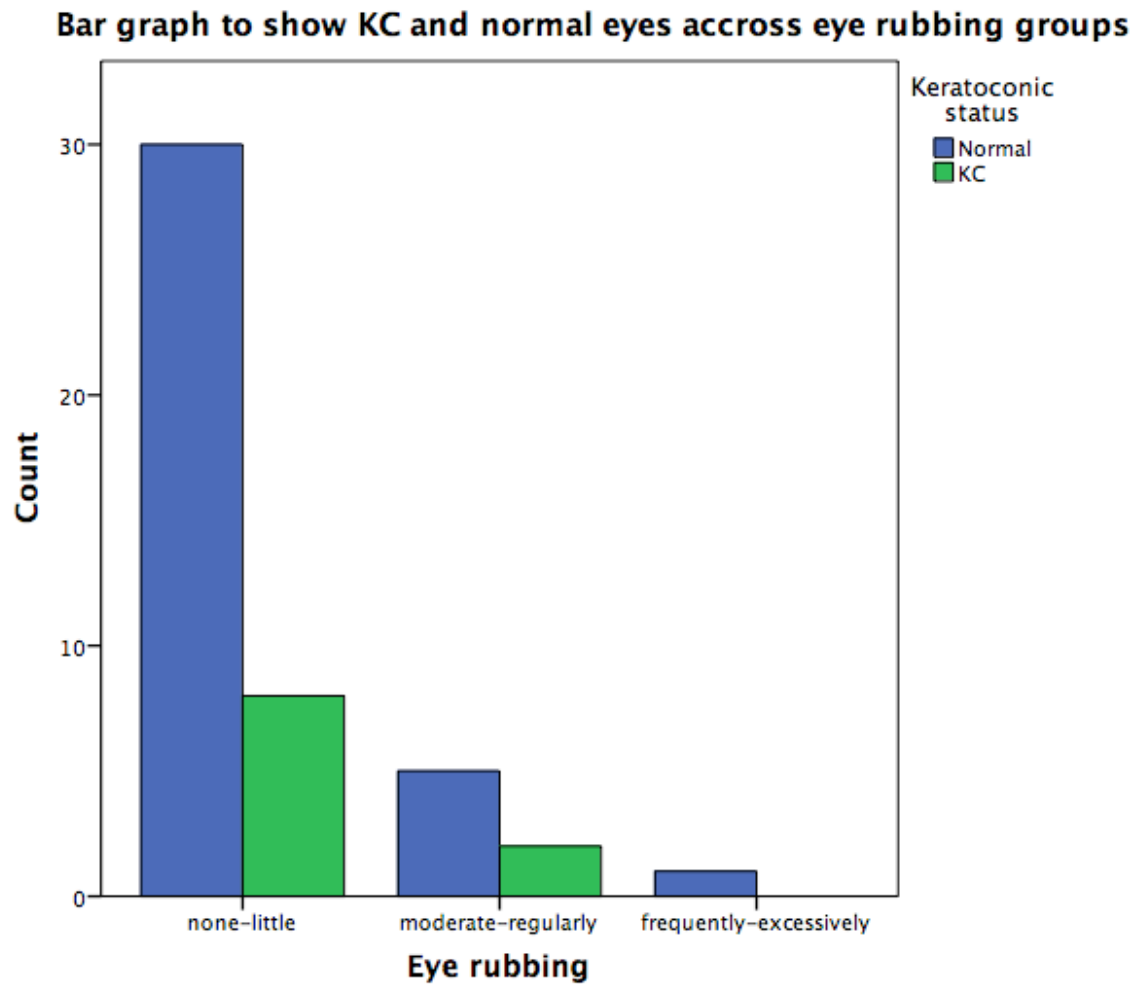


Figure 2. 4 The distribution of keratoconics and non-keratoconics across eye rubbing categories.

Proportion of eye rubbing category comprising normal and KC groups

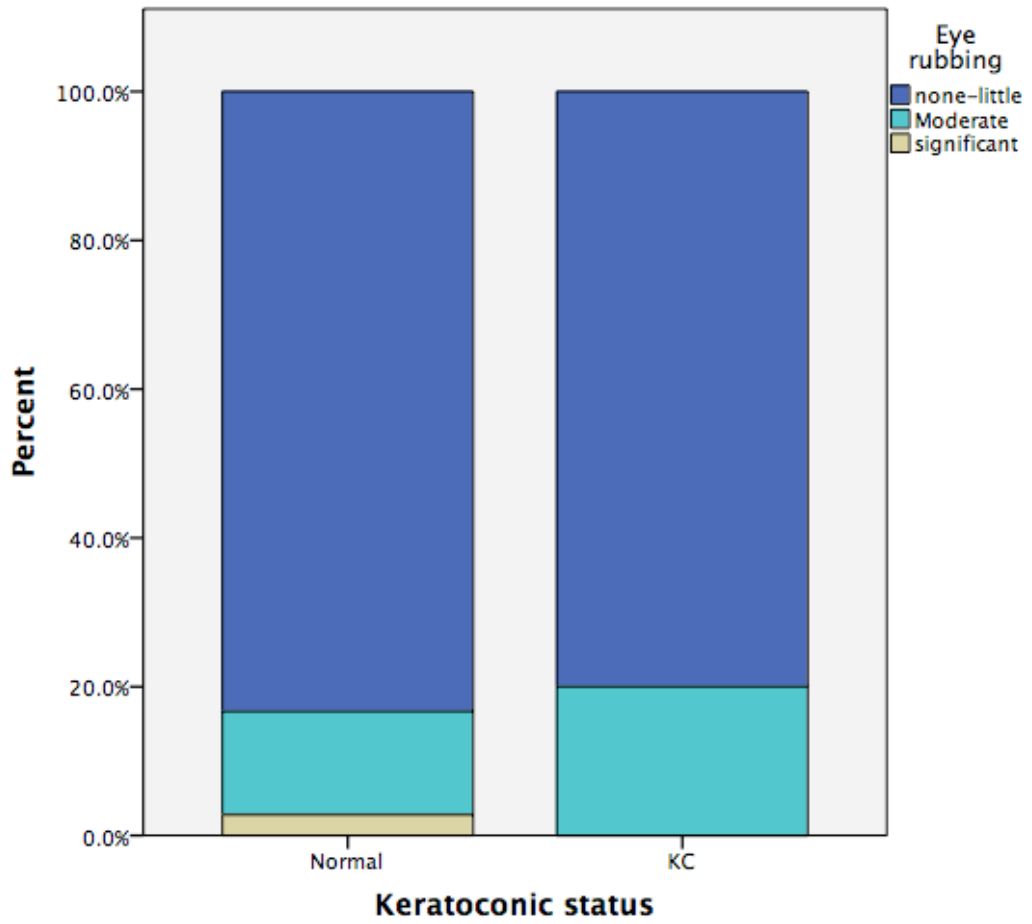


Figure 2. 5 An alternative presentation of the data in figure 2.4. The bar chart highlights the relative proportions of eye-rubbing in the KC and non-KC groups.

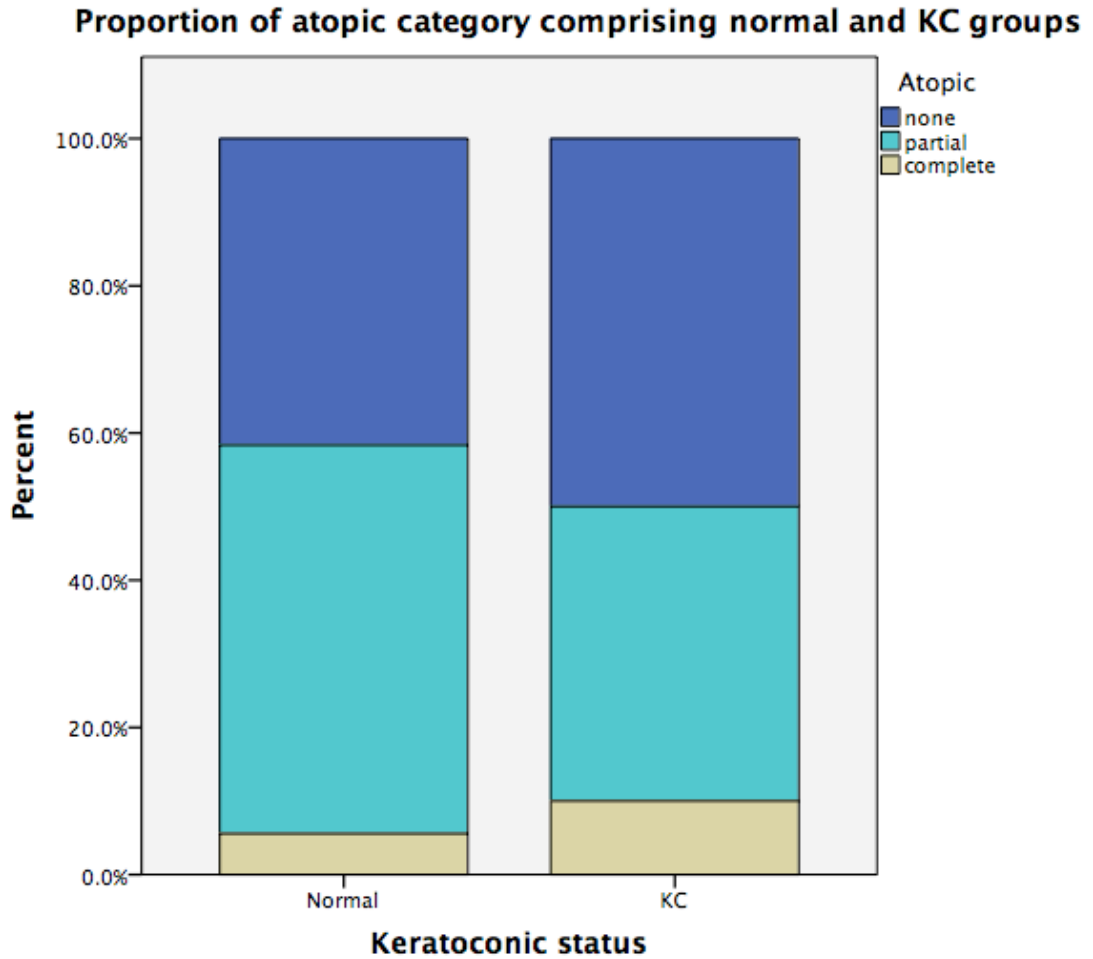


Figure 2.6 The proportion of atopic category comprising normal and KC groups.

Figure 2.6 demonstrates that absent, partial and complete atopy are comparably represented in the keratoconus and non-keratoconic Down's syndrome population. Those with partial or complete atopy are not over-represented in the keratoconic group.

Hay fever, as a component of atopy most likely to affect the ocular surface, is strongly associated with KC in non-DS individuals (Rahi et al 1977). It was therefore evaluated separately to investigate a relationship with KC. Results are shown in figure 2.7. No association was found ($P=0.80$), indicating that the presence and absence of keratoconus is represented similarly in those with and without hay fever.

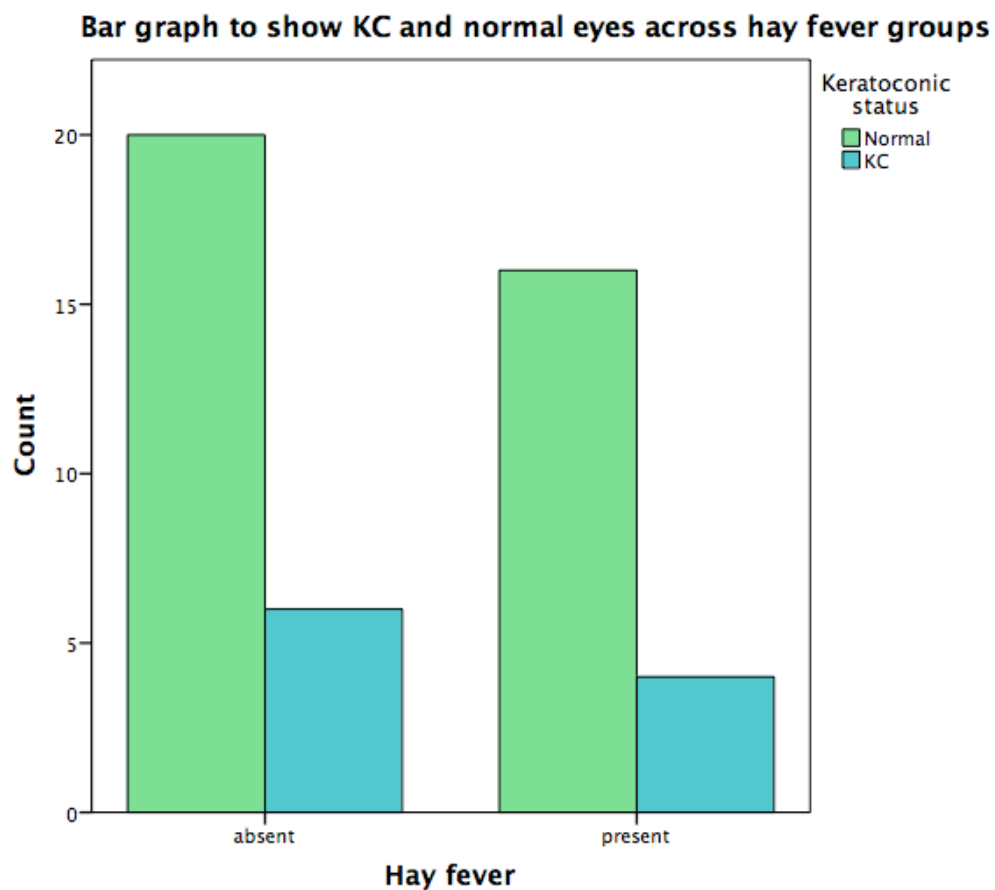


Figure 2.7 Bar graph to show KC and normal eyes across hay fever groups.

2.3.3 *Retinoscopy*

Retinoscopy assessment was performed successfully on all eyes of all 44 subjects. An abnormal ret reflex was found in 21 of 21 KC eyes and in 2 out of 8 KC suspect eyes. Of the eyes of participants with no KC, all (57) had a normal ret reflex (by definition). For the identification of KC eyes, this provides a sensitivity of 100% and a specificity of 96.9%. For the identification of KC and KC suspect (combined), this provides a sensitivity of 79.3% and a specificity of 100%.

2.3.4 *Refractive error*

Objective refractive error was attempted on all subjects, and successful in 57 out of 57 healthy eyes, 8 out of 8 suspect eyes and 10 out of 21 KC eyes. Failure to obtain refractive data was due to moderate & severe KC eyes having a reflex too distorted to gain a meaningful refraction.

Scatter graph to show the spread of spectacle-derived cylindrical refractive error in healthy and KC/KC suspect eyes

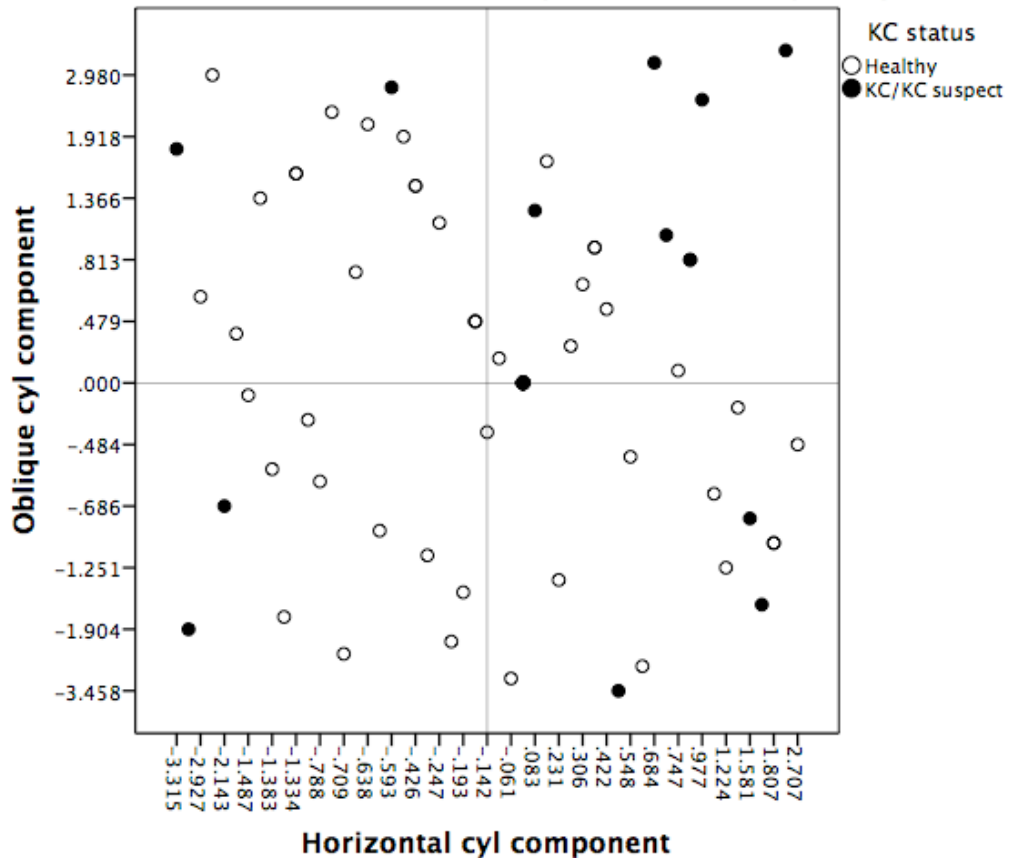


Figure 2.8 Scatter graph to show vector analysis of the astigmatic component of spectacle refraction

Figure 2.8 shows astigmatic decompensation of the cylindrical components of the prescription for all eyes. The keratoconic prescriptions (closed circles) are, on average, further from the origin of the graph, denoting that the astigmatic value is higher, as is expected in keratoconus. Data points around the horizontal indicate that the cylinder lies around 180° or 90°, and the vertical axis indicates an oblique prescription at 45°. No apparent patterns of this nature exist, and astigmatic values appear randomly spread. The extent of the overlap in the magnitude of astigmatism in healthy and keratoconic eyes is shown in figure 2.9.

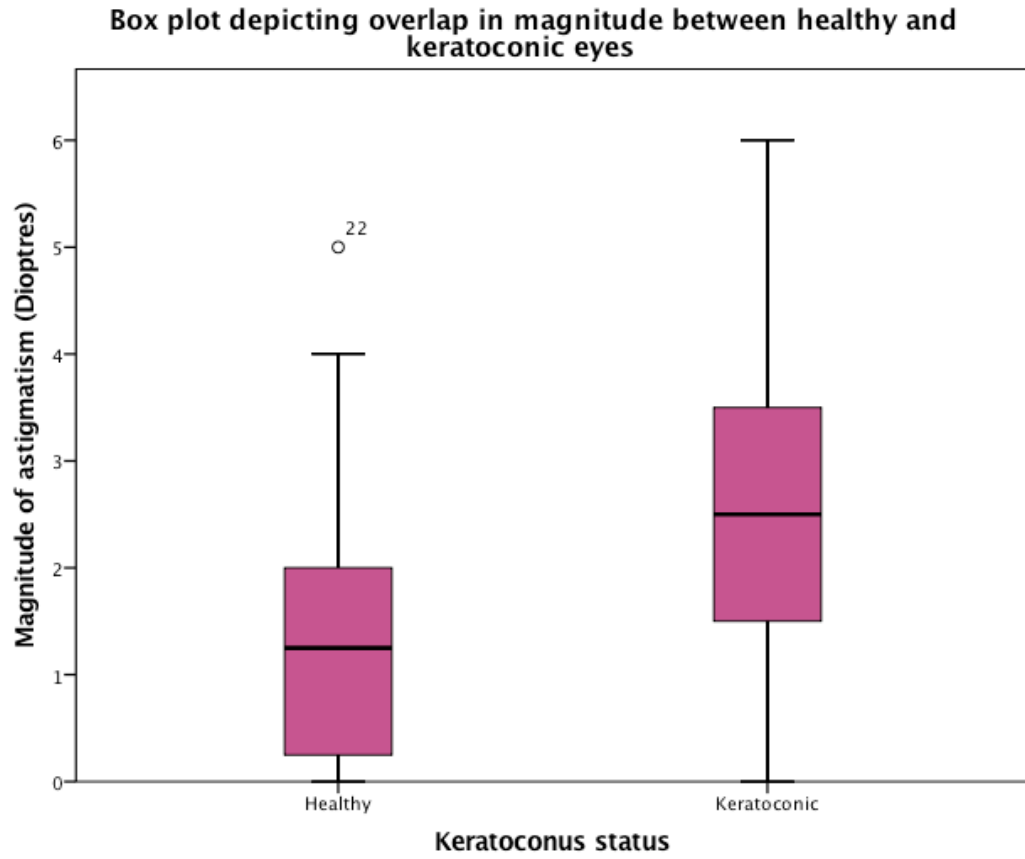


Figure 2.9 Box plot demonstrating the overlap between the magnitude of astigmatism in healthy and keratoconic DS eyes. Note that although the means are significantly different (by 1.2D), the extent of the overlap at the higher values provides poor discrimination between physiological and pathological cases.

2.3.5 Visual Acuity

Best corrected LogMAR visual acuity using crowded Kay Picture test was attempted for all subjects, except for 3 participants whose ability meant that they were able to perform a detection acuity task only and Cardiff Acuity Picture test was used. Kay picture test was used at 3 metres and Cardiff Acuity test results were converted to comparable measurement through their respective logMAR values.

For analysis, eyes that had ‘counting fingers’ or ‘perception of light’ were excluded from the analysis. Two such eyes had study grade 4 KC and two had study grade 5 KC, and were removed from analysis. This left 81 eyes.

The significant overlap in VA between healthy, and KC and KC suspect eyes (figure 2.10 and figure 2.11) demonstrates that the two groups cannot be separated by VA alone, and that VA is a poor predictor of KC.

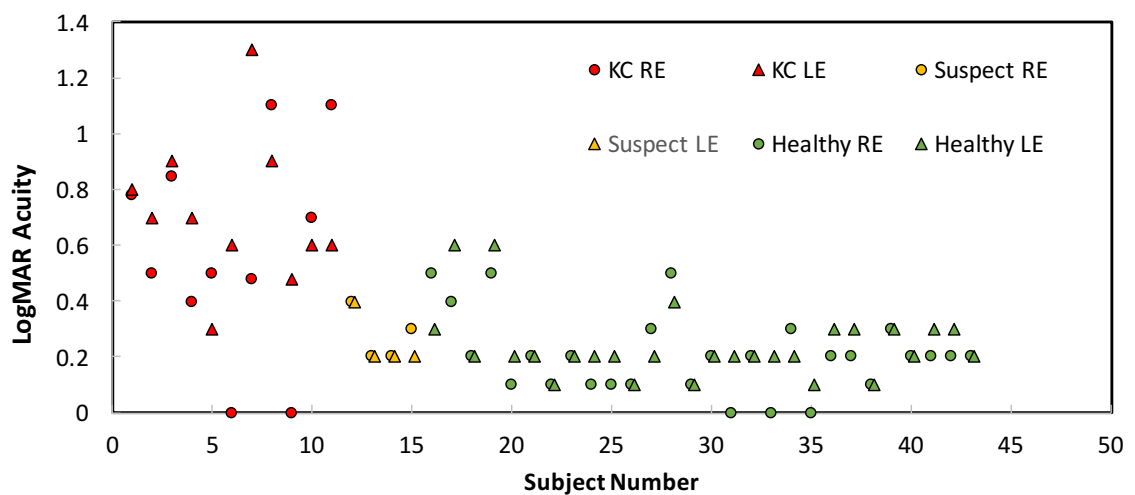


Figure 2.10 Spread of Visual Acuity in KC/KC suspect and healthy eyes. Note the significant overlap in the LogMAR 0.2 - 0.6 region, showing that this level of acuity represents patients with KC, KC suspect and healthy eyes).

Box plot to show VA spread in Healthy and KC/KC suspect eyes

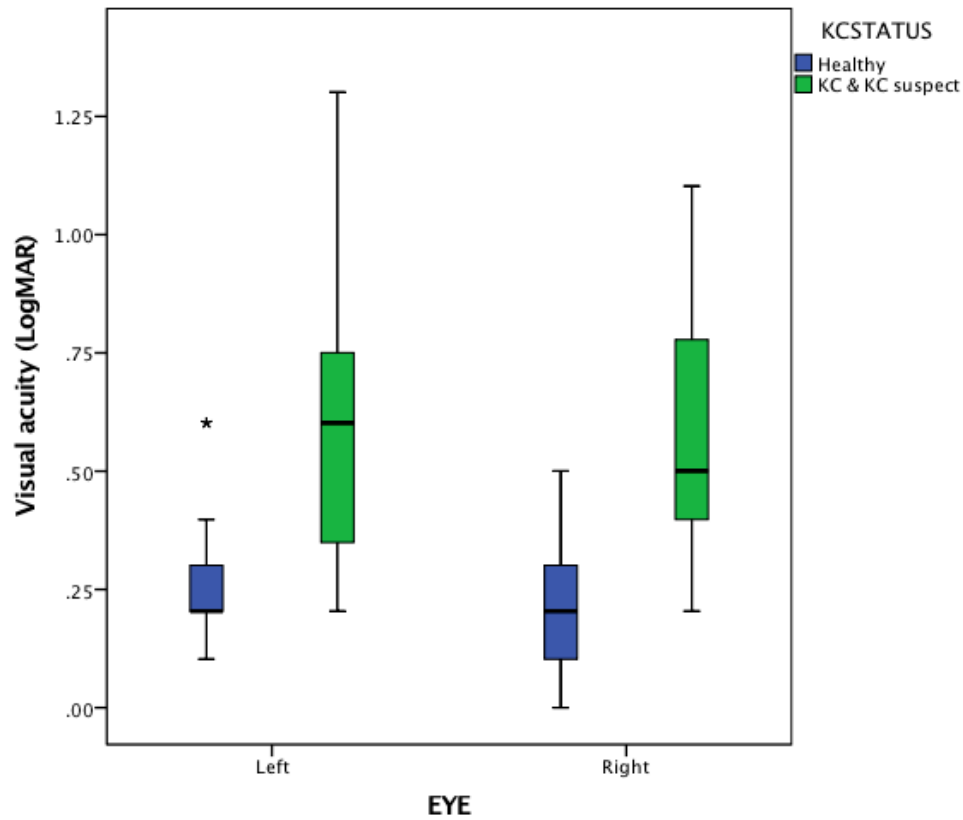


Figure 2. 11 Box Plot depicting the overlap of VA in healthy and KC/KC suspect eyes.

2.3.6 Contrast Sensitivity

Contrast sensitivity analysis carried the same exclusions as for the visual acuity, leaving 81 eyes for analysis. Figures 2.12 and 2.13 mirror VA findings such that a significant overlap between the healthy and KC/KC suspect eyes and thus does not carry suitable value as a screening test component.

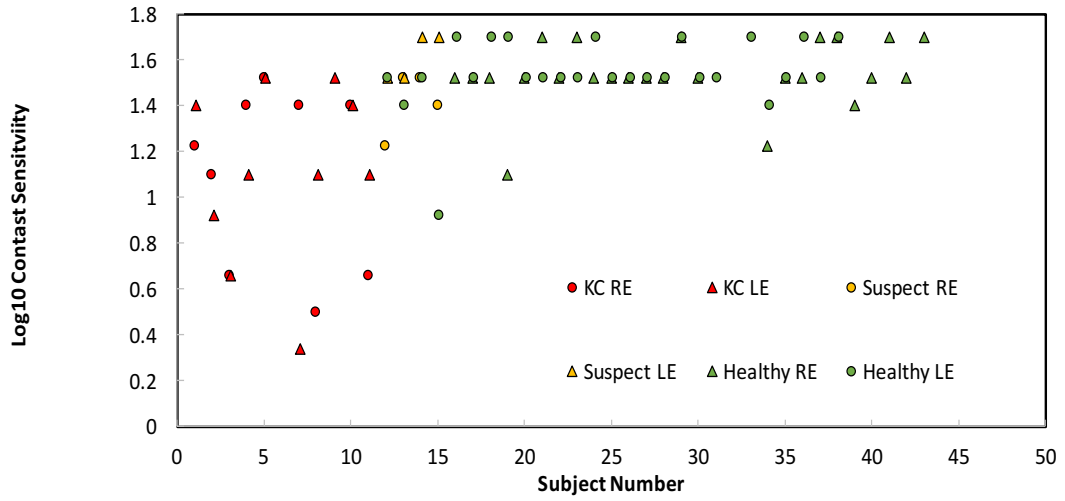


Figure 2. 12 Spread of contrast sensitivity KC/KC suspect and healthy eyes. Note the significant overlap in the LogMAR 1.1 – 1.5 region, showing that this level of acuity represents patients with KC, KC suspect and healthy eyes).

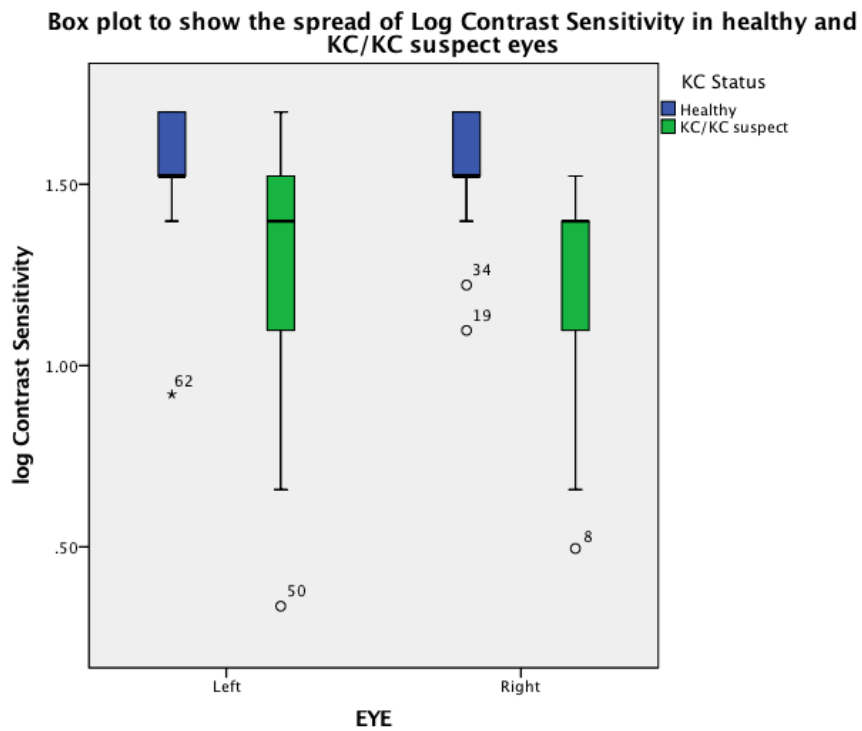


Figure 2. 13 Box plot depicting overlap of contrast sensitivity between healthy and KC/KC suspect eyes.

2.3.7 Congenital heart defects

Cardiac history data was available for 41 out of 44 participants (figure 2.4). There does not appear to be a positive association between those with congenital heart defects and those with KC. Rather, when the statistics are examined with Chi-squared analysis, there appears to be a negative association ($p=0.03$, Fisher's exact test).

Table 2.9 Proportion of congenital heart defects within each KC group

| | Healthy | KC suspect | KC | Total |
|---------------------------------|---------|------------|-----|-------|
| Number of subjects with CHD | 20 | 2 | 3 | 25 |
| Number of subjects with no CHD | 8 | 1 | 7 | 16 |
| Proportion of subjects with CHD | 71% | 67% | 30% | 61% |

2.3.8 Slit Lamp Biomicroscopy

Slit lamp examination was performed on 44 remaining participants. By definition, all 29 healthy participants and all KC suspect participants exhibited a clear cornea with no slit lamp signs of KC. Slit lamp results from the remaining 21 of 22 KC eyes are shown in table 2.5.

Table 2. 10 Table to show the numbers of subjects presenting with various slit lamp signs, stratified by study grading scale

| Clinical sign | Apical thinning | Scarring | Vogt's striae | Fleischer's ring |
|--|-----------------|----------|---------------|------------------|
| Mild KC (study grade 2) N=4 | 0 | 0 | 1 | 0 |
| Moderate KC (study grade 3) N=13 | 3 | 7 | 10 | 3 |
| Severe KC (study grade 4) N=2 | 1 | 2 | 2 | 2 |
| End stage KC (study grade 5) N=2 | 1 | 2 | 1 | 1 |

For eyes with early KC, corneae appeared normal in most cases, indicating a predominance of confirmed topographical KC in spite of the absence of slit lamp signs. Fleischer's ring and apical thinning was uncommon in moderate KC (study grade 3). Vogt's striae was seen in many corneae but not in the early stages. Scarring was exhibited in a number of corneae but only at study grade 3 and above. One eye had corneal hydrops that masked likely apical thinning, Vogt's striae and Fleischer's ring.

2.4 Discussion

In general, two main hypotheses for the high prevalence of keratoconus in Down's syndrome exist. The predominant hypothesis is that of the 'environmental' influence of eye rubbing (Pierse and Eustace 1971; Koenig and Smith 1993). This suggestion is furthered by the 'cascade hypothesis' wherein subsequent mechanical micro-trauma leads to inflammatory changes and the possible degeneration of the collagen scaffold (Kenney and Brown 2003). The second is that the DS eye is biomechanically weak, as a result of its altered shape or hypothetical ultrastructural defects. This is furthered by the 'two-hit hypothesis', suggesting KC develops in eyes that have an underlying genetic predisposition accompanied by an external environmental cause (McGhee et al. 2015).

2.4.1 *Eye rubbing*

The current study is the first to use a structured questionnaire to prospectively study KC, eye rubbing and atopy in the DS population. There appears to be only one study that prospectively considers eye rubbing, KC and DS, and the results of the current study are in contrast to that work (Haugen 1992). Haugen found that of the 16 institutionalised patients with learning disabilities who had keratoconus, 6 had Down's syndrome. Of these, 3 were noted to rub their eyes 'very often' relative to their peers whilst three did not rub their eyes at all. The study did not investigate the presence or absence of atopy. Whilst Haugen found that eye rubbing was correlated with keratoconus to some degree, it is certainly unclear if eye rubbing is indeed causative of KC, contrary to what the author suggests. The study of eye rubbing is known to be fraught with the possibility of inaccurate response, and patients may feel guilty about 'admitting' that they rub their eyes and so are thought to under-report (McMonnies and Boneham 2003). In the current study, the majority of the young people participating in this study lived with their parents, or had carers that were very familiar to them, the overwhelming majority were comfortable and confident to fill in the questionnaire appropriately. It is certainly a limitation of this

study that, short of filming the subjects for an extended period of time, no objective measure of eye rubbing exists. However, the positive association of atopy with eye rubbing is clinically reassuring and expected. Of the 11 patients who were keratoconic, one had both atopy and exhibited moderate eye rubbing. Another subject with keratoconus exhibited moderate eye rubbing in the absence of atopy. The other 9 subjects did not exhibit any significant eye rubbing signs to their parent or carer, and had little or no observed eye rubbing. This conflicts significantly with currently published literature, and demonstrates that the usefulness of eye rubbing history in the screening or diagnosis of keratoconus may be limited. Likewise, an absence of eye rubbing in clinical history taking should not lead the clinician to assume that keratoconus is unlikely.

Eye rubbing results from the current study may reflect a general shift towards better eye care and early intervention resulted in less clinically significant blepharitis being evident in the study group than reported in older literature (Jaeger 1980). Only two cases of substantial blepharitis were observed, and neither case exhibited eye rubbing behaviours. Better health care, education and better socialisation (since the institutionalisation of young people with DS is now exceptionally rare) in recent decades may mean that young people with Down's syndrome are more integrated with their society, and more engaged with their daily activities. Therefore, they may exhibit less stimming behaviours, such as eye rubbing or ocular massage (which blind and intellectually disabled people are sometimes known to engage in for sensory stimulation). It is likely that clinically significant blepharitis is now be picked up earlier by an optometrist and managed responsibly by the parents or carers. Another factor that has changed in the last two decades is that a large proportion of young people with Down's syndrome now wear spectacle correction and so it is quite possible that spectacles act as a physical barrier that detracts from unnecessary eye rubbing.

The low observed power (4.8%) of the eye rubbing result is expected since no significant difference was found between the two groups with the high p-value of 0.81 (Goodman and Berlin 1994, Hoenig and Heisey 2001). However, this does not negate the possibility of making a type II error, since the small sample size was

employed in the current study. While the current study carries a larger DS group than published studies to date, a significant limitation of the current study is that it is still small in size when compared to larger KC studies of typical individuals (eg Bawazeer et al. 2000). Although unlikely, it is statistically possible that the sample studied in the current study does not represent the population of keratoconics with DS throughout the nation. However, in practical terms, this is a large study sample from a niche population and in clinical terms, a significant effect size would have been required even in this limited sample in order to reject the null hypothesis. It therefore appears reasonable to accept the null hypothesis based on the data above.

2.4.2 *Atopy*

The association of atopy with eye-rubbing approached significance in this DS group ($p=0.05$), reflecting data seen from the typical non-DS population (Balasubramanian et al. 2013; Weed et al. 2007). Existing literature suggests that atopy is more prevalent in DS (Daneshpazhooh et al. 2007; Dourmishev et al. 2000), and as previously discussed KC also appears more prevalent in DS. In non-DS subjects, Rahi et al (1977) found a 3 fold increase in prevalence of atopy in the keratoconic cohort of 35%. It was therefore important to investigate if the rates of atopy were higher in the DS group with KC than the DS group with healthy corneae. In the current study overall, an atopic history was given in 44% of cases, higher than the levels of atopy generally reported in typical individuals (Copeman 1965), and in agreement with other studies (Daneshpazhooh et al. 2007; Dourmishev et al. 2000). However, atopic disease is no more prevalent in the KC group than the DS group ($p=0.73$). These results suggest that although atopy is more prevalent in DS as a whole, and that atopics have a tendency to rub their eyes, there is no evidence that eye rubbing or atopy are more common in, or indeed causative of, KC in DS. Whilst young people with DS may rub their eyes, it may not necessarily lead to KC in isolation. McMonnies suggests that atopy, itch and eye rubbing are relevant in the pathogenesis of KC only when ‘the highest levels of these factors are present’ (McMonnies and Boneham 2003). This hypothesis appears maintained even in DS, because a higher propensity to eye rub or be atopic is seen in the keratoconic group.

2.4.3 *Astigmatism*

Patients presenting to corneal clinics with higher magnitudes of regular astigmatism are more likely to progress to KC than those with lower astigmatism (Shirayama-Suzuki et al. 2009). Since, in the author's experience, a useful subjective response is unlikely in a patient with DS, the reliance on objective measures is greater and thus it was important to establish if a clinical cut-off level could be useful in identifying those with pathological corneae. Whilst, as expected, the results demonstrate that higher magnitudes of regular astigmatism exist in the keratoconic eyes, the results indicate that establishing a useful cut-off is not possible. This is likely a feature of the high refractive error seen generally in eyes with DS (Woodhouse et al. 1997), likely masking early astigmatic changes due to KC. Because the current study evaluated an asymptomatic population already undergoing regular eye examinations, it likely revealed the earliest levels of disease possible (when ectatic changes and thus induced astigmatism would be minor). However, the clinical implication of these results is that a young person with DS does not have to demonstrate a significant 'cyl' in order to have keratoconus.

2.4.4 *Visual function*

The significant overlap in the visual acuities and contrast sensitivities of healthy and keratoconic eyes demonstrate that a measures of acceptable visual function in DS do not exclude the possibility of KC. This result agrees with others from the non-DS population (Kanellopoulos and Asimellis 2013) and is likely the result of the overlap of the decrease of vision in the KC eyes with the level of visual impairment that exists naturally in DS. KC in DS is therefore a relatively asymptomatic disease, at least in the early stages when indeed there is a need to prioritise CXL (where available) in this group. Kanellopoulos and Moustou suggest that VA in KC is so variable that topographic irregularity indices should be more highly weighted in specific disease staging.

2.4.5 *Slit lamp biomicroscopy*

In agreement with others, slit lamp signs were not always present in early KC (Zadnik et al. 1996). The current DS study was unique in that the diagnosis of KC (in the absence of slit lamp findings) was confirmed by progressive topographical changes with time. This differentiated the early keratoconics from those ‘suspects’ with abnormal topography who had remained stable for a long period of time (>12 months). Although the current study was not longitudinal, many longitudinal observations were possible and it was notable that early keratoconus could progress clinically (and through the automated keratoconic staging) whilst maintaining a clear cornea. This mirrors the author’s general experience in non-DS patients, and the assumption in general ophthalmic practice that some patients maintain a relatively clear cornea until very late stages. The modern studies examining the DS cornea typically confirm a diagnosis of KC in DS only by abnormal topography in the presence of slit lamp signs (Aslankurt et al. 2013; Vincent et al. 2005). These studies are not able to differentiate their ‘suspect’ cases or ‘abnormal topography’ cases into actual KC or those with atypical but stable corneae. As such, these studies may overestimate the incidence of sub-clinical keratoconus.

2.4.6 *Retinoscopy*

The preferential use of autorefraction over retinoscopy in busy clinical practice is a modern phenomenon in order to save time and skill. Although modern autorefractors are generally reliable and accurate for spectacle prescriptions, they do not detect the high-order aberrations that are seen in very early KC using retinoscopy. Results of the current study indicate that retinoscopy provides an excellent screening and diagnostic tool for identifying KC in DS. This mirrors the work by (Goebels et al. 2015), and relies on the fundamental principles of Sir William Bowman who in 1859 who considered the use of the retinoscope primarily a technique of detecting keratoconus .

Conclusions

Results from the current study do not support the hypothesis that eye rubbing is associated with keratoconus, and it is therefore unlikely to be a causative factor.

Results from the current study are impactful in the screening and diagnosis of keratoconus in someone with Down's syndrome since it is no longer useful to assume that an absence of eye rubbing reduces the likelihood of the patient having keratoconus.

The results have implications for those being considered for corneal grafting or corneal cross-linking, where previously those with DS were often considered poor candidates for treatment due to the perceived likelihood of inadvertent trauma. While eye rubbing is likely to put the patient at risk of inflammation, infection and ultimately corneal graft rejection – the data highlight that each patient should be looked at on a case-by-case basis, especially with the knowledge that the keratoconus was not self-inflicted.

This research adds some weight to the hypothesis that the high prevalence of keratoconus in Down's syndrome may be due to an underlying collagen abnormality, causing a structurally weaker cornea that is predisposed to developing KC.

The current study reinforces the clinical need for the use of retinoscopy on all patients with DS. The sensitivity and the availability of this technique in the primary care setting makes it the most suitable tool for any screening. Although the sample size of keratoconic subjects was relatively small compared to non-DS studies, it was large in DS terms and provides enough overlap in the data to understand clinically that VA, contrast sensitivity and the measurement of astigmatism are not useful measurements in the identification of KC in DS. Further, waiting for slit-lamp signs is likely to prove too conservative a treatment plan for already-thin DS corneae, in areas where CXL assessment is available.

Chapter 3a

Topographical correlates of keratoconus in Down's syndrome

3.1 Introduction

The DS cornea is known to be thinner and steeper than controls. Vincent (2005) found that 61% of DS subjects had an average corneal power 2 S.D. outside the normal non-DS range, in corneae that otherwise appeared healthy. Vincent also presents a case of a DS subject with inferior steepening and yet normal topographic parameters. Since a keratoconic cornea inherently thins and steepens, and topographic systems are sensitive to such shapes, the attributes of the normal DS cornea create the capacity to provide false positive results if automated indices based on a non-DS population are used. In addition, the DS eye is known to have increased levels of whole-eye aberrations when compared to controls. Data from 44 DS eyes showed increased overall aberrations, increased coma, but these did not reach pathological proportions (McCullough et al. 2013). The small levels of abnormality reported, however, may account for the degradation in optical quality found in DS eyes, and the demonstrable improvement in vision found when the optics of the eye are bypassed using interferometric acuity (Little et al. 2007). It is not known to what extent any baseline aberrations in the healthy DS cornea differ from those eyes with both DS and KC, and how this influences the rate of false positive topography results. Aslankurt et al. (2013) excluded subjects with clinical signs of keratoconus and relied only on the automated topography diagnosis of keratoconus by Pentacam in a group of 27 children with DS (aged 8.9 ± 2.4 years), finding 'early, subclinical' KC in 11 eyes (21.1%), and abnormal topographic parameter in a total of 20 eyes (38.4%) overall, each proportion approximately ten-fold that of the control group. It is difficult to imagine KC being present in such epic proportions in such a young group, but rather it seems more likely that the Pentacam indices were producing a large proportion of false positives. In an older group (25 ± 9.6 years), Anderson et al. (2014) found 23.6% of DS corneal topographies to be abnormal (versus just 0.7% of non-DS topographies). Vincent (2005) proposes that people with DS have abnormalities of corneal shape even in the absence of clinical evidence of KC. In clinical ophthalmology, such 'suspect' cases are monitored over time in order to examine the reliability and progression of the initial

topography data - a cornea is not considered to have keratoconus unless the cornea is becoming more ectatic with time. To date, no DS studies have done this to confirm 'suspect' or 'sub-clinical' cases of KC by means of any prior or subsequent topographical mapping. This is essential in order to differentiate whether the abnormality of corneal shape in KC is truly representative of keratoconus ectasia, or if the abnormality of corneal shape is simply an inherent but stable finding in DS (such as the failure of emmetropisation and the resulting wide-range of refractive error seen in the DS population).

3.2 Aim of study chapter

To establish the normative topographical associations for healthy DS corneae, in combination with suspect/abnormal but demonstrably stable corneae (study grade 0-1); finding the useful topographical parameters that differentiate this group with established KC, in particular from early-moderate KC (study grade 2&3). By virtue of this, the current study will investigate the diagnostic capacity of the metrics available from the Oculus Pentacam for a DS population.

3.3 Methods

3.3.1 *Oculus Pentacam Images*

An accurate Pentacam image relies on excellent fixation. In a group of young people with learning disabilities this was aided through significant encouragement and minimising the task duration. Prior to introduction to the Pentacam, the subject had an eye exam with the researcher and was given plenty of time to feel at ease in the unusual environment. The subject was positioned until comfortable on the chin and forehead rest, and asked to concentrate on the blue fixation light. The shorter of two Pentacam settings was utilised, such that 25 component images were captured in 1.0 seconds. A display was available for the examiner to align the eye in the x, y and z directions. It was ensured that the subject had the opportunity to blink shortly before the alignment that initiated the capture, so that a broken tear film did not cause patterns of corneal irregularity. The participant was asked to look steadily at the blue fixation light and automatic capture was used when the alignment was deemed acceptable by the software. In some cases, the ‘auto-capture’ had to be overridden manually in order to gain a capture. In such cases, measurements were repeated until acceptable quality imaging was obtained (Aslankurt et al. 2013).

In most circumstances, scans were taken until at least 3 images with good co-operation for each eye were obtained. Such multiple images often required a high degree of co-operation and stamina, and the child or young person with DS was asked if they felt able to continue with each new capture. Occasionally, if the subject became fatigued, they would be invited back to complete this aspect of the study on another occasion. If the scan proved too difficult for someone with a particularly significant learning disability, or if a subject appeared to be becoming distressed, then this aspect of the study was abandoned.

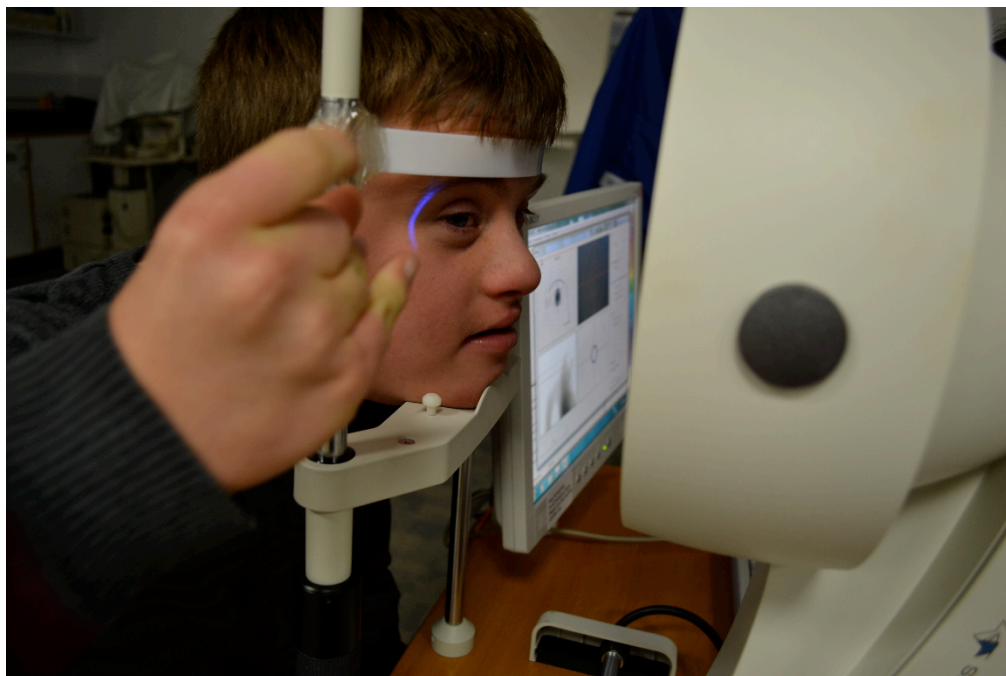


Figure 3.1 A young participant being lined up for tomography by Oculus Pentacam.

Those scans that demonstrated loss of fixation or a blink during the process were removed. Even the normal cornea will simulate the appearance of a cone if the line of sight (and therefore the optical axis) is not directly coincident with the fixation target (Hubbe and Foulks 1994). By the same principle, an eye with inferior corneal ectasia could appear relatively normal if the gaze was directed above the horizontal, pointing the cone in the forward direction and minimising the appearance of the true inferior steepening. In order to ensure that the participant understood the fixation instructions and to ensure that the scans were representative of the cornea in question, many images were taken from each eye, when possible. The effect described above applies primarily to front-surface topography but to mitigate for this effect, an observation of the location of minimum corneal thickness was employed to exclude variable gaze position. This appears to have been a method employed by others (Aslankurt et al. 2013). If in doubt, those scans with non-concentric contours were treated with caution, and excluded where more regular corneal thickness patterns were seen in the same eye. When this was inconclusive, a reference back to the position of the eye in the Scheimpflug images was made. Finally, the first scan for each eye to be found quantitatively sound (denoted by an 'OK' in the Pentacam reliability index) and/or qualitatively sound (manual examination of the component Scheimpflug images by eye) was shortlisted for analysis.

Since clinical signs such as Munson's sign or a Fleisher's ring are diagnostic of KC using a slit lamp, the clinical usefulness of Pentacam in diagnosis is to confirm KC in these cases (and in general, to monitor for progression over time). Therefore, Pentacam is more useful for the subtle clinical abnormalities that do not clearly manifest themselves on slit lamp examination, and in particular for identifying early and subclinical cases. Whilst many papers incorporate the latter KC stages into analyses, this has the detrimental effect of skewing the data, and the increasing the significance of diagnostic parameters against controls. Therefore, stages 4 and 5 KC have been removed from most analyses, leaving only early and moderate KC corneae included.

3.3.2 Statistical analysis of data

Statistical analysis of the differences between groups was carried out using the Mann-Whitney U test, or the Chi-squared test in SPSS (Version 20.0, IBM, Chicago, Illinois, USA). Receiver operating characteristic (ROC) curves derived from the same software were used to analyse the performance of selected parameters as diagnostic indices when used as binary classifiers. The graphs plot sensitivity (y-axis) against the specificity (x-axis) at various threshold settings. ROC 'Curves' that edge toward the upper-left of the plot are superior in their discrimination between healthy and diseased groups. The point (0,1) is a 'perfect classification', representing 100% sensitivity and 100% specificity.

3.4 Results

3.4.1 *Subjects*

The same study group as detailed in Chapter 2 were invited to complete this aspect of the study. Thirty-eight subjects were able to complete this aspect, including all 11 of those who had keratoconus. As with the previous chapter, two eyes were excluded (one due to a history of retinal detachment and one due to a history of a corneal ulcer). This left 74 eyes for analysis, and they fell into the study grades as set out in table 3.1.

Table 3. 16 Breakdown of keratoconic status in DS eyes

| <i>Study Grade</i> | <i>Status</i> | <i>Number of eyes</i> |
|--------------------|----------------|-----------------------|
| 0 | Healthy | 45 |
| 1 | Suspect / FFKC | 8 |
| 2 | Mild KC | 4 |
| 3 | Moderate KC | 13 |
| 4 | Severe KC | 2 |
| 5 | End stage KC | 2 |
| | Total | 74 |

3.4.2 *Corneal Power Indices*

All corneal power indices explored in eyes with KC were significantly different from control eyes (table 3.2). Even though KC eyes were not defined by the magnitude of

their curvature in this study, as expected, an increase of curvature was found in the pathological eyes. Each anterior surface index maintained its significance even when severe KC (SG 4-5) was excluded from analyses, as shown in table 3.2. The maximum corneal curvature (K_{max}) carried the greatest area under the curve 0.993 (see figure 3.2) and hence held the greatest diagnostic capacity. The box plot in figure 3.3 and the data in table 3.3 demonstrates that there is still significant overlap (3.70D), thus its capacity as a diagnostic indicator (when used alone) is limited.

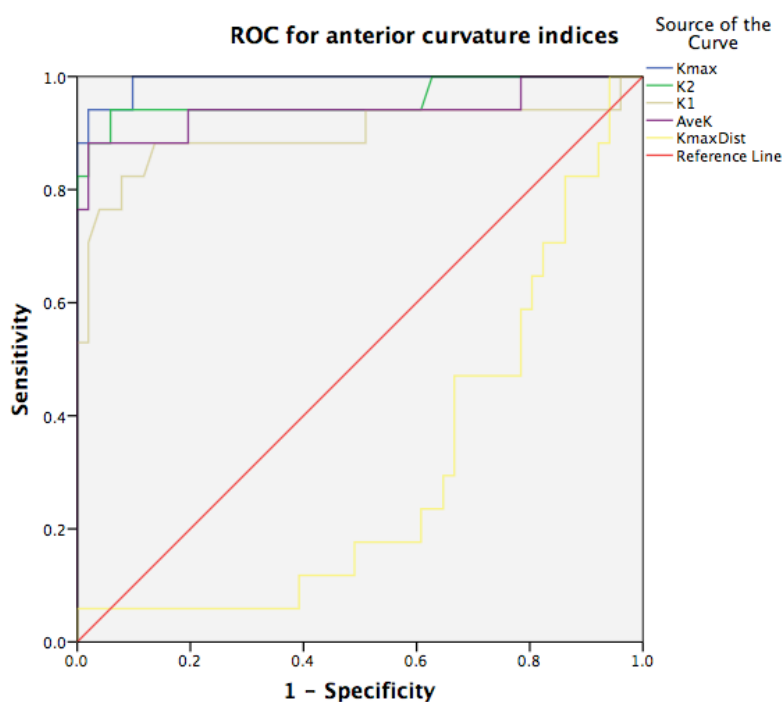


Figure 3. 2 ROC curve for curvature indices. This figure depicts the blue 'curve' (K_{max}) as the closest to the top-left corner, indicating the greatest sensitivity and specificity of the indices examined. This is a pictorial representation of the 'area under the curve 'AUC'.

Table 3.17 Table to show the significance of various corneal power indices using the Mann-Whitney U test when grouped by KC status (normal and suspect versus early KC)

| Index | Mann-Whitney U | Significance (p-value) | AUC | Significance (p-value) | Asymptotic 95% Confidence Interval | |
|---|----------------|------------------------|-------|------------------------|------------------------------------|-------------|
| | | | | | Lower Bound | Upper Bound |
| Maximum K, K_{max} | 6 | 0.00 | 0.993 | 0.00 | 0.979 | 1.000 |
| Steepest K, K2 | 35.5 | 0.00 | 0.959 | 0.00 | 0.889 | 1.000 |
| Flattest K, K1 | 90 | 0.00 | 0.896 | 0.00 | 0.777 | 1.000 |
| Average K, AveK | 52 | 0.00 | 0.940 | 0.00 | 0.850 | 1.000 |
| Distance from K_{max} to corneal centre, $K_{max_{dist}}$ | 313 | 0.05 | 0.302 | 0.02 | 0.163 | 0.441 |

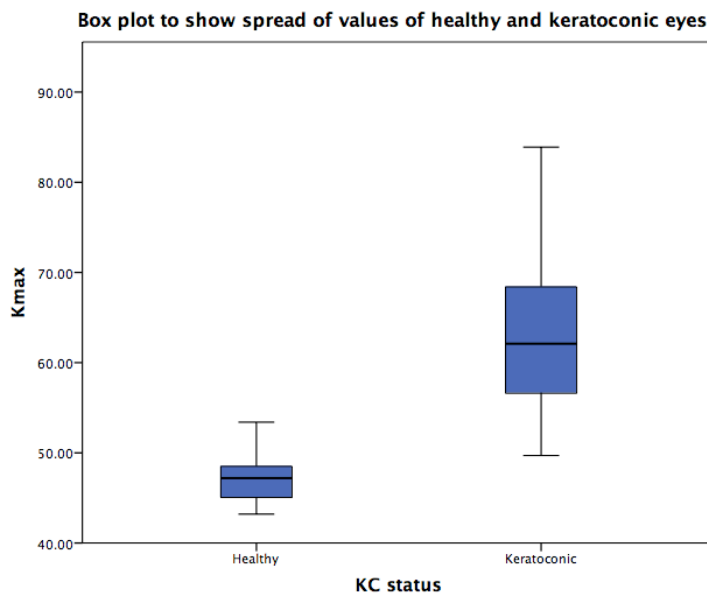


Figure 3. 3 Box plot to show spread of values for healthy and KC eyes

Table 3. 18 Box plot statistics for Kmax between the two groups

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2 & 3) |
|---------------------|--------------------|------------------------|
| Mean | 46.97 | 63.19 |
| Standard deviation | 2.37 | 8.41 |
| Minimum | 43.20 | 49.70 |
| Maximum | 53.40 | 83.90 |
| Total range | 10.20 | 34.20 |
| Interquartile range | 3.50 | 13.15 |

In Chapter 1, the potential shortcomings of current grading scales were discussed with respect to their suitability for use in DS, and therefore all eyes were split into study groups according to the clinical severity and likely visual rehabilitation need. In order to validate that method decision, table 3.4 and figure 3.4 document the *mean* and *standard deviation* of the anterior corneal surface variables for each study grade. It can be seen that each dioptric curvature index has increasing corneal power with increasing severity grade. As expected clinically, the smallest difference is found between healthy and suspect eyes. This suggests that the study grading scale does

indeed categorise KC eyes into appropriate clinical categories with respect to increasing corneal distension. In the KC group, increasing distension (characterised by increased corneal curvature), is correlated with a decreasing CCT. In the healthy group, however, the correlation between corneal curvature and CCT was minimal (figure 3.5).

Table 3. 19 Table to show mean and standard deviations for various corneal curvature indices with increasing study grade severity

| KC study grade | N= | Kmax | Kmax _{dist} | K2 | K1 | AveK |
|----------------|----|-------------|----------------------|------------|------------|------------|
| | | Mean ± S.D. | | | | |
| 0 | 40 | 46.67±2.10 | 1.66±0.82 | 45.82±1.88 | 44.29±1.40 | 45.04±1.59 |
| 1 | 13 | 48.60±3.15 | 1.39±0.93 | 47.18±2.63 | 45.26±2.32 | 46.19±2.35 |
| 2 | 3 | 60.50±8.58 | 1.24±0.58 | 54.10±7.02 | 50.60±8.35 | 52.27±7.71 |
| 3 | 12 | 65.82±7.39 | 0.87±0.50 | 59.19±6.05 | 54.07±7.20 | 56.47±6.61 |
| 4 | 3 | 87.73±12.73 | 0.30±0.08 | 73.23±6.53 | 65.33±6.26 | 69.03±6.01 |

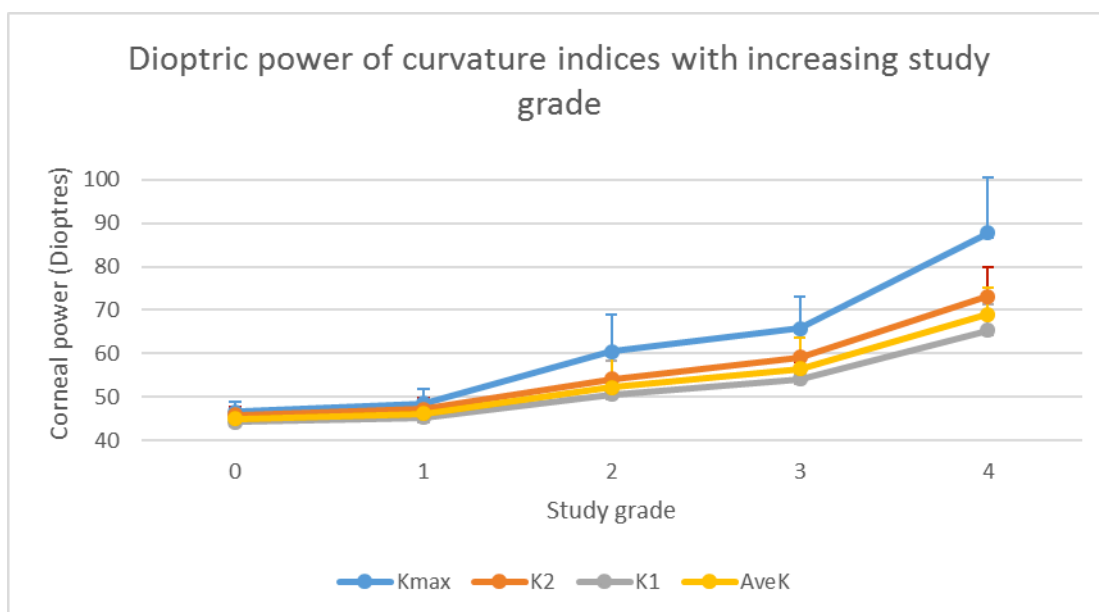


Figure 3. 4 Graph to show the mean and stand deviations for curvature indices with increasing study grade severity.

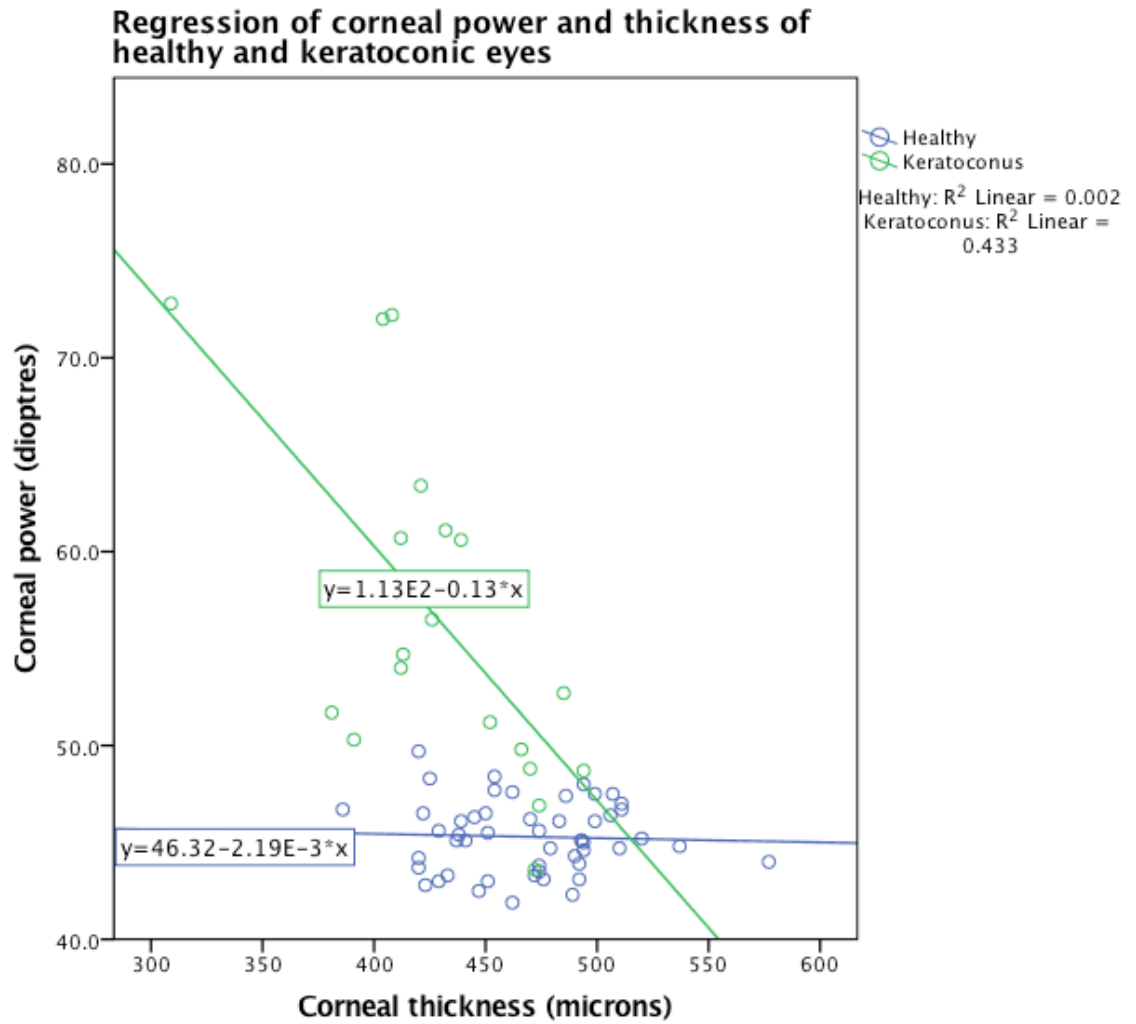


Figure 3. 5 Correlation and associated regression of corneal power (AveK) and corneal thickness in the healthy DS corneae (blue) and the keratoconic DS corneae (green). Note the significant difference in the gradient of the regression lines, and the relative steepening of the KC cornea. Evidently, there are a significant number of pathological corneae that share a CCT range with those of healthy eyes. In contrast, the pathological corneae are more readily separated by corneal power, however a significant proportion of overlap is still evident.

3.4.3 Astigmatism

Regular astigmatism from the anterior corneal surface was measured directly from the Oculus Pentacam - derived as the difference in dioptric value between the two principle meridians represented by the simulated keratometry values. The angle of the flat axis was also taken directly from the Pentacam, representing the location of a 'minus cylindrical correction'. As per the previous chapter, in order to preserve the directionality of the corneal astigmatism found, vector analysis was used (Thibos et al. 1997), breaking down the astigmatism into its vertical and horizontal components. If an eye were free of astigmatism, the points would be expected to collapse around (0,0).

Scatter graph to show the spread of cylindrical refractive error in healthy and keratoconic eyes

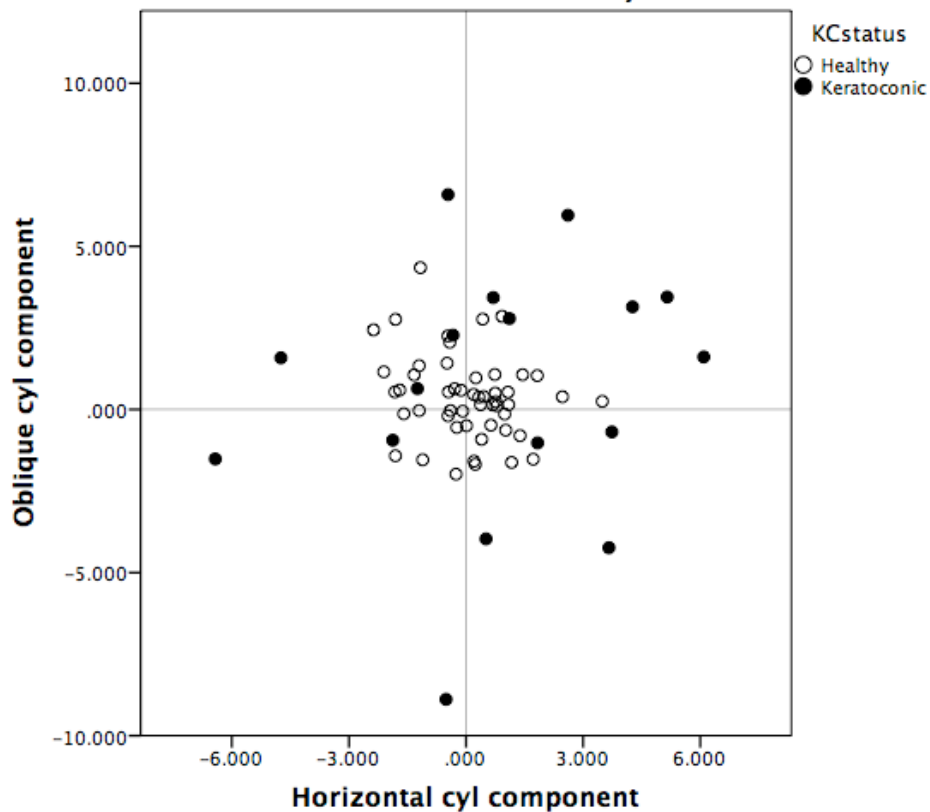


Figure 3. 6 Topographical cyl spread in Healthy and KC eyes.

Figure 3.6 shows the spread of astigmatism of healthy (open circles) eyes and those with KC (closed circles). As is the norm in KC, greater magnitudes of astigmatism are associated with KC. There appears to be no preferred orientation of the physiological astigmatism found in the healthy eyes nor the pathological astigmatism found in the eyes with KC. Since 7 eyes with KC are intermingled with healthy eyes, it is clear that an obvious cut-off value for either direction or magnitude is not available (see also box plot below, figure 3.7). From Pentacam data, the smallest magnitude of corneal astigmatism in a clinically keratoconic eye was 1.40DC. Clinically, this value would be considered within a normative range for a healthy population of DS or typically-developing individuals. A cut-off guideline to indicate pathology is therefore not possible.

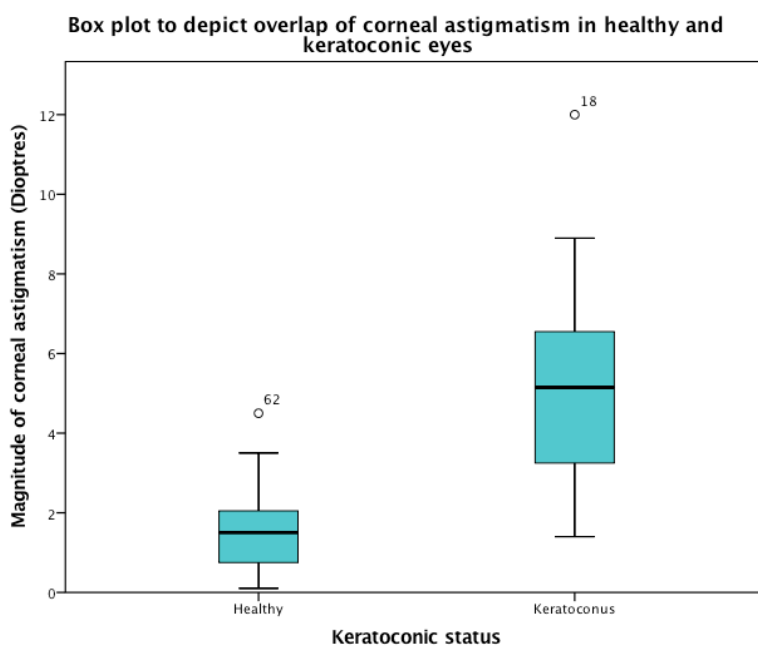


Figure 3.7 Box plot of corneal astigmatism in healthy and DS eyes.

Table 3.20 Box plot statistics for corneal astigmatism.

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2 & 3) |
|---------------------|--------------------|------------------------|
| Mean | 1.54 | 5.14 |
| Standard deviation | 0.95 | 2.59 |
| Minimum | 0.10 | 1.40 |
| Maximum | 4.50 | 12.00 |
| Interquartile range | 1.40 | 3.45 |

3.4.4 Surface irregularity indices

Two anterior surface irregularity indices were taken directly from the Oculus Pentacam output: the ‘Index of Surface Variance’ (ISV) and ‘Index of Vertical Asymmetry’ (IVA). A simulated ‘I-S’ value was derived by subtracting the paracentral inferior measurement from the superior measurement (Aslankurt et al. 2013). Using the Mann-Whitney U test, each irregularity index maintained significance when healthy/suspect eyes were compared to those with early KC. ROC analysis was used to investigate the diagnostic power of each irregularity index. Both ISV and IVA had an AUC of 1 and so differentiated completely between healthy and diseased groups (table 3.6). This is further demonstrated in box plots by the gap between the lowest ISV/IVA of the KC group and the highest ISV/IVA of the healthy group (figures 3.9 and 3.10). One outlier was detected (subject 15) and this datum was verified as correct and repeatable as per the other scans obtained at the same visit. However, I-S was not useful at discriminating healthy eyes from those with KC and was dropped from further analysis. This mirrors the findings of Aslankurt et al. (2013), who found that ISV and IVA were more reliable than I-S.

Table 3. 21 Table to show the significance of irregularity indices and their diagnostic capacity

| Index | Mann-Whitney U | Significance (p-value) | AUC | Significance (p-value) | Asymptotic 95% Confidence Interval | |
|-------|----------------|------------------------|-------|------------------------|------------------------------------|-------------|
| | | | | | Lower Bound | Upper Bound |
| ISV | 0 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| IVA | 0 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| I-S | 118 | 0.00 | 0.864 | 0.00 | .715 | 1.000 |

Since ISV and ISA both have such strong sensitivity and specificity for the early keratoconus, the correlation of both is shown in figure 3.8. They are strongly correlated especially toward the lower values.

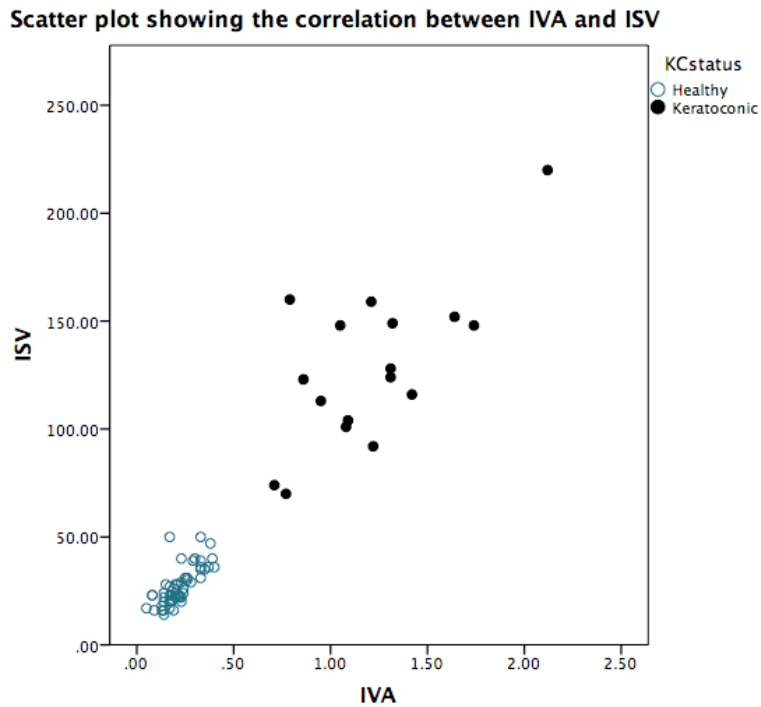


Figure 3. 8 Scatter plot correlating IVA and ISV. Keratoconic corneae are shown as closed black circles and each have a much greater magnitude of both IVA and ISV than healthy eyes (open blue circles). The correlation between IVA and ISV in healthy eyes is strong and whilst the correlation in the keratoconic eyes is weaker, a significant relationship is observed.

Table 3. 22 Box plot statistics for ISV between the two groups.

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2 & 3) |
|---------------------|--------------------|------------------------|
| Mean | 22.37 | 128.29 |
| Standard deviation | 9.03 | 36.70 |
| Minimum | 14.00 | 70.00 |
| Maximum | 50.00 | 220.00 |
| Total range | 36.00 | 150.00 |
| Interquartile range | 14.00 | 48.00 |

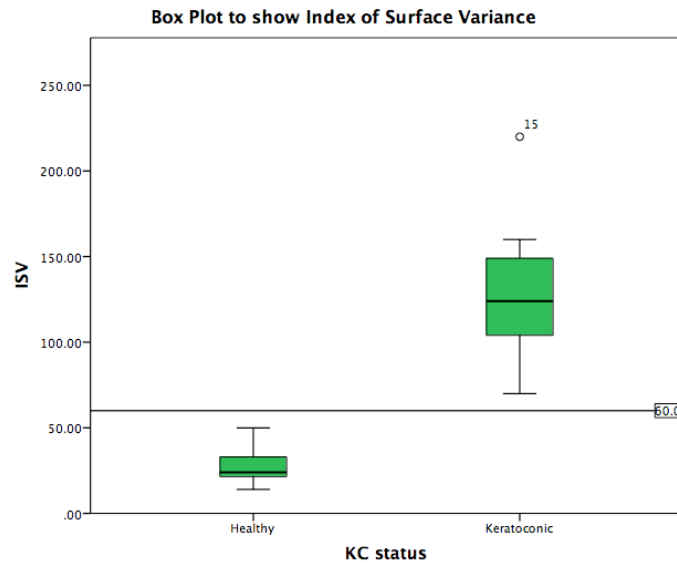


Figure 3. 9 Box Plot to show Index of Surface Variance

Table 3. 23 Box plot statistics for IVA between the two groups

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2&3) |
|---------------------|--------------------|----------------------|
| Mean | .22 | 1.21 |
| Standard deviation | .09 | .37 |
| Minimum | .05 | .71 |
| Maximum | .40 | 2.12 |
| Total range | .35 | 1.41 |
| Interquartile range | .11 | .47 |

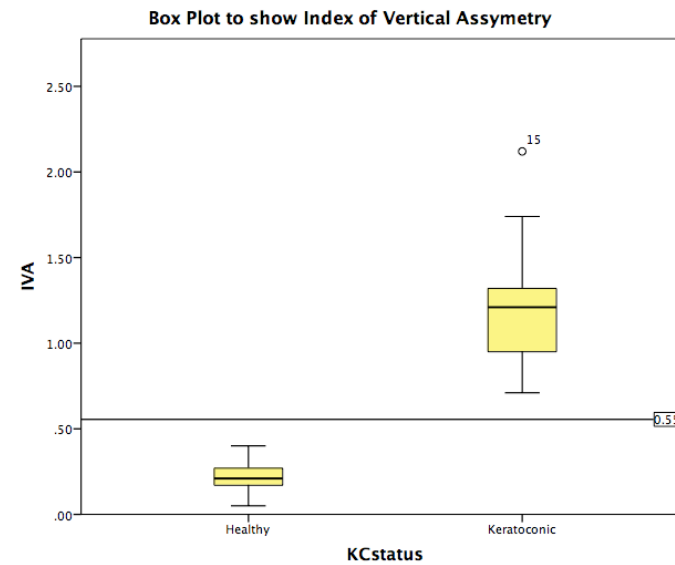


Figure 3. 10 Box Plot to show Index of Vertical Asymmetry

3.4.5 Keratoconus Indices

Pentacam-derived Keratoconus Index (KI), Central Keratoconus Index (CKI) and the total KC index from the Belin-Ambrosio display (D) were examined. All indices were significantly different between KC and healthy groups, and ROC and AUC analysis revealed that Keratoconus Index (KI) carried the most diagnostic power (table 3.9). When examined using box plot analysis (figure 3.10 and table 3.11), the KI index generates the possibility of creating a cut off value between the healthy/suspect and the keratoconic eyes, which could be used toward clinical guidance for the DS population. Such a value would lie between the maximum healthy KI of 1.11 and the minimum KC value found for KI of 1.15. This is in agreement with Goebels et al. (2015), who found that stage 2 (early KC) in the studied group also began at an index of 1.15, in non-DS eyes.

Table 3. 24 Table to show the significance of keratoconic indices in the two groups and their diagnostic capacity.

| Index | Mann-Whitney U | Significance (p-value) | AUC | Significance (p-value) | Asymptotic 95% Confidence Interval | |
|-------|----------------|------------------------|-------|------------------------|------------------------------------|-------------|
| | | | | | Lower Bound | Upper Bound |
| KI | 39.5 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| CKI | 6.5 | 0.00 | 0.993 | 0.01 | 0.976 | 1.000 |
| D | 0 | 0.00 | 0.997 | 0.00 | .988 | 1.000 |

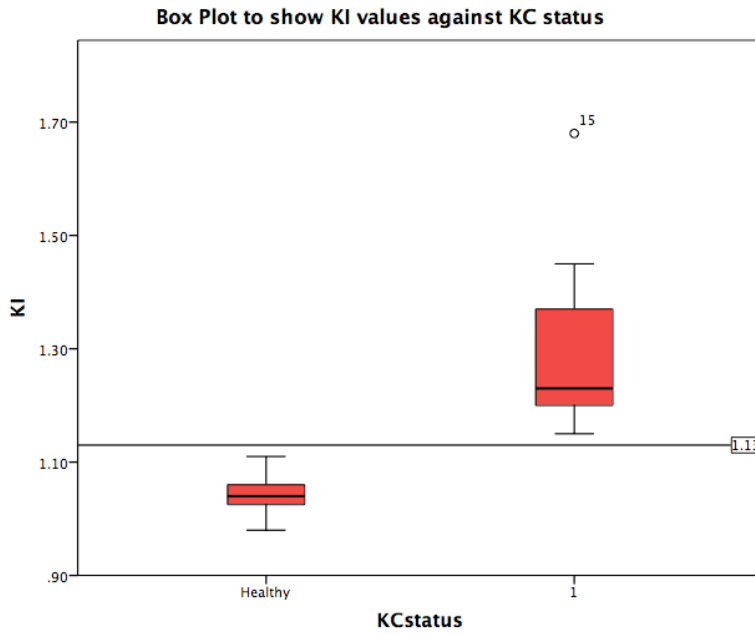


Figure 3. 11 Box plot of Keratoconus Index against KC status. A cut off value of 1.13 is present on the graph, showing the halfway point between the maximum KI value of the healthy eyes and the minimum KI value from pathological eyes.

Table 3. 25 Box plot statistics for KI between the two groups

| <i>Statistic</i> | <i>Healthy (SG 0 & 1)</i> | <i>Keratoconic (SG 2&3)</i> |
|---------------------|-------------------------------|---------------------------------|
| Mean | 1.04 | 1.29 |
| Standard deviation | 0.03 | 0.14 |
| Minimum | 0.98 | 1.15 |
| Maximum | 1.11 | 1.68 |
| Total range | 0.13 | 0.53 |
| Interquartile range | 0.04 | 0.20 |

The topographic keratoconus index (TKC) is the Pentacam method of topographic keratoconus classification. It does not place a grade on healthy eyes but grades supposedly keratoconic eyes as G1, G1-2, G2, G2-3, G3, G3-4, G4. Whereas the Pentacam graded an eye as a combination (eg displayed G1-2) the median was taken and recorded (in this case, G1.5). It was of interest to compare the clinically-based study grading with that automated by the topography only TKC method. Eyes with clinical KC received a Pentacam grade of G2 or higher, whereas healthy eyes received up to a grade G1-2 (analysed as G1.5). This results in a ‘clean split’ between healthy and KC, but as such the Pentacam appears to overestimate the KC grade in healthy DS eyes (with respect to the study grade), labelling them up to a G1-2. Clinically keratoconic eyes were labelled with a G2 and above. If in DS eyes the G1 and G1-2 is interpreted as suspect KC then this index becomes useful at differentiating between healthy/suspect and clinical KC.

The clinical study grade (SG) given to each subject at the end of the clinical examination was correlated with the automated measurement provided by the Pentacam (TKC). The regression of the data is $TKC = 1.3333(KC \text{ grade}) + 0$, indicating that the TKC systematically overestimates the level of KC with respect to the study scale, or that the study scale underestimates the level of KC found topographically.

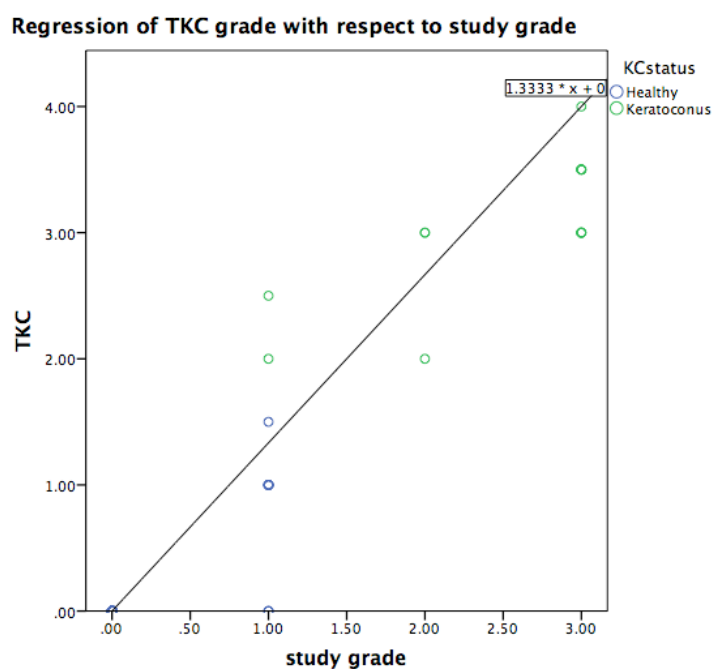


Figure 3. 12 Regression of Pentacam TKC grading and the study study grading scale.

3.4.6 *Elevation Indices*

Elevation indices obtained from the Pentacam were as follows:

- IHA = Index of height asymmetry (Pentacam output), IHD = Index of height decentration (Pentacam output)
- Standard Ft Ele Th = Caliper measure of the *front* surface elevation at the thinnest point (Pentacam derived)
- Standard Bk Ele Th = Caliper measure of the *back* surface elevation at the thinnest point (Pentacam derived)
- Belin Ft Ele Th = Caliper measure of the *front* surface elevation at the thinnest point using the Belin-Ambrosio display (Pentacam derived)
- Belin Bk Ele Th = Caliper measure of the *back* surface elevation at the thinnest point using the Belin-Ambrosio display (Pentacam derived).

All elevation indices were significantly different between healthy and KC groups (table 3.11). The IHD index of one healthy eye crossed into the KC range – this was identified as an outlier, but on post hoc it belonged to a subject who went on to develop map-dot keratopathy. Tables 3.12 and 3.13 and figures 3.13 and 3.14 show specific indices IHD and ‘Front surface elevation at the thinnest point’ respectively.

Table 3. 26 Table to show AUC data for elevation indices

| Index | Mann-Whitney U | Significance (p-value) | AUC | Significance (p-value) | Asymptotic 95% Confidence Interval | |
|--------------------|----------------|------------------------|-------|------------------------|------------------------------------|-------------|
| | | | | | Lower Bound | Upper Bound |
| IHA | 126.5 | 0.00 | 0.854 | 0.00 | 0.741 | 0.967 |
| IHD | 1 | 0.00 | 0.999 | 0.00 | 0.995 | 1.000 |
| Standard Ft Ele Th | 0 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| Standard Bk Ele Th | 2 | 0.00 | 0.998 | 0.00 | 0.992 | 1.000 |
| Belin Ft Ele Th | 0 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| Belin Bk Ele Th | 2 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |

Table 3. 27 Group box plot statistics for IHD

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2&3) |
|---------------------|--------------------|----------------------|
| Mean | .016 | 0.124 |
| Standard deviation | .011 | .042 |
| Minimum | .00 | .06 |
| Maximum | .07 | .25 |
| Total range | .06 | .20 |
| Interquartile range | .01 | .04 |

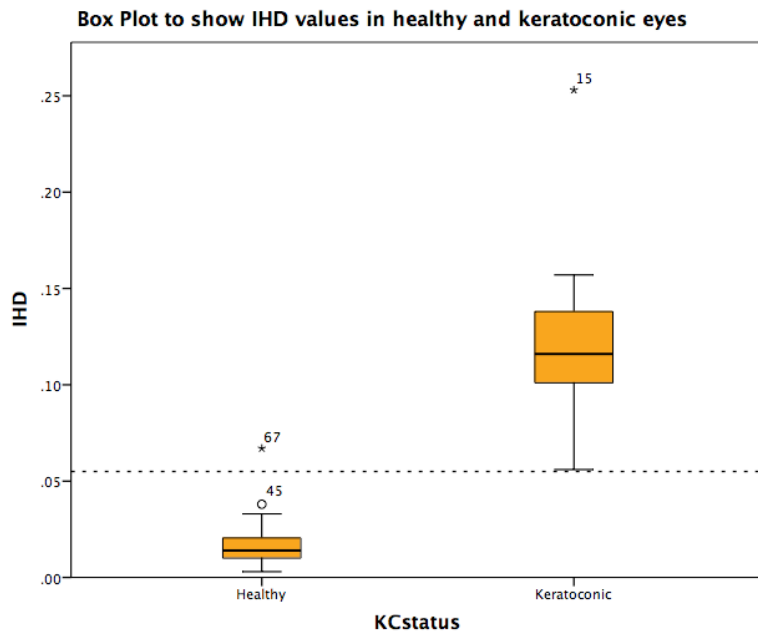


Figure 3. 13 Box Plot to show IHD values in healthy and KC eyes.

Table 3. 28 box plot statistics for 'Front surface elevation at the thinnest point'

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2&3) |
|---------------------|--------------------|----------------------|
| Mean | 7.55 | 51.65 |
| Standard deviation | 3.62 | 20.28 |
| Minimum | 1.00 | 22.00 |
| Maximum | 19.00 | 91.00 |
| Total range | 18.00 | 69.00 |
| Interquartile range | 4.00 | 34.50 |

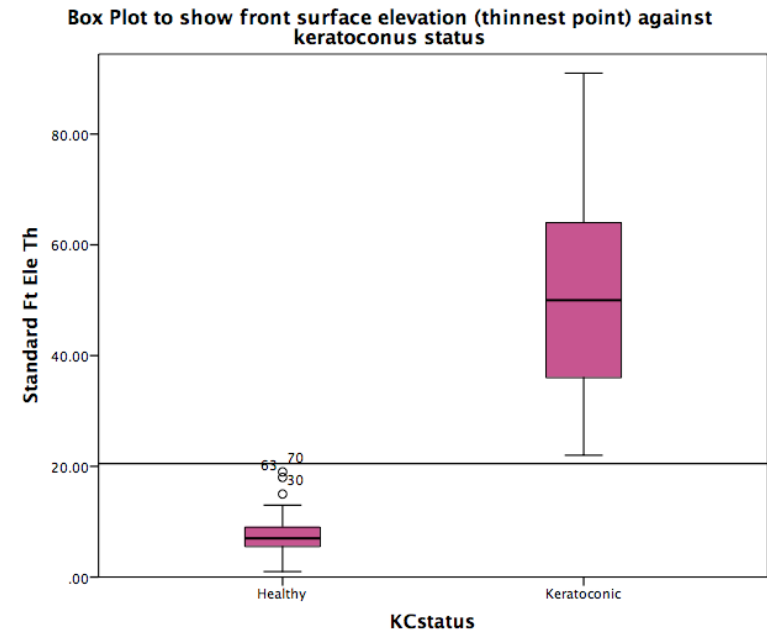


Figure 3. 14 Box plot to show front surface elevation (thinnest point) against KC status.

3.4.7 Aberration indices

Vertical coma and spherical aberration showed significant differences between healthy and keratoconic groups. Horizontal coma did not, and was removed from further analysis. Spherical aberration had a small AUC and therefore carries poor diagnostic capacity. Vertical coma had an AUC of 1 and was further examined using box plot analysis

Table 3. 29 Analysis of aberration indices using the Mann-Whitney U test when grouped by KC status (normal and suspect versus early KC)

| Index | Mann-Whitney U | Significance (p-value) | AUC | Significance (p-value) | Asymptotic 95% Confidence Interval | |
|-----------------|----------------|------------------------|-------|------------------------|------------------------------------|-------------|
| | | | | | Lower bound | Upper bound |
| Horizontal coma | 404 | 0.68 | - | - | - | - |
| Vertical coma | 1 | 0.00 | 1.000 | 0.00 | 1.000 | 1.000 |
| Spherical | 148 | 0.00 | 0.171 | 0.00 | 0.011 | 0.330 |

The relationship between vertical coma and spherical aberration was studied, with respect to healthy and keratoconic eyes. Figure 3.15 shows healthy eyes were clustered together and keratoconic eyes showed much higher spherical and vertical comatic aberration. Interestingly, some keratoconic eyes demonstrated a significant increase in negative spherical aberration in the absence of great comatic aberration, and vice versa.

Scatter Plot to show correlation between spherical aberration, comatic aberration with respect to keratoconic status

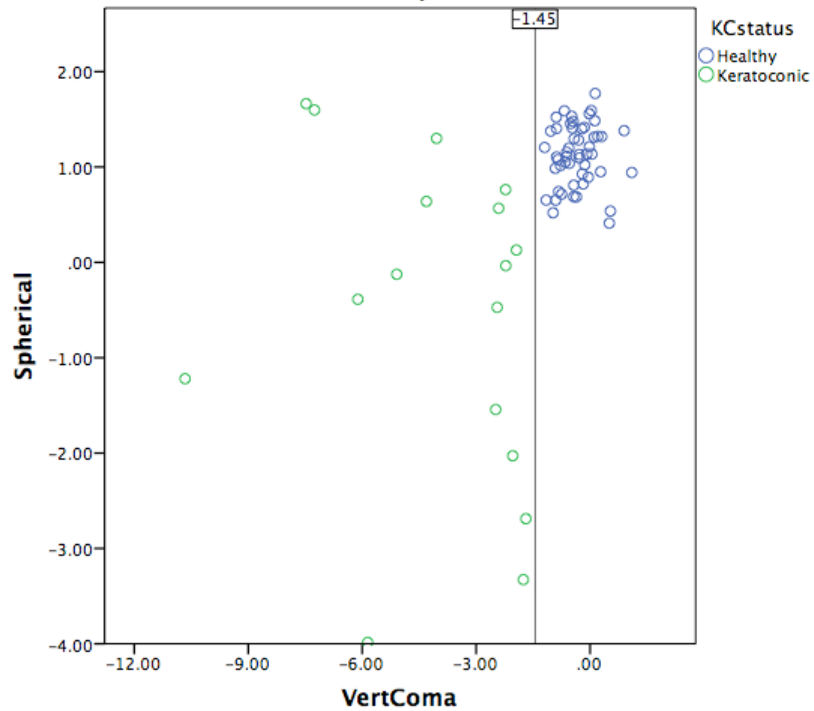


Figure 3. 15 Scatter plot to show correlation between spherical aberration and comatic aberration in KC and normal eyes. Examination of vertical coma reveals no overlap between healthy and keratoconic values, whereas such a cut-off is not possible for spherical aberration, with a significant degree of overlap occurring.

Box Plot to show vertical coma

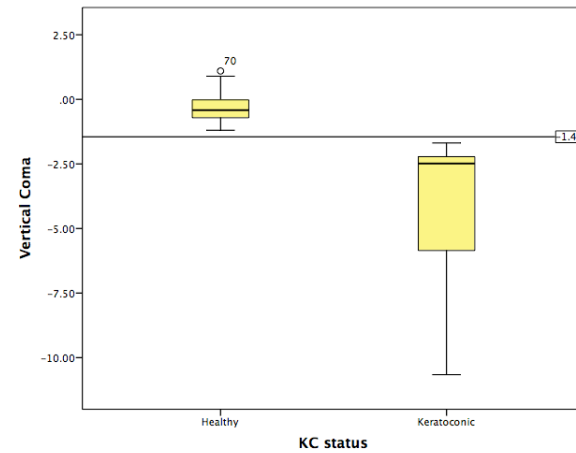


Figure 3. 16 Box plot to show vertical coma

Table 3. 30 Box plot statistics for vertical comatic aberration data between the two groups.

| Statistic | Healthy (SG 0 & 1) | Keratoconic (SG 2&3) |
|---------------------|--------------------|----------------------|
| Mean | -0.33 | -4.12 |
| Standard deviation | 0.51 | 2.60 |
| Minimum | -1.20 | -10.66 |
| Maximum | 1.20 | -1.69 |
| Total range | 2.29 | 8.97 |
| Interquartile range | 0.73 | 3.86 |

3.5 Discussion

It is clear from previous DS corneal studies that a significant proportion of the corneae provide anomalous topography results that may indicate the presence of keratoconus (Vincent et al. 2005; Aslankurt et al. 2013; Anderson et al. 2014). While some authors believe topographical abnormalities may exist in a healthy DS cornea, others suggest that all abnormal topography in DS is an early sign of ectatic disease in these patients. Ophthalmologically, the only way to determine if KC is present in such a case is to compare subsequent topographies over a period of time. While the current study was not intended to be a longitudinal one, previous topographical records were used when appropriate to confirm stability or progression. When historical records were not available, suspect cases of abnormal topography were followed up in clinic over a 12 month period to monitor for change, in line with standard corneal clinic protocol. However, although others were available for comparison, it was always only the first topographies during the timeframe of the current study that were used in the analyses of this thesis. This topographical study of KC in DS is therefore the first attempt to reliably distinguish true ectasia from 'abnormal but stable' corneae in those eyes which exhibit suspicious topography.

The current research used a specifically designed 'study grading scale' (SG) to stratify the study patients based upon clinical severity in a manner that was independent of visual acuity, contrast sensitivity, CCT and corneal power. The stratification of corneal curvature indices in figure 3.4 demonstrated that this approach was appropriate since there was a clear increase in the magnitude of the corneal power measures for each incremental step in severity. These results were in line with findings of the general population by Pinero et al. (2010), albeit with the DS eyes demonstrating higher baseline values. When SG was correlated with TKC, the automated Pentacam grading scale, the TKC was found to overestimate suspect keratoconus in a linear fashion. Any DS cornea that was graded by the Pentacam as TKC 2 or above was correctly identified (with respect to the clinical appearance, the ophthalmic history and the stability of topography). However, the Pentacam had a

tendency to grade healthy DS corneae up to TKC '1-2' (taken to be TKC 1.5 for the purposes of this study), indicating the possibility of false positives if the TKC grade is relied upon alone in DS. Revisiting Aslankurt et al. (2013), 'subclinical' was not specifically defined in TKC terms, but it is highly likely to have been TKC 'poss' or TKC 1, and in light of the current study may therefore have overestimated the prevalence of KC (at 38%) in this group of 5-13 year old children.

In addition to the automated Pentacam grading from the current study, other findings were of clinical and scientific use. Typically, an Index of Surface variance (ISV) is considered to be abnormal if >37 and pathological if >41 in the typical population (Oculus, 2006). Values of up to 50 were found in healthy eyes with DS and this indicates that healthy DS eyes may have more irregularity in their corneal surface as standard than that of the typical population. In the current study, the range of elevation index, IHD, in healthy DS eyes was also larger when compared to a prior study measuring IHD on healthy controls (Kanellopoulos and Moustou 2013). It is likely that larger measures of IHD and ISV in healthy DS indicate an abnormality in corneal topography, reflected in the overestimation of TKC grade. By their nature, higher ISV and IHD values are likely to correspond to increased aberrations from the cornea. As expected, spherical aberration and vertical coma were both significantly associated with KC in this study. Horizontal coma was not significantly different between healthy and KC groups, perhaps resulting from the tendency of cones to be vertically rather than laterally displaced. The current study found an increase in vertical coma in healthy DS eyes when compared to that of controls in a previous study eliciting the same Zernike coefficient data (DS controls, -0.33 ± 0.5 and non-DS controls, 0.00 ± 0.23 , Piñero et al. 2009). The DS control comatic aberrations did not reach the pathological aberrations seen in either DS (current study) or non-DS (Piñero study). Research by McCullough et al. (2013) found larger whole-eye HOAs in DS eyes, and the current study suggests that this may be, at least in some part, corneal. It is possible that an increased physiological threshold of aberrations may exist in DS, that these may be 'natural' aberrations in the DS cornea to some extent, a product of the fundamentally altered shape, rather than an indication of early ectatic disease, as suggested by others (Aslankurt et al. 2013).

While eyes with KC were associated with a higher magnitude of corneal astigmatism, significant overlap existed between healthy and diseased eyes in the low and moderate astigmatic range. This demonstrates that a diagnosis of KC cannot be reliably made upon the level of corneal astigmatism alone. Maximum corneal power (K_{\max}) provided the largest AUC values and hence had the most diagnostic value of the corneal power set. However, when examined further using box plot analysis, K_{\max} too demonstrated significant overlap between healthy and diseased eyes and thus a reliable cut-off point could not be established. This parallels the clinical view that physiologically steep corneae can occur in the absence of disease, and thus K_{\max} should be used as a descriptor of disease rather than a diagnostic indicator alone. This study did however provide some useful indicators of normal limits for demonstrably healthy corneae, some of which may be useful clinically when using the Pentacam with DS patients.

One metric, the Pentacam-derived Keratoconus Index (KI) separated the healthy and the diseased groups. The highest value for the healthy group was 1.11 and the lowest value for the KC group was 1.15. Goebels (2015) classified the beginning of stage 2 also at 1.15, lending validation to the results obtained in this study. A cut-off value could be chosen as 1.15, again to provide maximum specificity and to be in common with that of the general non-DS population.

When corneal power was correlated with CCT, and these results grouped by keratoconic status (confirmed KC or non-KC), the regression lines are markedly different between the groups (figure 3.5). A very small negative relationship between corneal power and CCT was found, but a much more significant relationship found in the KC eyes, indicating a divergence of pathological and physiological data. The weak relationship in healthy eyes demonstrated that those DS corneae which were steeper were not necessarily thinner, and vice versa. This is important, since it must be considered that DS corneae and their abnormal topography could represent a spectrum of keratoconus, with only those most genetically affected expressing the clinically manifest disease and the accompanying clinical signs. However, the very clear divergence in regression between the two categories, coupled with the weak correlation in healthy eyes, suggests a spectrum of

disease is not the case. The development of a much larger database would strengthen or weaken this argument, as the sample size is a limiting factor to the power of this statement.

Elevation indices in this study provide excellent discrimination, especially when Belin-Ambrosio display units are used. High elevation at the thinnest corneal point is considered highly indicative of keratoconus (Miháltz et al. 2009), and this was reflected in the AUC values across all 4 direct elevation measures. Results from this study are reflected in those from De Sanctis and colleagues, who found similar efficacy in detecting subclinical and early disease with and without the Belin-Ambrosio enhanced display (de Sanctis et al. 2013). The ability of elevation-based topography to measure the posterior surface is considered a great advantage in the study of KC. Posterior change is in some instances the earliest indicator of subclinical ectasia (de Sanctis et al. 2008; Belin and Khachikian 2009), yet in the current study, the posterior elevation at the thinnest point was not found to be more sensitive than the anterior elevation at the thinnest point. It is clinically useful to know that the anterior surface of the DS cornea can be relied upon to demonstrate early KC, particularly as some hospitals do not have access to posterior curvature data. This lack of difference found may be because of study sample size, or simply because the anterior surface of the DS cornea is not as able to mask early changes in the same way as a relatively thicker and flatter cornea from a typically-developing person. To utilise a parameter that is available in all Pentacams with or without Belin-Ambrosio display software, anterior elevation at the thinnest point was chosen. In order to gain maximum specificity, a cut-off should be set at 22 μ m.

In conclusion, the current study provides an indication of Oculus Pentacam limits for confirmed healthy DS eyes, and an indication of possible cut-off points that may be useful if validated with a further test set. This data are of scientific importance since it is the first DS study to have provided a considered grading scale to remove biasing aspects such as corneal power and CCT. Although the current sample size was limited, the divergence between the physiological and pathological was considerable. Since the corneal astigmatism alone was not sensitive or specific enough to differentiate healthy from KC eyes, the use of a keratometer to quantitatively assess

for KC in DS is unsuitable. Using the Oculus Pentacam, it was possible to separate the two groups using data from the anterior ocular surface parameters only. If such results were demonstrated to be transferrable between different topographers, the analysis of corneal 'tomography' using posterior surface data may not necessarily be required to identify KC in DS; standard topography may suffice. Similarly, the Belin-Ambrosio enhanced ectasia display is not essential to the determination of ectasia in this group. Finally, the healthy DS cornea carries levels of comatic aberration that may contribute to the increased whole-eye aberrations, and therefore poorer visual quality in DS eyes.

Chapter 3b

Morphology of cones in
keratoconus:

Down's syndrome and non-

Down's syndrome eyes

3.6 Introduction

Understanding how the morphology of the keratoconic cone in DS differs to that in a typical population is vital not just to the clinical understanding of KC in DS and its subsequent treatment, but also in the scientific application of the findings in the current study. That is, should the cone morphology in DS be comparable to that in non-DS eyes, KC in DS could serve as a potential model for the disease as a whole. The studies in this chapter will address the hypothesis that the cone morphology in DS has similar characteristics to that of typical individuals.

Studying the shape difference in cone morphology between DS and non-DS eyes could be compared to the study of morphology of mountains existing in two different countries. Initially, one might consider features such as absolute height, height above sea level, and slope steepness. Analogous to KC, these are features that are dependent upon the severity of the cone, but there exist other important features of corneal shape that are less dependent upon the KC severity and rather are more a function of the phenotype of the cone that has developed. Such features will be compared across a DS and a non-DS sample when the individuals in each group are matched for severity using an objective method such as the TKC index on the Oculus Pentacam.

Furthering the mountain analogy, useful morphological features might include:

1. Shape of the mountain when viewed with elevation contours from the top, with respect to (i) its classification, such as round, oval (analogous to the shape of the KC cone such as nipple, oval) and (ii) the location of the steepest point with respect to its base (analogous to the cone apex location with respect to the corneal centre). A full classification for (i) can be found at table 1.2 and figure 1.25, while the depiction of the steepest point location is given in fig 1.22, Chapter 1)
2. An observation of the cross-sectional profile of the mountain (analogous to the shape of the cornea when viewed from the side using Scheimpflug imaging).
3. Rate of incline of hill slope and how this changes from top of the mountain to bottom (analogous to asphericity).
4. The lateral variation in thickness of the different material layers (eg bedrock, soil) i.e. tomography (analogous to corneal tomography thickness profile).

The aim of this study is to test the hypothesis that KC in DS is comparable in shape to KC in the non-DS cornea.

3.7 Methods

Keratoconic corneae from the current study were selected for analysis. A decision was made to include eyes only from one side so as to not confuse any locational analyses. The left eye was chosen since a greater spread of severity was present. Non-DS eyes with the same Pentacam-derived TKC grade were randomly chosen from the university and hospital cornea clinics to provide a matched control for each DS keratoconus subject. Eyes with Pentacam grades between 2 and 4 were included in this section of the study in order to evaluate those with diagnosed early to moderate disease. In DS eyes, the same Scheimpflug scan from earlier analyses was used, and for the non-DS eyes, a selected Scheimpflug scan was selected in line with the protocol set out in Chapter 3a. Relevant derived topographical data were extracted from the clinical display and analysed using SPSS (version 20).

3.8 Results

3.8.1 Topographical classification

Eleven (left) eyes with DS and KC were selected for analysis with matched keratoconic controls. Each eye was matched to classification criteria set out by Rabinowitz et al. (1996) and is represented by the classification guide symbol. A symmetric bow-tie with skewed radial axes (SB/SRAX) was the modal presentation in both groups. Round and oval cones were found in each group. Superior and inferior steepening were only found in the control group, whilst asymmetric bow-tie with skewed radial axes (AB/SRAX) was found in the DS group only.

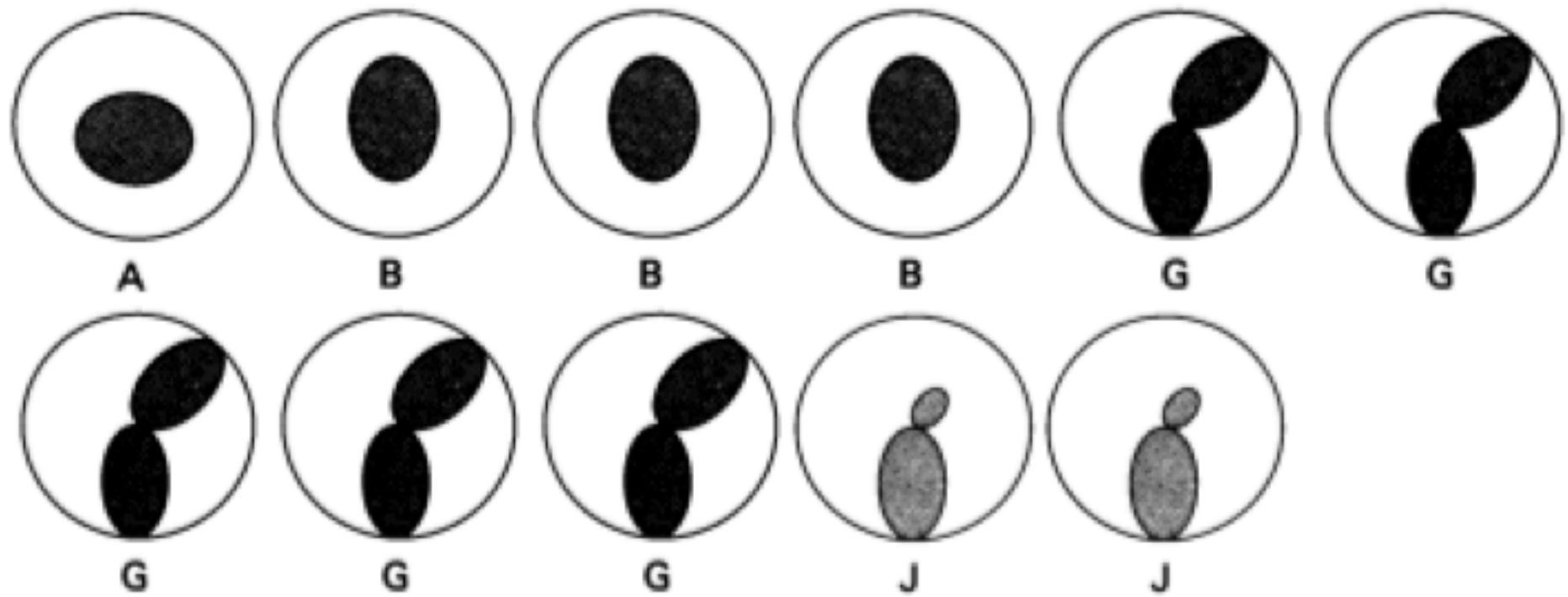


Figure 3.177 Representation of the quantity of each topographical classification present in left DS eyes with KC in the current study, using the classification key from Rabinowitz et al. (1996).

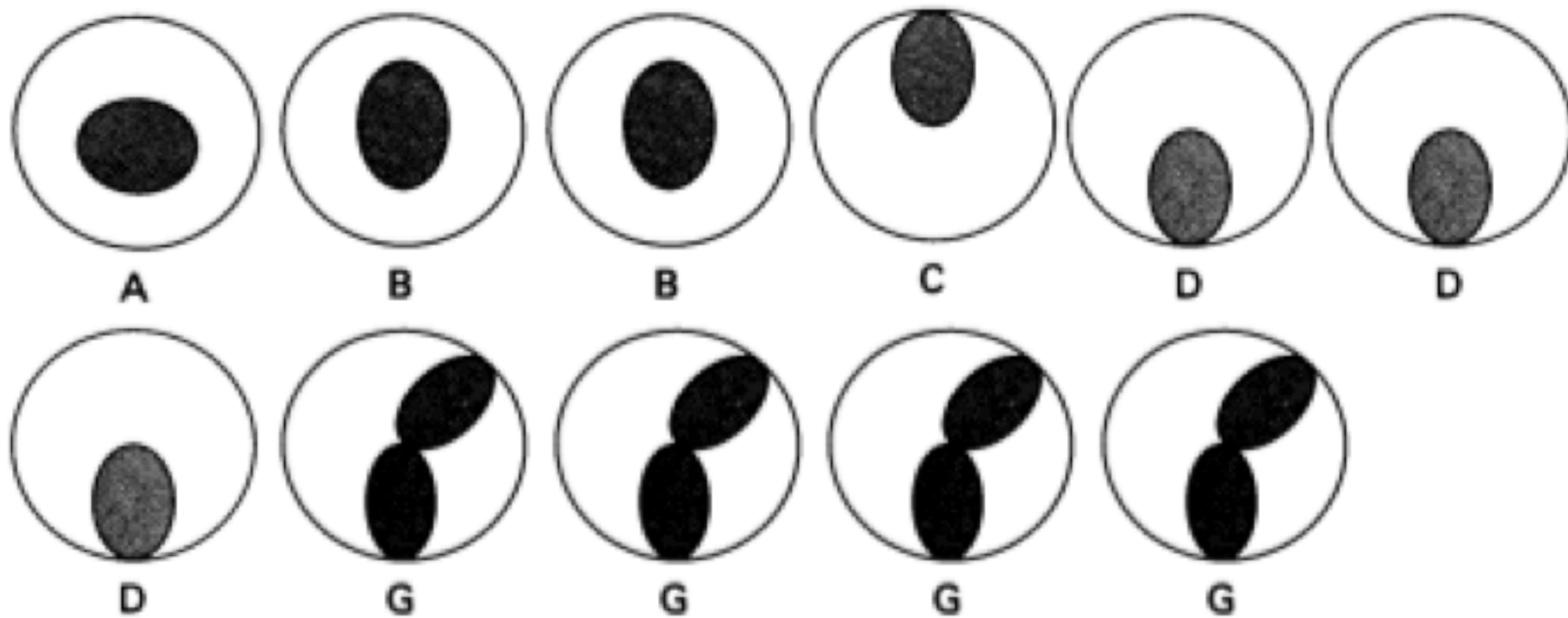


Figure 3.18 Representation of the quantity of each topographical classification present in left non-DS control eyes with KC in the current study, using the classification key from Rabinowitz et al. (1996).

3.8.2 Apical location

The apical locations from the sagittal curvature maps (denoted by the location of K_{\max}) are shown in figure 3.19. Apices are predominantly in the inferior quadrants in both groups, and toward the vertical midline.

Scatter graph showing the locations of apical curvature (K_{\max}) in DS and control eyes

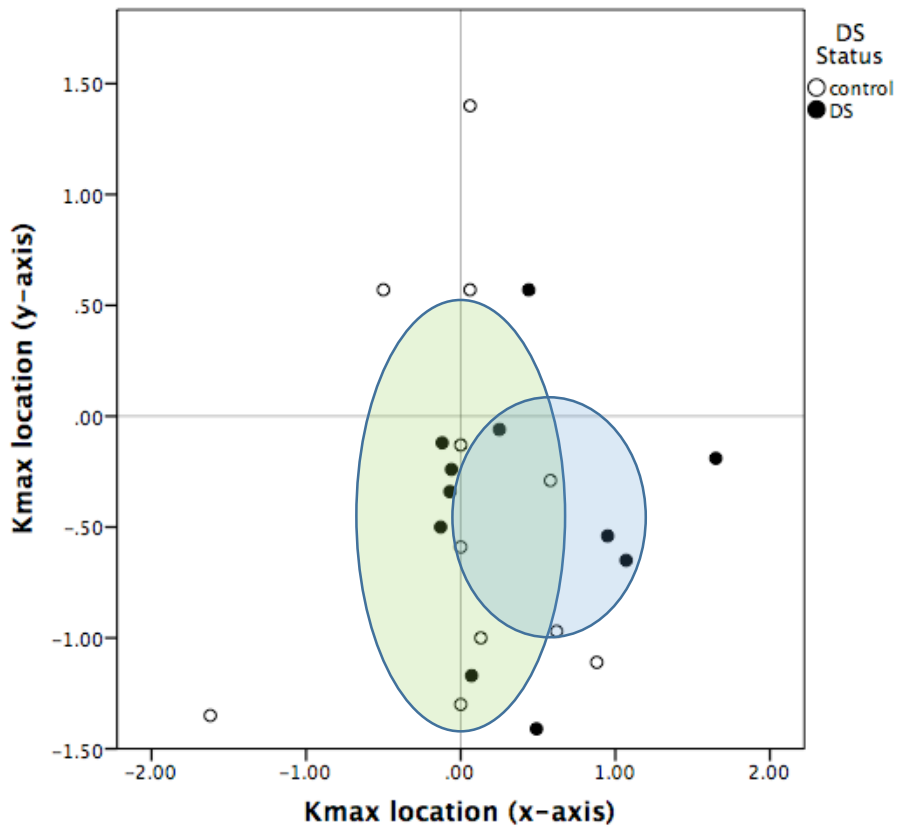


Figure 3. 19 Apical locations from sagittal curvature maps, DS eyes (closed circles) and control eyes (open circles). The apical locations by curvature of both groups are scattered along the vertical and towards the inferotemporal quadrant, indicating a similar pattern of apical location and a similar pattern of spread. The green oval denotes the standard deviation of the control eyes, whilst the blue oval denotes the standard deviations of the DS eyes.

The apical locations of the cones provided by the point of maximum corneal elevation is given in figure 3.20 for both groups. It is apparent that the majority of cones of both groups lie in the inferotemporal quadrant. There appears to be no demarcation between the location of DS cones and those of controls.

Scatter graph showing the locations of elevation apex in DS and control eyes

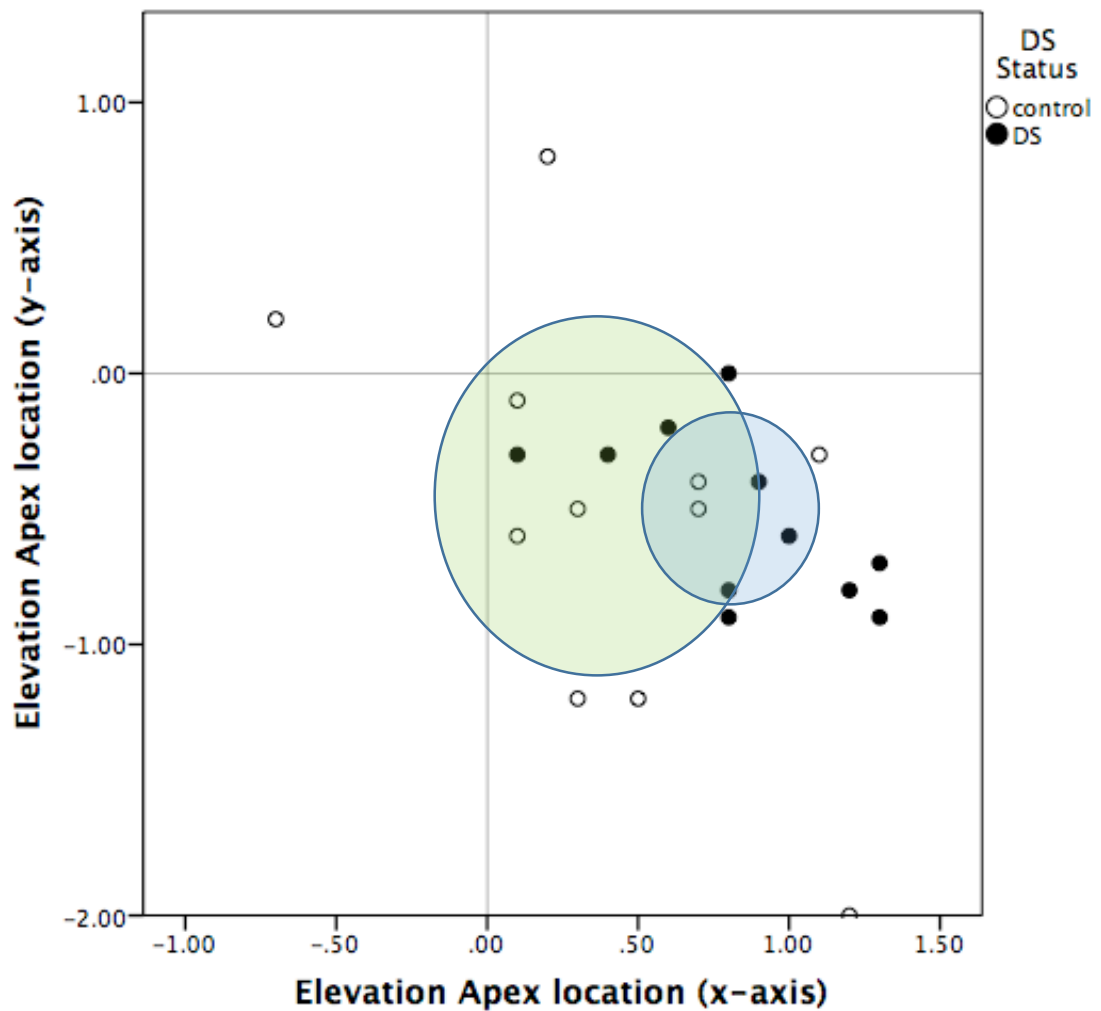


Figure 3. 20 Apical locations from elevation maps. While the control eyes indicate a slightly greater spread than DS eyes, most subjects in each group have an elevation apex in the inferotemporal quadrant. The green oval denotes the standard deviation of the control eyes, whilst the blue oval denotes the standard deviations of the DS eyes.

3.8.3 *Scheimpflug imaging*

Still shots of Scheimpflug imaging were obtained from all subjects. For each integer TKC grade, a DS eye and a control eye were studied to qualitatively compare the anterior segment morphology. An example of a DS eye is shown in the upper figure and a control eye below.

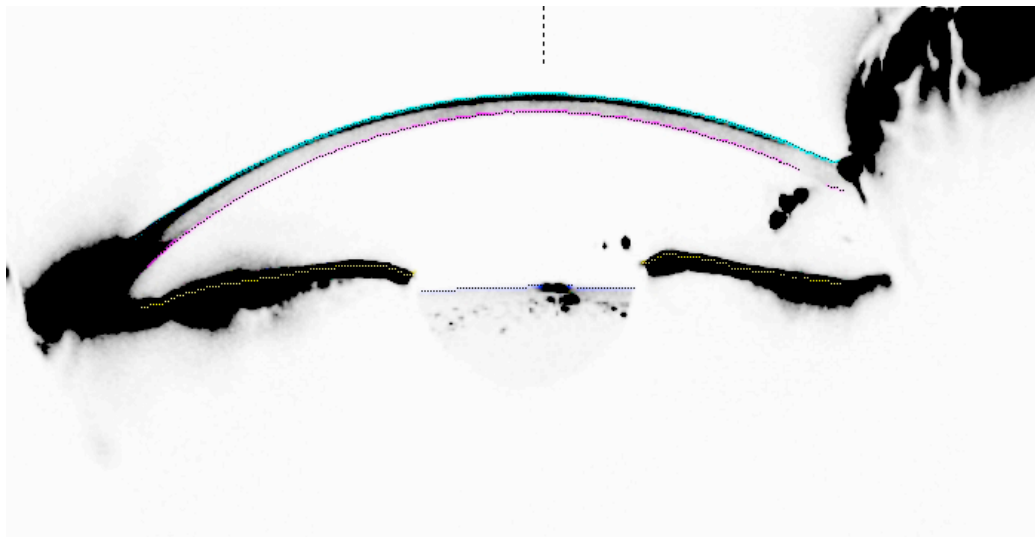


Figure 3.21 Scheimpflug image of a DS eye with Pentacam TKC grade of 2. Note the dotted appearance of lens opacities in this subject.

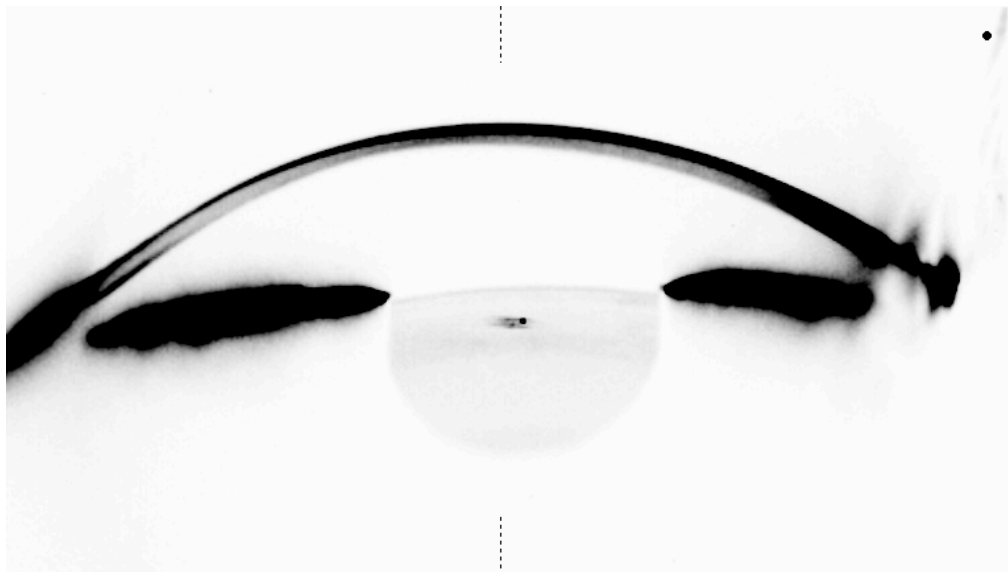


Figure 3.22 Scheimpflug image of a control eye with Pentacam TKC grade of 2. Note that the cornea appears grossly normal and the stromal thinning is not yet visible from this view.

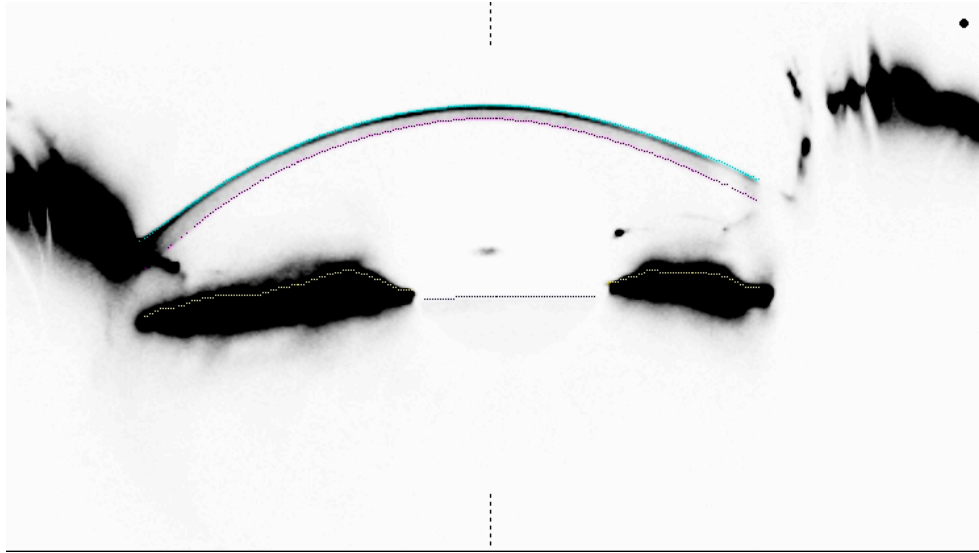


Figure 3.23 Scheimpflug image of a DS eye with Pentacam TKC grade of 3. Note that some bowing of the cornea is visible, particularly on the back surface.

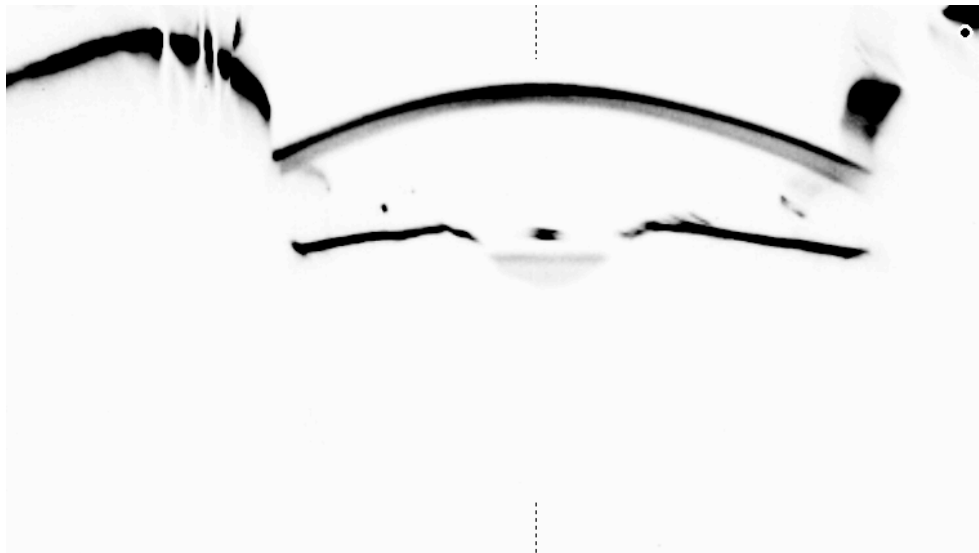


Figure 3.24 Scheimpflug image of a control eye with Pentacam TKC grade of 3. Note that some early bowing of the cornea is visible.

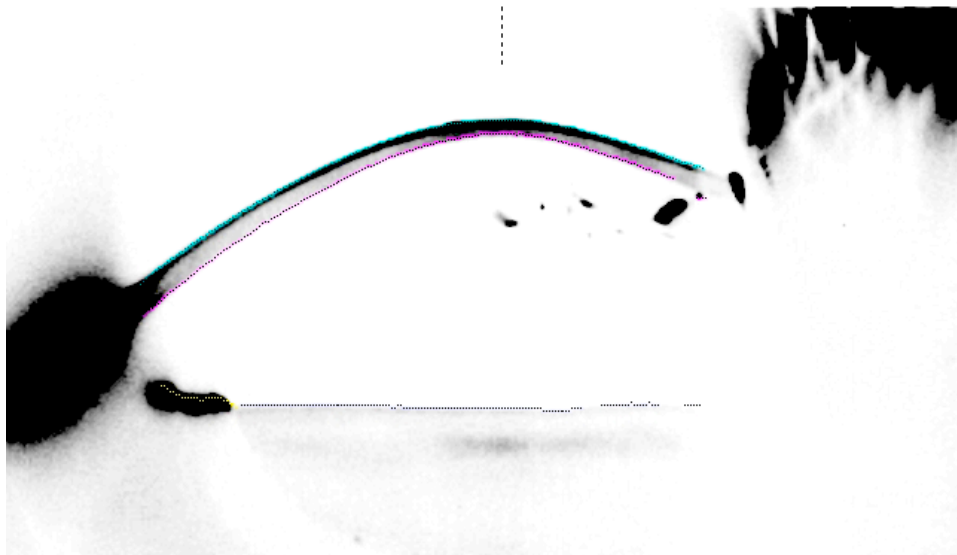


Figure 3.25 Scheimpflug image of a DS eye with Pentacam TKC grade of 4. Note the thinned central cornea and the irregularity on the anterior corneal surface.

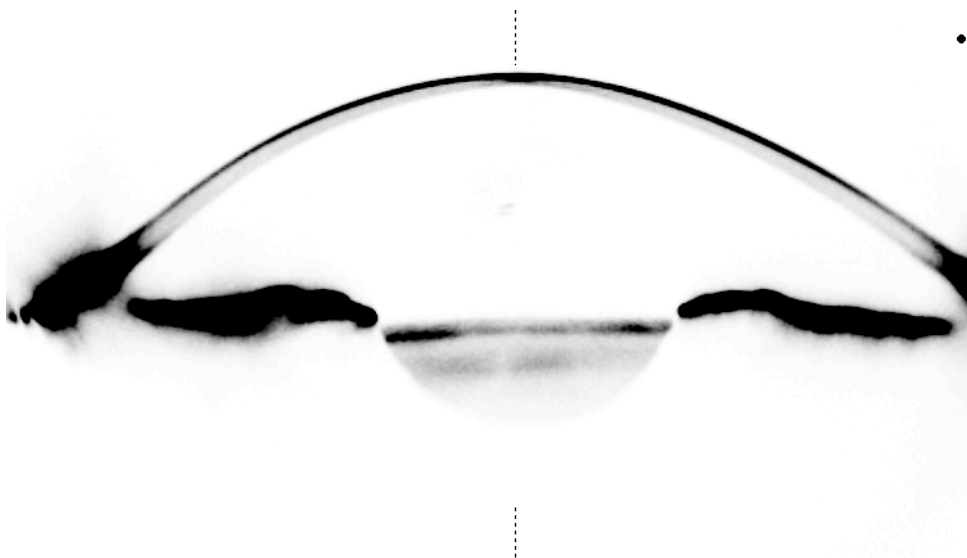


Figure 3.26 Scheimpflug image of a control eye with Pentacam TKC grade of 4.

3.8.4 Asphericity

Asphericity was derived from mean eccentricity values taken at 8mm for all subjects. The results are shown in table 3.16 and 3.17. As can be seen from figure 3.27, there is a much greater range of asphericity values in the DS than the control group. Despite this, there was no significant difference in asphericity between the two groups.

Table 3. 16 Box plot statistics for asphericity of DS and controls.

| Statistic | Control | DS |
|---------------------|---------|-------|
| Mean | -1.69 | -2.34 |
| Standard deviation | 0.49 | 1.47 |
| Total range | 1.52 | 4.05 |
| Interquartile range | 0.80 | 3.01 |

Table 3. 17 Analysis of asphericity using the Mann-Whitney U test between DS and control groups.

| Index | Mann-Whitney U | Significance (p-value) |
|-------------|----------------|------------------------|
| Asphericity | 48 | 0.412 |

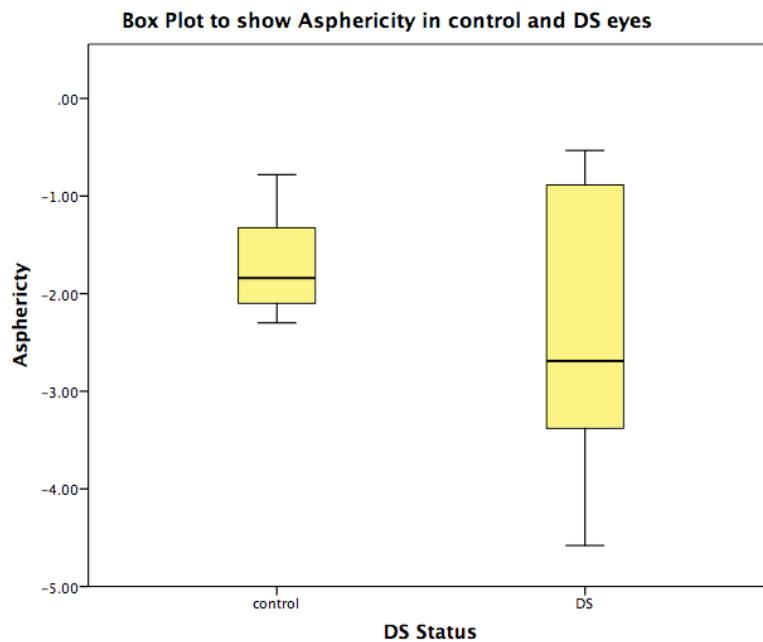


Figure 3. 27 Box plot to show the range of asphericity found in control and DS eyes with KC.

3.8.5 Pachymetric profiling

Percentage Increase Thickness (PIT) scores were available for all analysed corneas at 4mm ring around the thinnest point. Table 3.18 shows that the control and DS groups had a PIT of 31.81 ± 19.04 and 28.91 ± 18.64 respectively. There was no significant difference in this value between groups (table 3.19), and little difference in the spread of values (figure 3.28).

Table 3.18 Box plot statistics for PIT between the two groups.

| Statistic | Control | DS |
|---------------------|---------|-------|
| Mean | 31.81 | 28.91 |
| Standard deviation | 19.04 | 18.64 |
| Total range | 67 | 63 |
| Interquartile range | 19 | 14 |

Table 3.19 Analysis of PIT using the Mann-Whitney U test between groups

| Index | Mann-Whitney U | Significance (p-value) |
|-------|----------------|------------------------|
| PIT | 49 | 0.450 |

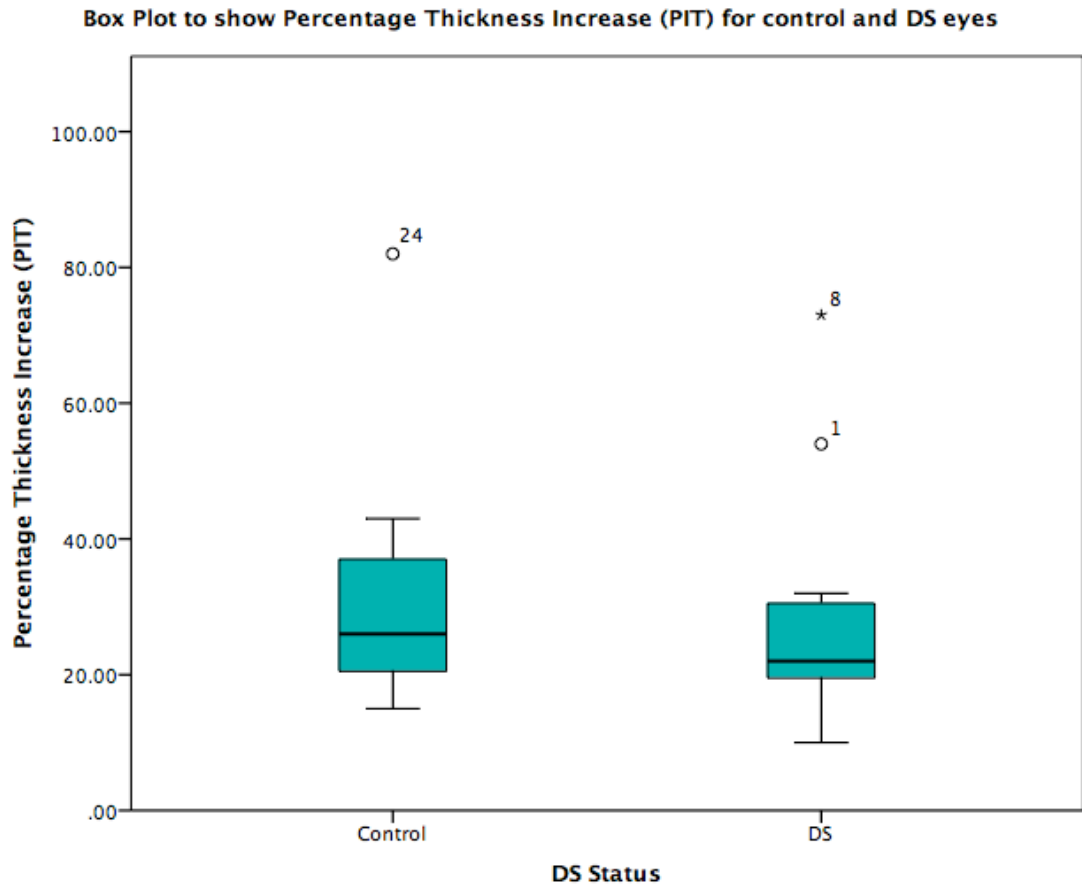


Figure 3.28 Box plot to show the range of PIT found in control and DS eyes with KC. Both group means, lower interquartile ranges and standard deviations are similar.

For each integer TKC grade, a DS eye and a control eye are presented over the following pages to qualitatively compare and contrast the anterior segment morphology. The DS is shown in the upper figure, and the control eye below. The plots depicting both the spatial thickness of each matched cornea (CTSP) and the PIT are shown.

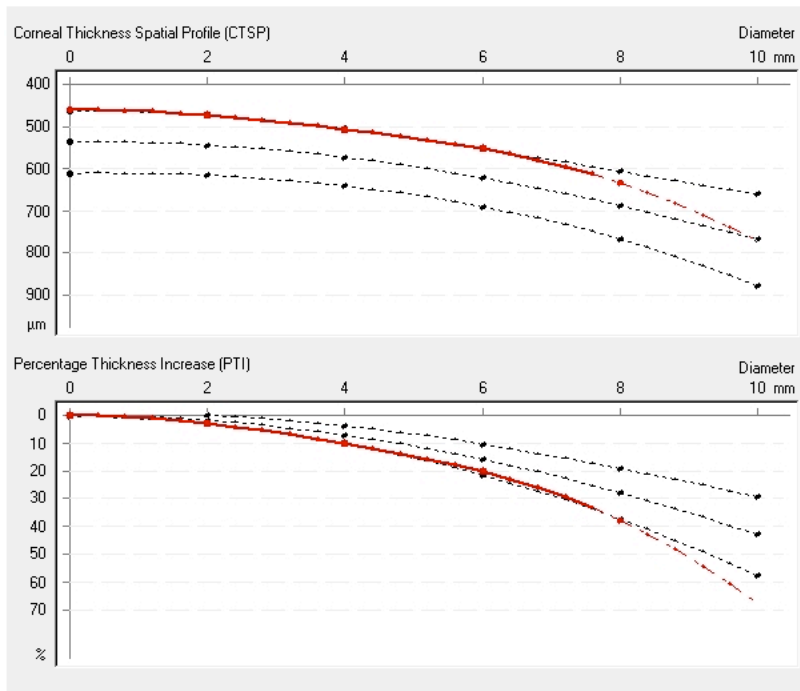


Figure 3.29 CTSP chart (top) and PIT chart (bottom) of a DS eye with TKC grade of 2. Note that both charts of this early keratoconic eye fall just within the expected normative range as denoted by the dotted lines representing 2 standard deviations from the mean.

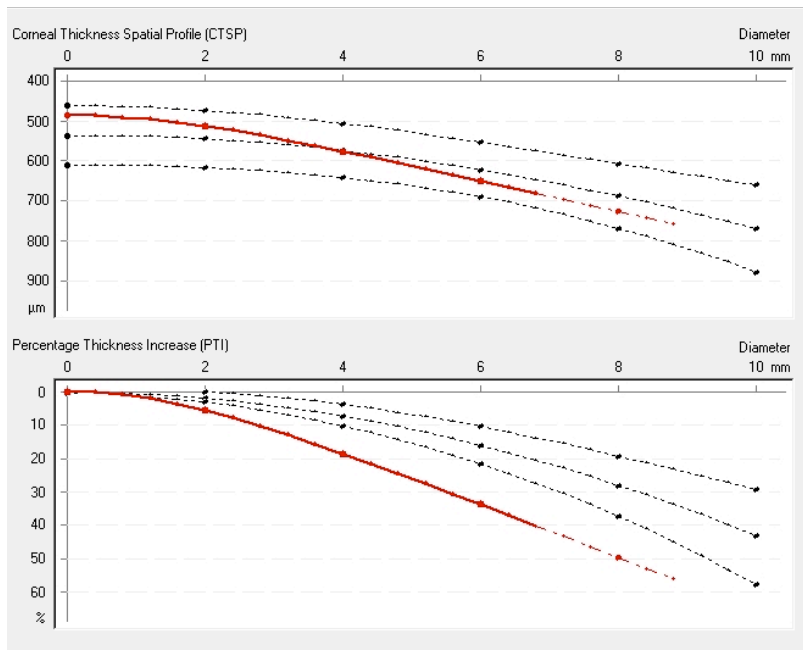


Figure 3.30 CTSP chart (top) and PIT chart (bottom) of a control eye with TKC grade of 2. Note again that both charts of this early keratoconic eye fall just within the expected normative range as denoted by the dotted lines representing 2 standard deviations from the mean.

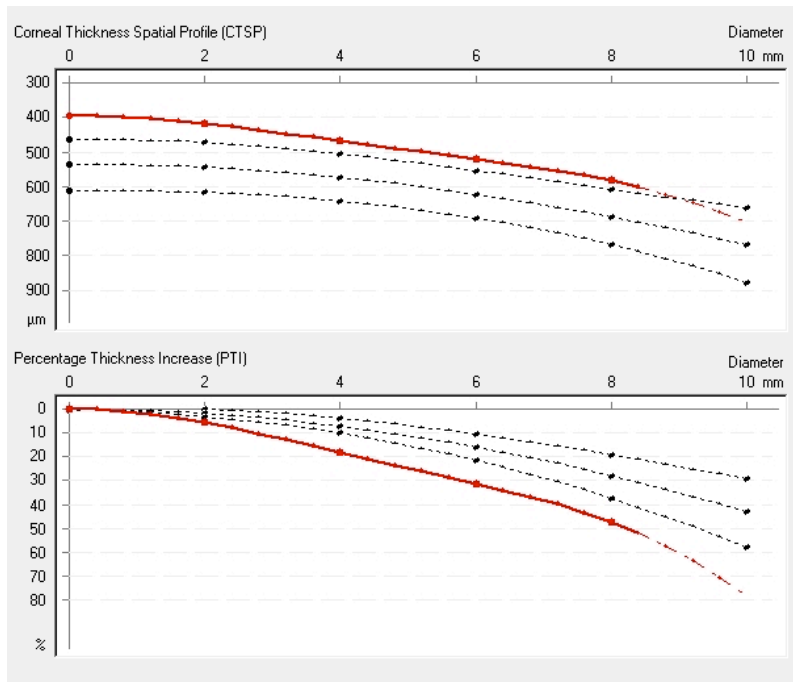


Figure 3.31 CTSP chart (top) and PIT chart (bottom) of a DS eye with TKC grade of 3. Notice the significant decrease in corneal thickness from the expected norm value, that in part results from the thinner starting point of the DS eye.

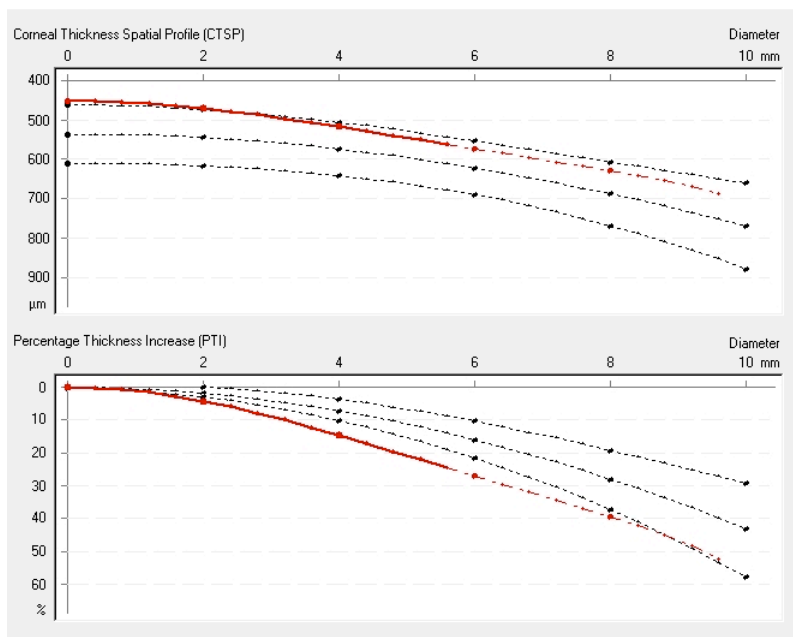


Figure 3.32 CTSP chart (top) and PIT chart (bottom) of a control eye with TKC grade of 3.

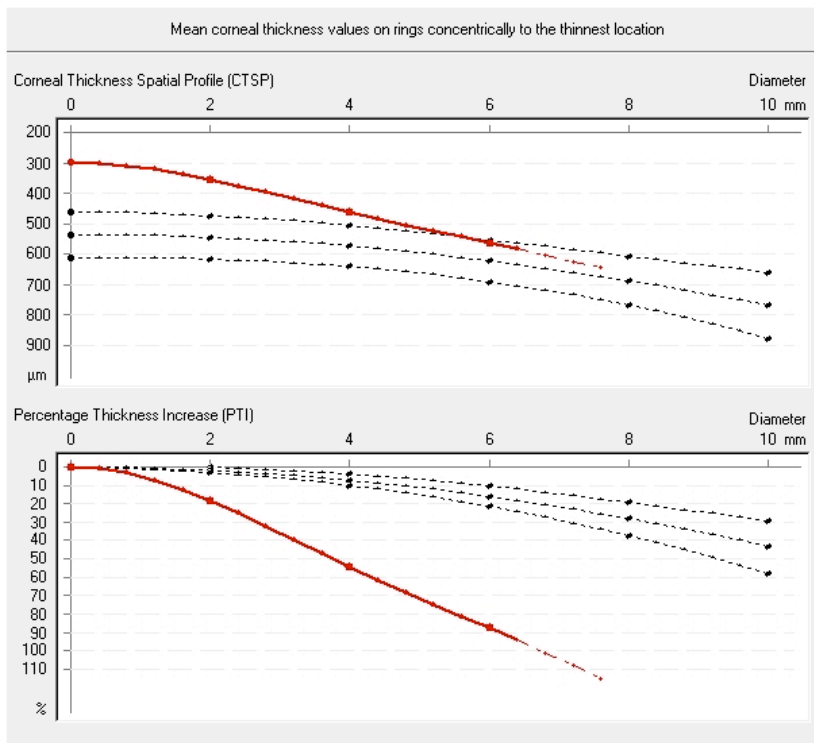


Figure 3.33 CTSP chart (top) and PIT chart (bottom) of a DS eye with TKC grade of 4. Note the gross thickness change from the normative values depicted by the dotted lines.

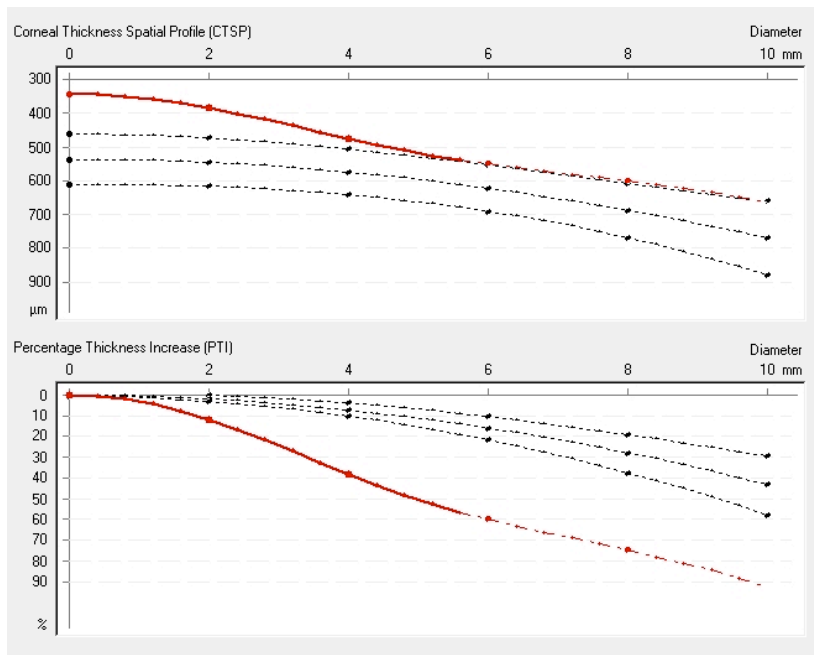


Figure 3.34 CTSP chart (top) and PIT chart (bottom) of a control eye with TKC grade of 4.

3.9 Discussion

This section of the current study aimed to identify whether the morphology of the DS cone was comparable to that of the non-DS eye, when matched for severity. This appears to be the case, and that taken together, the results above support the hypothesis.

As has been discussed at length in previous chapters, the DS cornea has a naturally thinner and steeper profile, even in healthy eyes. Because of the altered starting point, it must be considered that the underlying pathogenesis of KC may or may not be the same as that in typically developing eyes. Nevertheless, it is vital to understand if the morphological phenotype is the same. In considering if a common underlying genetic basis exists between DS and non-DS eyes, the shape of the cone is of aetiological importance. Rabinowitz (1990) suggests that cone morphology is a genetic trait, expressed in family members as a variable phenotype. If the KC in DS was predominantly a different cone type from the control eyes, then the evidence for DS KC being the same genetic disease as non-DS KC would be much weaker. While the study group size was relatively small with just 22 eyes, significant similarity exists between the cone morphology existing in DS eyes (figure 3.17) and non-DS control eyes (figure 3.18). The majority of common cone type in each group was (SB/SRAX), reflecting the irregular nature of the astigmatism produced and the typical decentering of the corneal apex from the corneal centre.

Asphericity data was statistically comparable between the groups, albeit with a large spread of values in the DS group. Mean values for asphericity -2.34 ± 1.47 (DS) and -1.69 ± 0.49 (control) data are in line with that expected from significant KC (Alio et al. 2011). PIT was closely matched between DS and non-DS eyes, for the 4mm zone studied. This value was chosen to represent the thickness difference between the mid-stroma and that of the minimum thickness. These results demonstrate that for the given initial reduced thickness of the DS cornea, ectatic thinning is still similar to non-DS eyes when matched for severity.

Closer analysis of apical decentration revealed that the vast majority of both DS and non-DS cones lay in the infero-temporal quadrant, when measured in elevation terms. Elevation based localisation of corneal apex is preferable and more clinically useful over axial curvature localisation, since it is less prone to influence by local variations in corneal thickness across the cornea. Both elevation apex location and curvature apex location are consistent with results from Demirbas and Pflugfelder (1998), with axial curvature data demonstrating an cluster toward the inferior vertical midline. Clustering and overlapping of DS and non-DS cone locations indicates that spatially the cones are indistinguishable. Comparable elevation mapping suggests that the high-order aberrations of coma and the resultant visual degradation is likely to be comparable, if the severity of the cone is matched. These data can be used to show that contact lenses suitable for correcting KC in typically developing individuals are at least topographically suitable for those with DS, since the cone morphology is comparable.

Despite similar morphological phenotype, there is an important observed clinical difference of KC in DS. People with DS progress to corneal hydrops more often and more readily than those in the general KC population (Grewal et al. 1999). This could be of scientific significance, as it is evidence of an altered phenotype at the later stages of disease. This study did not seek to examine the severity of the disease between DS and non-DS KC, but it is a distinct possibility that the underlying disease process might be accelerated in some way in DS. Another variable not investigated in the current study is the age of onset of the disease. The earliest reported DS case of KC is in that of a 4 year old child (Sabti et al. 2015) and in non-DS an 8 year old (Jiminez et al. 1997). In non-DS eyes, the presentation of KC at a young age is usually accompanied by severe and uncontrolled allergic eye disease and thus may be considered a separate entity, in part. Such a genetic relationship was investigated by Adachi et al. (2002), reporting an increase of HLA antigens in blood samples of subjects 12-20 years over those 21 years and above. Men were also found to be significantly younger at diagnosis than women. Genetics therefore likely play a key role in the onset and severity of KC, the biochemical basis for which is not yet known.

In summary, this sample of DS and non-DS KC did not establish any significant morphological differences between the studied groups and thus KC in DS may be a suitable morphological model for KC as a whole.

Chapter 4

In-vivo biomechanics of healthy Down's Syndrome eyes

4.1 Introduction

The healthy DS cornea is, on average, thinner and steeper than a healthy non-DS cornea (Haugen et al. 2001). Haugen postulates that corneal rigidity may be lower in DS eyes because of their generalised reduction in corneal thickness leading to steeper corneae, and that such altered shape may be of aetiological importance to the high levels of KC in DS. Aslan (2013) found that corneal volume is reduced in DS subjects, and goes further than Haugen to suggest that the thinner cornea in DS is early evidence of degenerative corneal disease such as KC. However, not all people with DS go on to develop KC and since this study was not longitudinal, no evidence of progressive disease is available to credit this opinion. In order to investigate the biomechanics of the DS cornea, deformation parameters should be compared against healthy non-DS controls, and any difference in results be assessed against non-DS keratoconic data to assess if any difference is of pathological proportion.

To date, the biomechanics of the DS cornea have not been examined either *in vivo* or *ex vivo*. As such, it is not yet possible to know if the DS cornea is weakened as a result of its reduced corneal thickness, or if it is pathologically weakened beyond thickness changes. The aim of this pilot descriptive study is to examine deformation parameters in healthy DS and non-DS eyes that are matched for age and IOP, and controlled for CCT in statistical analyses, in order to elicit if the shape of the healthy DS cornea impacts upon its biomechanical strength and, if so, to what extent.

4.2 Methods

4.2.1 *Patients*

A sub-group of known non-keratoconic subjects with DS from the DS cohort at Cardiff University were invited to take part. Non-DS subjects with healthy corneae were recruited from staff, students and siblings of patients at the same centre. Inclusion criteria for healthy subjects (both with and without DS) were the absence of any known corneal abnormality or prior ocular surgery and high myopia (>5.00DS).

4.2.2 *Examination*

All subjects were determined not to have keratoconus through previously established methods, including the use of retinoscopy, a full eye examination, and when possible, both slit lamp examination and Oculus Pentacam measurements.

CorVis ST (Oculus, Wetzlar, Germany) examination was performed by positioning the subject comfortably in front of the equipment, encouraging them to put their chin on the chinrest and place their forehead against the forehead band. Where possible, a parent/carer was asked to assist in maintaining the contact of the forehead to the band in order to maintain safety distances between the machine and the subject's cornea. While aligning the device, further oral consent was taken from the subject to ensure that they were still happy to proceed. Where possible, a blink was encouraged shortly before auto-alignment and auto-release of the air-pulse.

4.2.3 *Data modelling*

The air pulse delivered by the CorVis is standardised with a peak pressure of 60mmHg, from a distance of 11mm. This study used software (6.07r24) that, in line with others examining confounding variables (Ali et al. 2014), did not include the ‘corneal compensated IOP parameter. The CorVis uses the positioning of the outer and inner edges of the corneal profile to measure biomechanical effects such as deformation. Under the force of the air-pulse, the cornea deforms, but in addition the whole eye globe retracts. The displacement of the eyeball must therefore be separated from the deflection of the cornea itself (Koprowski et al. 2014). In the first instance, the shape of the cornea visible at $t=0$ is removed. In the second instance, the shape of the peripheral cornea is monitored in position throughout the applanation. The waveform that represents this movement with time denotes the eyeball displacement response (shown in red in figure 4.1), and may therefore be excluded to facilitate the analysis of the corneal response only. For corneal analysis, the component representing the eyeball displacement is reduced to zero, and the resulting waveform is now termed a deflection (shown in green in figure 4.1).

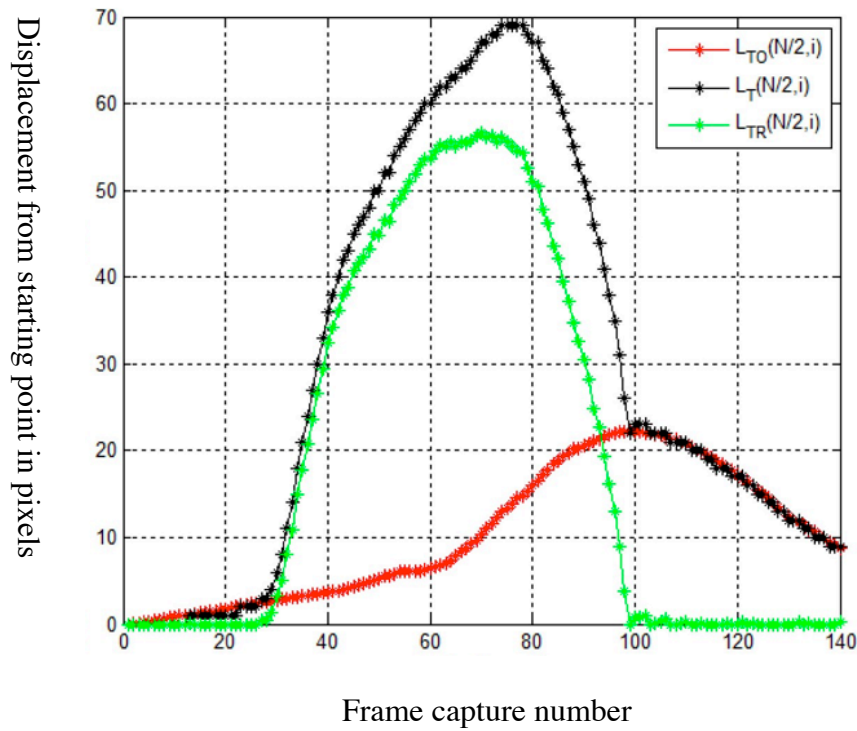


Figure 4.1 Graph to show the whole eye deformation (black, $L_T(N/2,i)$), during air-pulse tonometry with respect to the frame capture number (akin to time) and its subsequent decompensation into corneal deflection component (green, $L_{TR}(N/2,i)$) and eye globe component (red, $L_{TO}(N/2,i)$). Adapted from Koprowski et al. (2014) with permission.

In both groups, the results of the right eye were used for analysis, unless the data capture preview taken from the right eye was deemed too inaccurate on the first two occasions. In this case, a measurement from the left eye was taken (Ali et al. 2014). When measuring biomechanical properties, it is deemed important not to take repeated measures consecutively in order that the viscosity of the cornea is not adversely altered, such that it could unintentionally affect subsequent readings. When more than one measurement was taken, at least three minutes were given prior to any further measurements to allow for the aforementioned, and to ensure re-stabilisation of the tear film.

4.2.4 *Statistical analysis of results*

Deformation data was gathered primarily through the extraction of raw csv data (location points with respect to time). IOP and CCT data from the front-end software were taken, and when making comparative analyses with other studies, deformation amplitude (DA) and velocity at inward appplanation (V_{IN}) from the front-end screen were used. Standard parameters are listed in figure 1.19 and denote the typical deformation attributes used by research teams. In order to interrogate the data and determine the change in attributes such as DA with respect to time, it was necessary to utilise the raw data and rebuild the graphs presented on the front end display. The same extraction procedure enabled the mean attributes for each group as a whole to be calculated (with respect to time).

Statistical analysis was performed using Microsoft Excel version 15.23.1 (Microsoft, Richmond, Washington, USA) for the organisation of extracted data, the early descriptive testing of deformation characteristics, and the production of graphs. SPSS version 23.0 (IBM, Chicago, Illinois, USA) was used to perform descriptive testing, analysis of variance and multiple linear regression testing to assess confounding variables. Significance was taken to be $p < 0.05$.

4.3 Results

Nineteen subjects with DS were recruited for this section of the study, and 23 controls. (For an ‘a priori’ power calculation, see appendix E). All non-DS controls were confirmed to have healthy corneae but one subject was excluded due to the use of contact lenses on the day of visit (because of the theoretical risk of induced corneal swelling). In the DS group, 1 subject was excluded due to a clinical query about a corneal dystrophy. Two subjects changed their mind about participating before measurement, due to a fear of the new equipment. A further DS subject had one inaccurate measurement taken before deciding they no longer wished to participate. The results of 2 subjects who particularly struggled with fixation (and for whom no accurate measurement could be obtained), and 1 subject for whom the raw data extraction yielded corrupted results were removed from the analysis. This resulted in the inclusion of data for 22 non-DS controls and 12 DS subjects. The groups were matched for both age and IOP, and in doing so, data from 5 control subjects was excluded from the analysis, leaving 17 control subjects. Nine right eyes from the DS group were used for analysis (and 3 left eyes). Sixteen right eyes were used from controls (and 1 left eye).

The mean and standard deviations corresponding to age, IOP and CCT for each group can be found below in table 4.1. The p value corresponding to the student’s t-test is provided.

Table 4. 6 Table comparing group means of age, IOP and CCT

| | <i>DS</i> | <i>Control</i> | <i>Significance (p value)</i> |
|------------|------------|----------------|-------------------------------|
| Age | 19.1±9.7 | 19.1±8.8 | 0.86 |
| IOP (mmHg) | 14.0±2.0 | 15.0±3.0 | 0.32 |
| CCT (µm) | 507.3±42.1 | 555.9±23.3 | 0.00 |

Age and IOP were matched and therefore by nature not significantly different between the groups, but there was a significantly greater CCT in the control group.

Numerical deformation attributes may be considered in the following categories:

1. Deformation amplitude & deflection amplitude
2. Deflection area
3. Timing of applanations
4. Corneal deflection velocity
5. Globe displacement mechanics

Deformation is defined as the maximum displacement of the anterior corneal surface from its habitual convex shape to the inverted shape at the highest concavity between applanations. For visual reference, a DS cornea with a large deformation is seen in figure 4.1 (left) and a thicker non-DS cornea producing a smaller deformation amplitude is on the right.

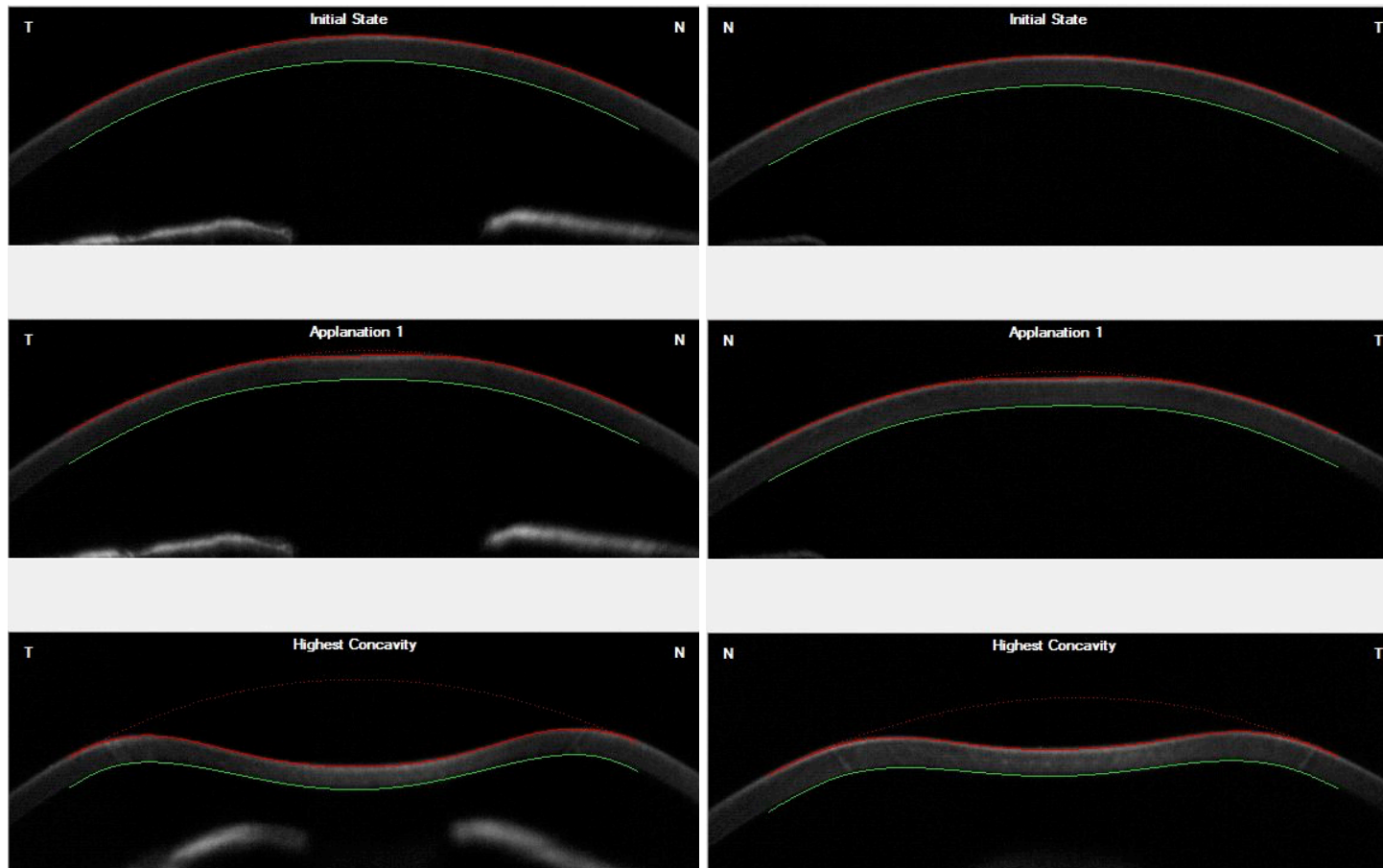


Figure 4.2 The left image shows a DS cornea with a high deformation amplitude. Note the visible iris as a function of captured position and a shallow anterior chamber. The image on the right shows a control cornea with a significantly larger corneal thickness and much reduced deformation amplitude.

4.3.1 Total deformation amplitude & corneal deflection amplitude

Table 4. 7 Table showing group deformation amplitudes and corneal deflection amplitudes

| | <i>DS</i> | <i>Control</i> | <i>Significance (p value)</i> |
|---------------------------------|-----------|----------------|-------------------------------|
| Total deformation amplitude/mm | 1.21±0.17 | 1.03±0.11 | 0.01 |
| Corneal deflection amplitude/mm | 0.95±0.12 | 0.84±0.09 | 0.02 |

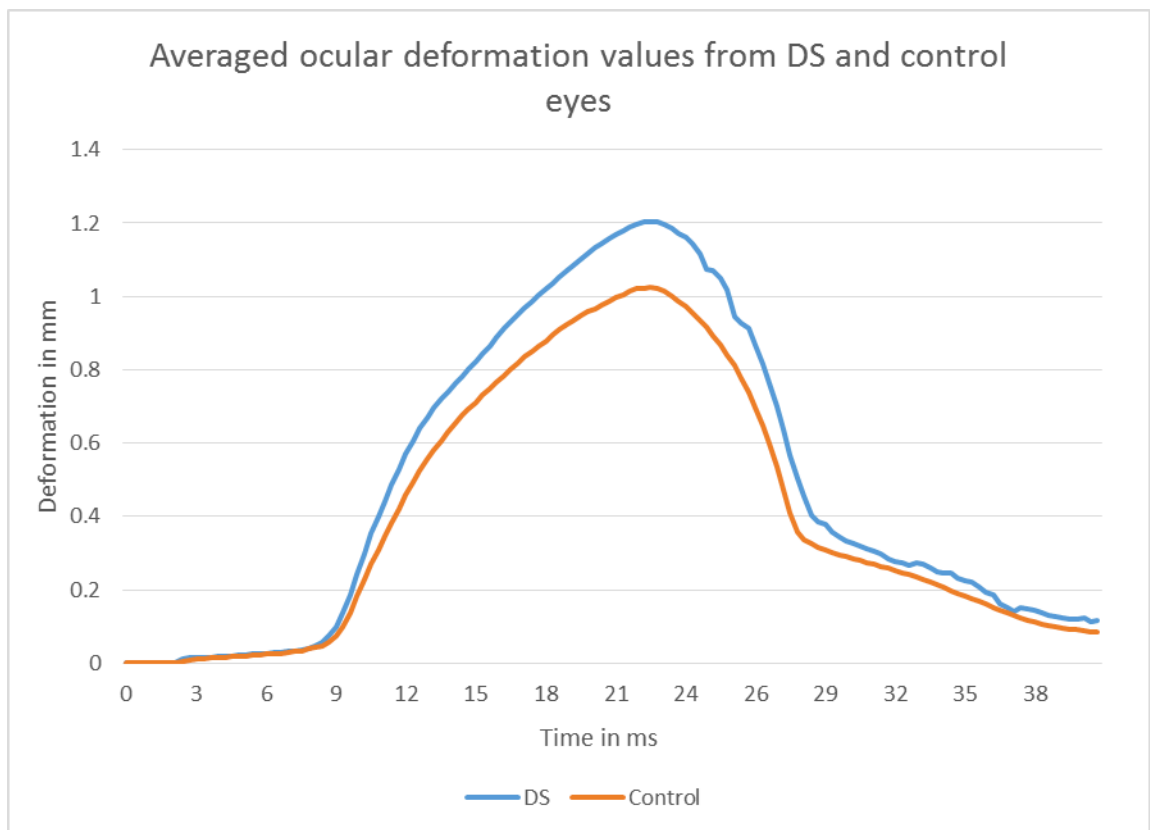


Figure 4. 3 Averaged ocular deformation values for DS eyes (blue) and control eyes (orange) with respect to time. The corneal spatial location sampled over time for each subject was that corresponding to the peak amplitude. The Y-axis denotes deformation in mm, the x-axis denotes time in ms.

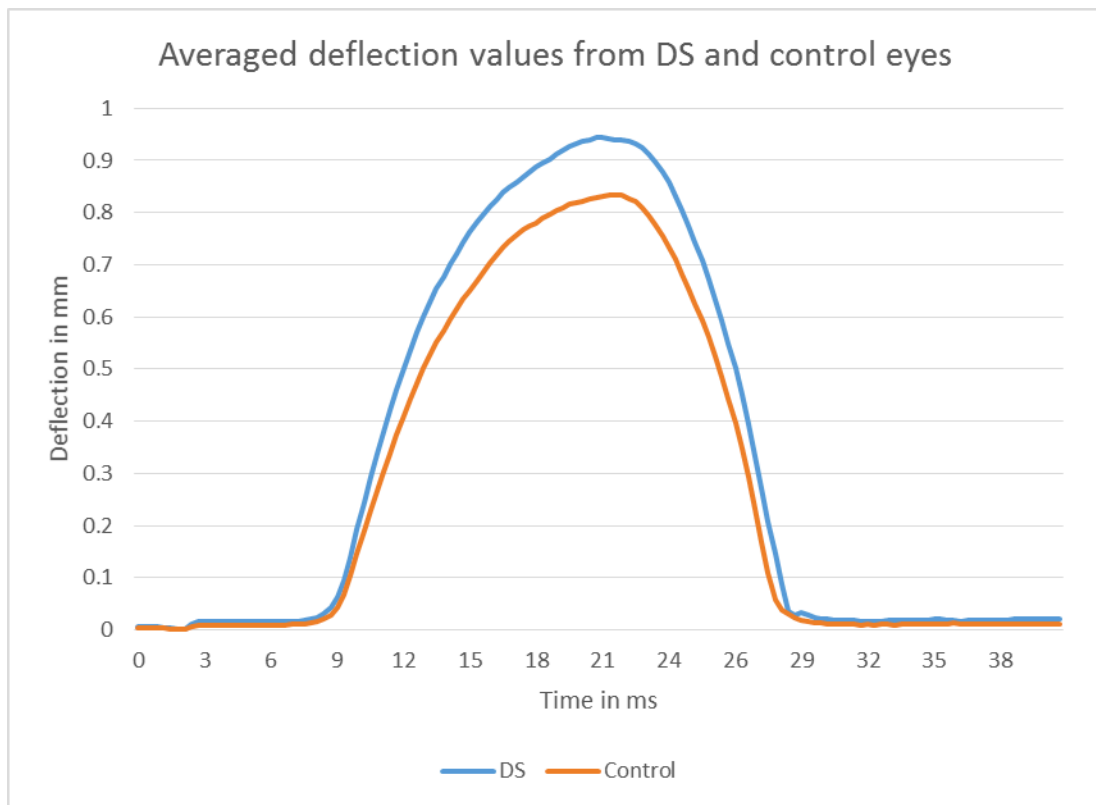


Figure 4. 4 Averaged corneal deflection values for DS eyes (blue) and control eyes (orange) with respect to time. The corneal spatial location sampled over time for each subject was that corresponding to the peak amplitude.

Removal of the ‘globe displacement component’ allows for analysis of the cornea specifically (figure 4.4). The analysis of the deflection curves indicated up to 0.12mm difference between DS and control groups – equivalent to an increase of 17.0% in DS corneal deflection from control eyes ($p=0.02$). It is apparent from table 4.2 that corneal deflection accounts for a significant proportion of the total deformation response, but that a significant proportion of the deformation is due to the displacement of the globe itself.

In order to examine the impact of confounding variables upon the difference in deformation between the DS and non-DS eyes, two statistical methods were employed, both multiple linear regression analysis and analysis of variance. ANOVA testing confirmed a significant difference between the DS and the non-DS groups (in the absence of confounding variables. Principal component analysis was used when testing the ‘homogeneity of regression’ assumption in order to mitigate for the small sample size in testing. The resulting significance of this test was 0.683, and at this level, failed to reject the null hypothesis. Therefore, the assumption of homogeneity of data holds and it is acceptable to progress with

the analysis of variance. ANCOVA was used to re-estimate the means in order to account for the effect of the covariates. When CCT was corrected for, the difference in deformation amplitude was no longer significantly different between the two groups, meaning that CCT accounts for a significant proportion of the variance between the two means (17.9%) leaving the DS/non-DS grouping now accounting for just 6.7% of the variance.

Multiple linear regression analysis was then used to establish if the DS/non-DS grouping could predict deformation amplitude above and beyond CCT. Since this produced a non-significant result ($p=0.08$), it could not.

4.3.2 Deflection area

Table 4. 8 Deflection area in DS and control eyes

| | <i>DS</i> | <i>Control</i> | <i>Significance (p value)</i> |
|---|-----------|----------------|-------------------------------|
| Maximum deflection area/mm ² | 3.02±1.20 | 2.94±1.15 | 0.63 |

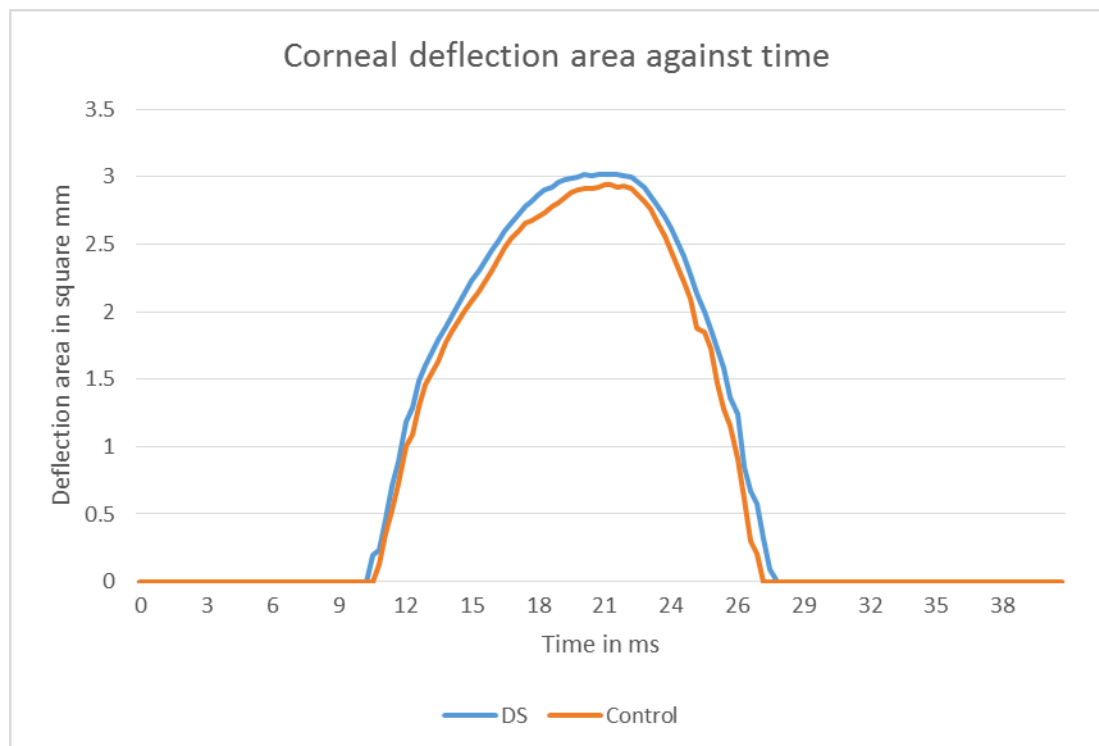


Figure 4. 5 Graphical representation of corneal deflection of DS and control eyes with respect to time.

Corneal deflection areas are comparable between DS and control groups ($p=0.63$) figure 4.5, indicating that any change in deformation or deflection is because of alterations in the depth of applanation (the z-axis) and not in the spatial distribution of the cornea (x, y-axis).

4.3.3 Timing of applanations

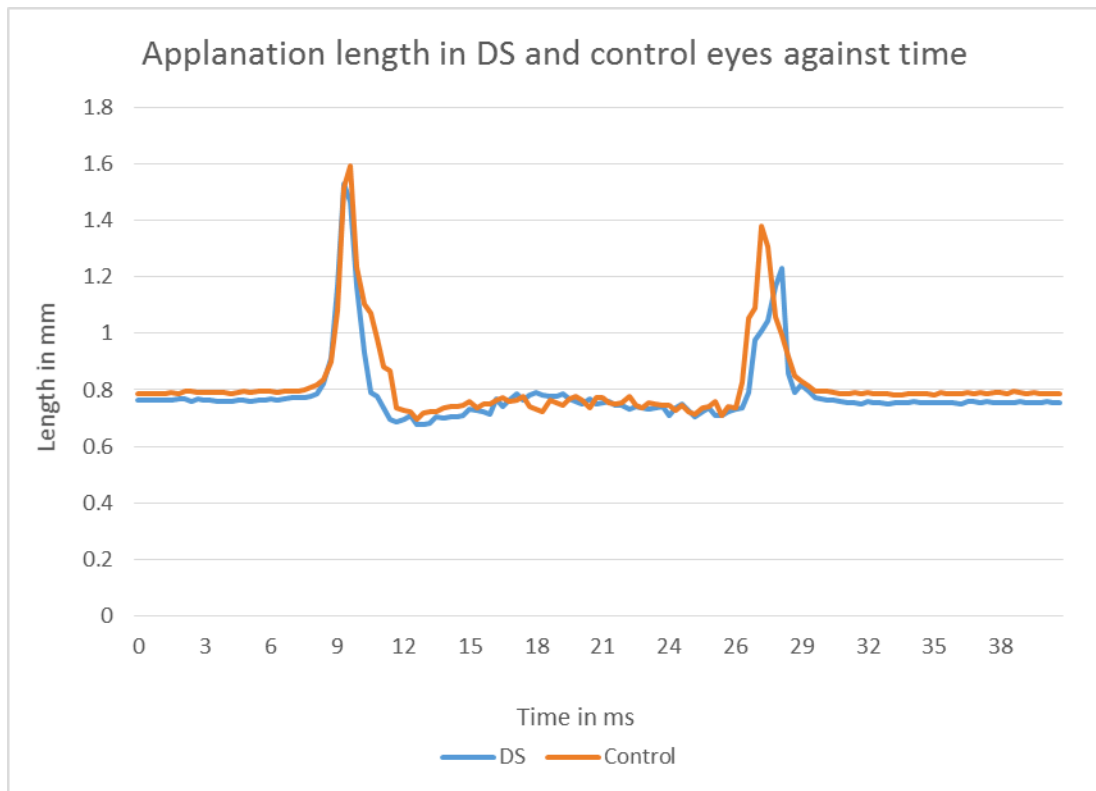


Figure 4. 6 Applanation length in DS and control eyes with respect to time. No significant differences in timings or amplitude of the peaks occur

The peaks in figure 4.6 depicts the time (in ms) in which the averages of DS and control corneae make their applanation. As individual groups, both DS and control eyes appear to follow a similar course until the second applanation (second applanation time 2AT), which appears slightly earlier in control eyes (not significant, $p=0.21$), and with greater applanation length (non-significant, $p=0.26$) (second applanation length 2AL). Data from this limited sample show no significant differences between DS eyes in the timings of either applanation time or amplitude. When the timings of each of the peak amplitudes were calculated for each subject individually, the distance between them (i.e. the lag time between the two amplitudes) were not significant ($p=0.15$). Further, difference in applanation amplitudes were not significant ($p=0.91$).

4.3.4 Corneal deflection velocity

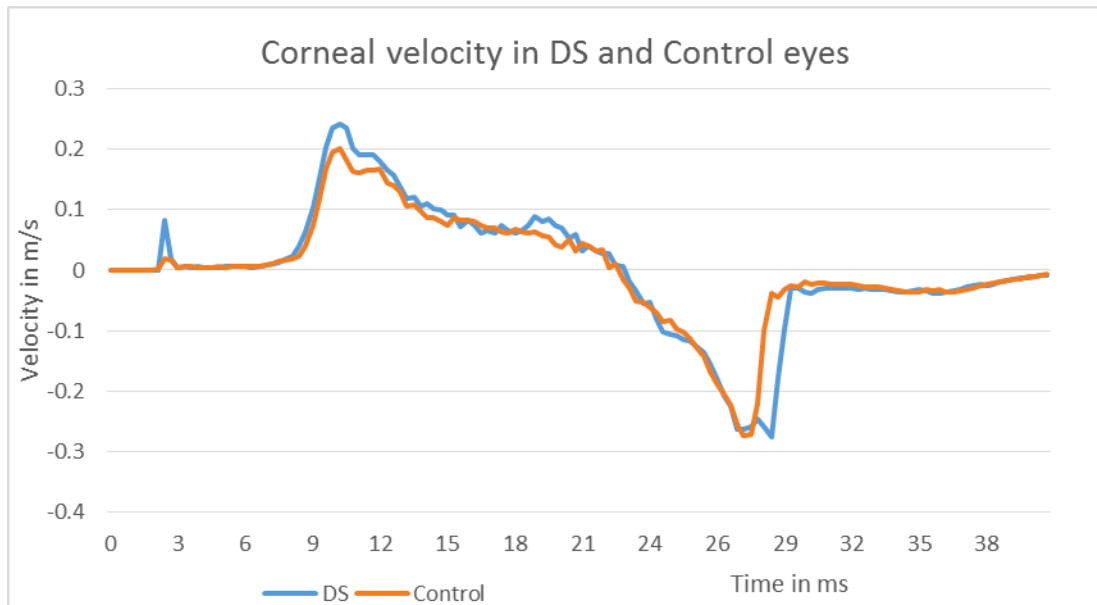


Figure 4.7 Corneal velocity of DS and control eyes. Note the comparable waveforms. The positive deflection at 3ms appears to be an anomalous noise peak of the extracted raw data.

The major positive peak in graph in figure 4.7 above denotes the peak velocity achieved by the deforming cornea following its inward appplanation, and the negative peak the peak velocity around the time of the recovering appplanation. The data have been interrogated between 6ms and 32ms to evaluate the significance of the graphical observations. From table 4.4, the difference between the DS and control velocities at the inward appplanation approach significance, with the DS cornea carrying greater velocity. The same is mirrored in the recovering velocities, and this is statistically significant.

Table 4.9 Maximum corneal velocity in the positive and negative directions for DS and control eyes.

| | DS | Control | Significance (p value) |
|---|--------------|--------------|------------------------|
| Maximum corneal velocity around inward applanation (m/s) | 0.251±0.048 | 0.216±0.037 | 0.05 |
| Maximum corneal velocity around outward applanation (m/s) | -0.465±0.138 | -0.330±0.062 | 0.01 |

The front-end software was used to compare the corneal velocity at the point of inward applanation (V_{IN}). V_{IN} in the DS group was 0.157 ± 0.016 m/s whilst V_{IN} in controls was 0.142 ± 0.021 m/s. The significance of the higher inward applanation velocity between the groups was significant ($p=0.049$, rounded to 0.05 in table 4.4) but fell away when CCT was factored in as a confounding variable in analysis of covariance ($p=0.18$).

4.3.5 Globe displacement mechanics

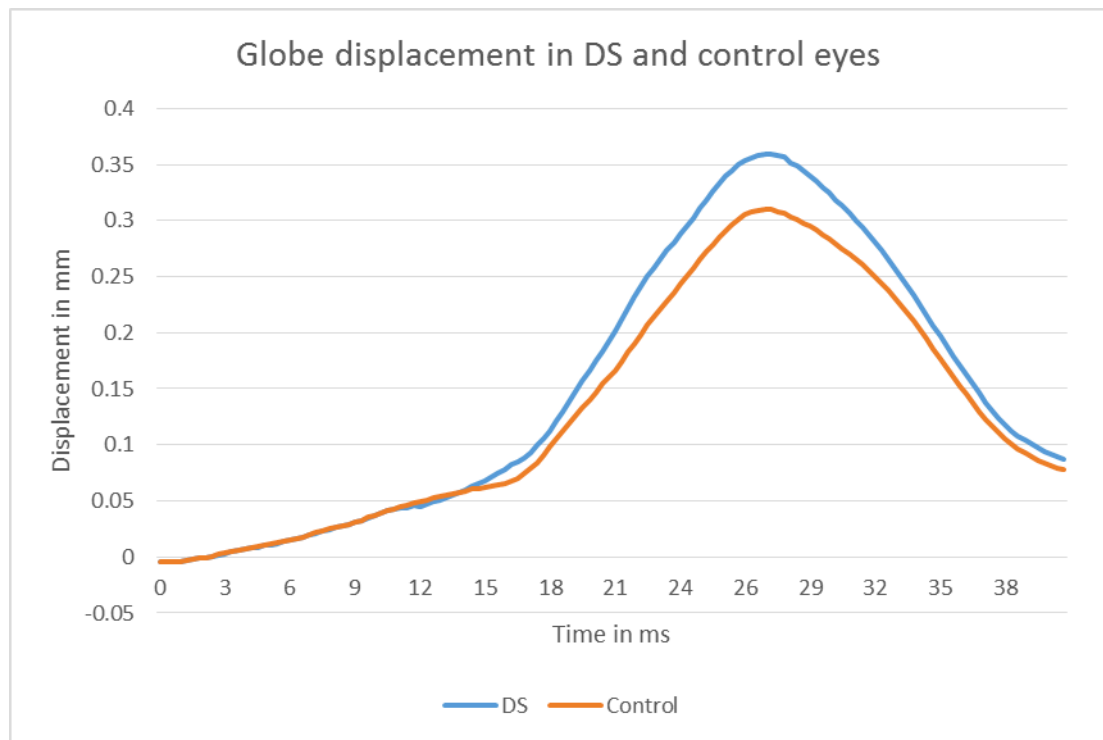


Figure 4. 8 Globe displacement in DS and control eyes. Note the larger displacement seen in the DS eyes

Table 4. 10 Average globe displacement in DS and control eyes.

| | DS | Control | Significance (p value) |
|-------------------------|-------------|-------------|------------------------|
| Average displacement/mm | 0.363±0.087 | 0.312±0.047 | 0.08 |

The difference between the means of the maximum displacements in the DS and control globes were compared (figure 4.8, table 4.5). DS globes were displaced 16% more than controls, although this result did not approach statistical significance (p=0.08).

4.4 Discussion

The CorVis analyses corneal deformation parameters under the assumption that these deformation characteristics are closely related to biomechanical properties. While this scientific relationship has not yet been explicitly clinically established, there is an abundance of *ex vivo* evidence to suggest that with a standardised force, the keratoconic cornea is less able to resist deformation and to withstand an applied force; by clinical definition the keratoconic cornea has lost its regular shape under the normal ocular forces. Therefore, the results from the current study measure deformation parameters that are an effect of corneal biomechanics, as opposed to a direct measure of biomechanics such as modulus of elasticity.

Since the cornea in DS is inherently thinner and steeper than controls, it is possible that the altered corneal structure presents weaknesses that predispose the eye to developing the high levels of KC that appear to be present in DS.

The current pilot study is the first to investigate biomechanical characteristics in DS eyes. Deformation amplitude (DA) measured with the high-speed Scheimpflug imaging was significantly greater in DS than non-DS eyes, indicating that, on average, the standardised air-pulse deforms the DS eye more easily. Considered alone, having DS accounts for 28.5% of the variability of the deformation amplitude. The effect size was significant at 1.30 [standard deviations], equivalent to 0.143mm, indicating that 90.0% of the control group would lie below the mean of the DS group. However, the statistically significant difference in DA between the DS and control group fell away when a known confounding variable, CCT, was accounted for. Having DS now accounts for only 6.7% of the variance in DA between the two groups and is non-significant.

Because the applanation area of the cornea is very comparable for both DS and non-DS eyes and the output pressure of the CorVis is standardised, the applanation force is comparable in the two groups. The load (stress) upon each cornea can thus be

related to the strain of the cornea when interpreting the deformation response (Ye et al. 2015). In keratoconic eyes, Ye and colleagues suggest that lamellar loss and collagen fibril distortion result in increased lamellar sliding; an inherent biomechanical weakness. Their results show that a statistically significant increase in DA (of keratoconic eyes) remained even after adjusting for IOP, CCT and age. In the current study, it is not possible to draw the same conclusion and deduce that a healthy DS eye has an inherent biomechanical weakness since CCT accounted for 18% of the variance between the two groups and the significant difference in DA was lost. It is entirely possible that the difference in corneal thickness alone is enough to account for a large proportion of the increased deformation in the DS cornea. In a group of healthy non-DS subjects, Asaoka et al. (2015) found that DA was affected more by IOP than by CCT, however this was an exploratory study in which confounding variables were not investigated, and a recommendation was that future studies control for IOP in the first instance.

Figure 4.6 depicts applanation length against time. This is akin to the waveform produced by the ORA (figure 1.14), albeit characterised by a different mechanism. Since no significant differences in the amplitudes of the peak applanation lengths or timings of the corresponding applanations were identified between the groups, this indicates that the groups were well matched for IOP (calibrated to correspond directly to the timing of the first applanation), and that IOP has a minimal contribution to deformation amplitude in this study. However, data from all tonometry and pachymetric studies highlight that, with current methods, IOP and CCT are inextricably (but not predictably) linked; and until the relationship between corneal biomechanics is derived independently of CCT and IOP, they will continue to be so. Since a lower CCT gives rise to a lower IOP reading, an unavoidable limitation of the current study (in initially matching controls for IOP) is the potential exclusion of normative subjects with a higher IOP (or higher CCT), who may have had a reduced DA and thus this study may have overestimated DA in the control group.

Although few papers separate the corneal deflection from the whole eye deformation, the ability to do so ensures that assumptions are not unduly made. In

the current study, the proportion of deflection due to the cornea alone could be elicited. As per DA, the corneal deflection was significantly different between the two eyes, confirming the majority of the deformation may correctly be attributed to the cornea. The data in figure 4.3 highlights the relative symmetry between the initial deformation movement and the recovery to the physiological state. Both groups of corneae recover more quickly than they deform, and other than a greater degree of deflection, there are no unexpected findings.

Inward corneal velocity is increased in KC corneae typically by 0.01m/s to 0.02m/s (Tian et al. 2014; Ali et al. 2014; Ye et al. 2015). In the current study, the corneal velocity in healthy DS eyes was 0.014m/s significantly faster than the controls, indicating that the DS cornea is moving at a faster rate. This is consistent with the ectatic velocities in the aforementioned studies that did not control for CCT when measuring velocity. However, the current study found that like DA, the significant difference between the two groups fell away when CCT was accounted for. It is possible that the increase in corneal velocity as it passes through the first appplanation is, at least in part, a feature of corneal thickness.

It is known that the biomechanics of the cornea behave differently when attached to the rest of the globe. In a whole-eye situation, the limbus and the peripheral strain appear to take up a significant proportion of the strain (Boyce et al. 2008), likely as a result of the circumcorneal annulus of collagen fibrils in this area (Meek and Newton 1999). The peripheral cornea and limbus plays a key role in the absorption of applied force and should therefore be considered in an in-vivo setting where possible. The separation of the corneal deflection from the whole-eye deformation leaves data regarding the displacement of the globe itself. The extent to which the globe displaces is likely related to the ability of the cornea to absorb the force applied, the extent to which it is transmitted through to the posterior chamber, and the extent to which the movement of the globe is resisted by the orbital fat and muscles. Whilst the current study found a larger displacement in the DS globe, this did not reach statistical significance.

Ultimately, the aim of this study was to investigate the deformation parameters in the DS group and establish to what extent these might impact upon the corneal biomechanics. It is not yet clear to what degree deformation represents elasticity or hysteresis of the cornea, or indeed the mechanism by which keratoconic corneae appear to deform more when imaged on the CorVis. Like the KC corneae from prior studies, the DS cornea deforms more, and it may be less biomechanically robust. Given the high prevalence of KC in DS, data from the current study suggest that the DS cornea may be biomechanically weak due to reduced corneal thickness alone.

The progression of keratoconus certainly leads to a reduction in corneal thickness and an increase in steepness, therefore studies using subjects with established keratoconus certainly do, on average, exhibit a reduced corneal thickness compared with controls. The current study raises the question of whether a naturally thinner cornea in the first instance (both in DS in the general population) predisposes to KC. It was beyond the scope of the current study to track the progression of the disease in DS subjects, but the nature of the cohort meant that there were two patients who now have keratoconus that happened to have undergone Scheimpflug imaging approximately 10 years before the current study began. It was notable that the patients who subsequently developed KC were not necessarily those who originally had the thinnest corneae – the averaged original corneal thickness of the 4 eyes was $470\mu\text{m}$, comparable to the averaged healthy corneae in the current study - $469\pm 36\mu\text{m}$. This reflects the clinical picture seen in ophthalmology clinics, whereby often patients with a surprisingly thick mid-peripheral cornea are developing paracentral ectasia. If subsequent longitudinal DS research fails to demonstrate a causative link between original corneal thickness and the likelihood of developing keratoconus, it is highly likely that there are additional biomechanical issues involved in the development of ectasia, such as those indicated by Ye (2015).

Although it is now well established that DS corneae are thinner than controls, and the current study shows this significantly impacts upon deformability, the mechanism for this has not yet been investigated. Fundamentally, it is not yet clear if the thinner DS cornea has fewer collagen fibrils, or if they are simply more closely packed. Like in DS skin (Brand-Saberi et al. 1994), it is possible that an altered

collagen and GAG presence exists, or that the natural cross-linking is altered in some way.

While it is not yet possible to elicit corneal rigidity, modulus of elasticity or corneal hysteresis from CorVis measurements, it is clear that the reduced CCT impacts significantly upon the ability of the DS cornea to resist applied force. Should a calibration for established biomechanical measures such as elasticity become available, the results of the current study should be reanalysed.

A limitation of the current study is the small sample size it contains. Despite this, the post hoc power of this study (for deformation amplitude) was 89.4%. In order to ensure reproducibility of these results and to draw further inferences on the proportions of confounding variables giving rise to corneal deformation, it will be important to repeat this exploratory study on a much larger sample size, perhaps allowing for analysis of sub-groups, and possibly the matching for (rather than the statistically controlling for) CCT.

Chapter 5

Ultrastructural study of the Tc1 mouse model of Down's Syndrome

5.1 Introduction

5.1.1 *Mouse models*

Mice, *Mus Musculus*, are the most widely studied organism in the study of human biology (Gharib and Robinson-Rechavi 2011). The genetic, biologic and behavioural systems of mice resemble humans to some degree, because of their divergence from a common mammalian ancestry around 75 million years ago, representing a relatively late-branching lineage. Specifically, 80% of amino acids synthesised by mouse genes have strict 1:1 orthologues in the human genome (Chinwalla et al. 2002). Yet, striking differences in the fundamental biology exist. The primary known limitation of the use of murine cornea is the difference in the appearance of proteoglycans seen in the corneal stroma. Typical, fine filaments associated with collagen fibrils are present. However, long complexes are seen to traverse the fibrillar network, and these are thought to be unique to mouse cornea (Young et al. 2005). These interweaving structures, thought to be CD/GS GAG chains, predominate in the mouse cornea, in contrast to the human cornea in which KS is most abundant. Despite this, mice are still used in research because they are small, low-cost, and breed quickly with a short generation time (10-12 weeks) - the phenotype of offspring are therefore easily studied (Guénet and Bonhomme 2003).

5.1.2 *Mouse models of DS*

Targeted gene mutation in mouse can take 3 forms (Crawley 2007).

1. Additional copies of normal mouse genes to study overexpression
2. Inserted copy of a diseased gene from human (humanised mouse)
3. Knockout model where the specific genes in mice are identified and inactivated

In the early modelling of Down's syndrome, it was identified that a large proportion of genes produced by human chromosome 21 were produced by mouse chromosome 16, with the remainder across 10 and 17 (see figure 5.1). A trisomy 16 mouse therefore demonstrated many characteristics of Down's syndrome, morphologically and biochemically, and was used throughout the 1980's and early 1990's (Kola and Hertzog 1998). Unfortunately, this trisomy 16 mouse rarely survived to term, and thus the mouse had huge limitations as a therapeutic model – particularly as several key health challenges are not present until adulthood.

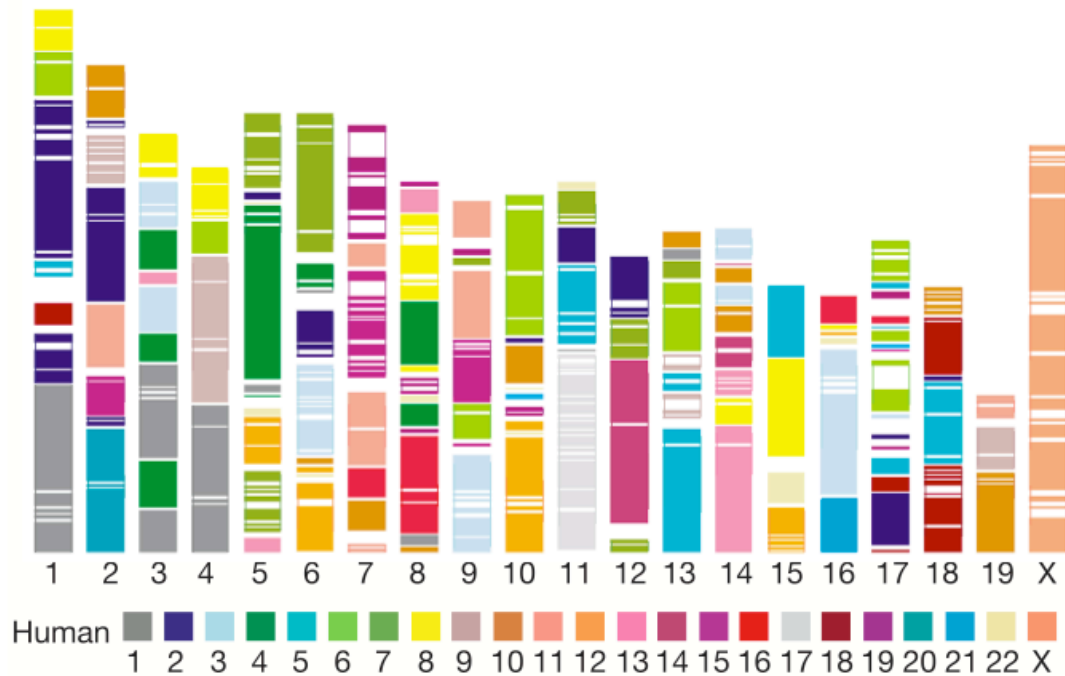


Figure 5. 1 Segments and blocks of human genetic material (>300kb in size with conserved synteny) superimposed on the mouse genome. Each colour corresponds to the human chromosome as shown Chinwalla et al. (2002)

Ts65Dn mouse

The Ts65Dn mouse was released in 1993 as a segmental, or partial trisomy 16 mouse, that is significant genetic material from the distal aspect of mouse chromosome 16 (Holtzman et al. 1996), containing up to 50% of human chromosome 21 genes (Reeves 2006). This mouse has the advantage of surviving into adulthood and thus was a more feasible model. While initially appearing phenotypically normal, the Ts65Dn mouse exhibited some important features of DS in humans:

- I. Impaired vision (Scott-McKean et al. 2010)
- II. Craniofacial abnormality (Richtsmeier et al. 2000)
- III. Impaired hearing (Kuhn et al. 2012)
- IV. Impaired muscle strength (Cowley 2011)

- V. Spatial learning and memory impairment (Escorihuela et al. 1995; Reeves et al. 1995)
- VI. Small cerebellum volume (Delabar et al. 2006) with septohippocampal cholinergic neural degeneration and astrocytic hypertrophy that are common findings in Alzheimer's disease (Holtzman et al. 1996)

Ts1Cje mouse

The Ts1Cje mouse represents a translocational mutation akin to that of Robertson's translocation, resulting in a partial trisomy 16 mouse model (Sago et al. 1998). The mutation is considered to cause triplication of 21q22 – what previously was considered the DS region. Correlating and contrasting the phenotypes and the triplicated genes that are present in Ts65Dn and absent in Ts1Cje helps to narrow the chromosomal region for specific features of DS such as craniofacial defects, auditory impairment and forebrain degeneration (Sago et al. 1998; Delabar et al. 2006).

Tc1 mouse

The Tc1 mice typically express triplication of 92% of the genes occurring on chromosome 21 with analogues for human genes across mouse chromosome 10, 16 and 17. Two small gaps are present in the genetic material, although this is the smallest gap of any known DS mouse model (O'Doherty et al. 2005). Tc1 is therefore currently the most complete model for DS in terms of genetic representation, originally having been engineered for the study of Alzheimer's disease.

Tc1 mouse shows deficits in short-term and long-term memory tasks (Dunlevy et al. 2010), hearing defects (Kuhn et al. 2012), and a reduction in tumour angiogenesis (Reynolds et al. 2010). It is the only mouse model to show abnormal cardiac development (O'Doherty et al. 2005), a crucial feature required for the study of life threatening defects in DS. That the Tc1 mouse demonstrates a congenital heart defect where the Ts65Dn mouse does not, indicates that the genes contributing to this defect likely lie in the region not triplicated in Ts65Dn.

5.2 Methods

5.2.1 *Transmission electron microscopy (TEM)*

Transmission electron microscopy is used to provide much greater magnification and resolution beyond that of light microscopy utilising the small de Broglie wavelength of the electrons – permitting the viewing of details within the collagen lamellae and therefore imaging at the molecular level. A stream of electrons is generated from an emission source, attracted down through a vacuum whilst focused using electromagnetic lenses and electrostatic plates. Apertures are used to narrow refine the beam and prevent loss of image contrast as a whole. An ultra-thin sample is positioned in the path of the electron stream and since electrons are displaced by the molecules that they interact with in the sample, an image is formed beneath owing to the interaction with the sample as the stream of electrons pass through. In the study of collagen, the variation in the greyscale image that allows viewing of the sample is predominantly caused by density and sample thickness differences. This effect is enhanced by using heavy metal elements throughout the preparation process that ‘stain’ aspects of the tissue by increasing electron scatter. Because the human eye is insensitive to electrons, the electron intensity distribution is imaged on a fluorescent screen from which digital images can be obtained.

Thirty-six eyes were obtained from 18 mature 12 – 14 month old Tc1 mice (n=9) and relevant wild type littermates (n=9) being held and sacrificed in Cardiff University for neurological and psychological studies of the Tc1 Alzheimer’s phenotype, for another research project. Eyes were obtained after live perfusion fixing of study mice with 4.1% paraformaldehyde. Live perfusion carries particular advantages over post-fixation:

- (i) Fixation begins immediately after the arrest of the systemic circulation, and there is no time delay and minimal artefacts due to post mortem enzymatic cell changes and autolytic effects.
- (ii) Rapid and uniform penetration of fixative throughout the tissue is gained, utilising the vascular system rather than solely from the sides as with immersion fixation.
- (iii) Tissue is fixed and hardened prior to direct handling and thus the reduction of traumatic artefacts.

The cornea, of course, does not have the standard vascular supply of other tissues and organs and therefore may not benefit directly from the advantages above. It is likely that the fixative indirectly meets the cornea through release into the anterior chamber and into the peripheral cornea through the limbus, albeit more limited in effect than that seen in the brain and connective tissue surrounding the eyes.

Eyes were removed using curved forceps and post-fixed in vials of 0.5% paraformaldehyde (PFA) in Sorensen's buffer within 60 seconds of death. Corneae were dissected out along the limbal region and cut into four quarters. Two quarters were reserved for the study of collagen fibril architecture and two for the study of proteoglycans, and will be discussed respectively.

5.2.2 Preparation for TEM of collagen fibril architecture

Fixation

Samples were fixed overnight in 2.5% glutaraldehyde in 0.1M Sorensen's buffer pH 7.0-7.4 at room temperature in order to cross-link proteins to prevent deterioration of the specimen after death. Prior to preparation for embedding in resin, the fixative was rinsed off with 2 washes of 10 minutes each, in buffer.

Samples were then placed in 1% Osmium tetroxide in 0.1M sodium cacodylate buffer for 1 hour. Osmium is an additive fixative that also has a strong affinity for unsaturated cellular proteins and lipids, rendering it a broad-spectrum electron dense stain. The use of osmium as a fixative and a stain prevents coagulation during dehydration with alcohol.

Samples were then rinsed in distilled water and placed in 0.5% aqueous uranyl acetate (UA) for 1 hour. Uranyl acetate is again both a stain and a fixative, and is the highest density electron stain, such that even light staining results in the scattering of electrons required for EM imaging. Treatment with UA following double fixation with glutaraldehyde and osmium provides optimal staining of membranes and other structures since UA binds strongly to glycoproteins that are found in cell membranes, nuclei and proteoglycans.

Embedding

Samples were then dehydrated to remove all water, by using increasing steps of ethanol at 70%, 90%, 100% (x2), each step for 15 minutes. Two 15-minute changes of propylene oxide were then used to remove the ethanol from the samples, followed 1 hour in a 1:1 mixture of the propylene oxide and Araldite resin mixture that the sample is to be embedded into.

Samples were then placed into 6 changes of Araldite resin over 2 days, before placement in TEM embedding moulds and final curing for 24 hours in a polymerisation oven at 60 °C and subsequent storage at room temperature before sectioning.

5.2.3 *Preparation for EM of proteoglycans*

Fixation

Immediately after dissection, samples for proteoglycan study were fixed and stained overnight in 2.5% glutaraldehyde within 25mM sodium acetate buffer at a pH of 5.7, containing 0.1M of magnesium chloride and 0.05% Cupromeronic blue (CB). (The pH of the sodium acetate buffer was reduced to 5.7 with the addition 0.1M hydrochloric acid).

Cupromeronic blue (CB) is a cationic dye developed specifically for localisation and characterisation of proteoglycans and sulphated glycosaminoglycans (such as keratin sulphate, chondroitin sulphate) and hyaluronan (Scott 1992). Its cationic charge provides a very high affinity for the negatively charged GAGs found in the corneal stroma. It is used for PG staining in conjunction with Scott's "Critical Electrolyte Concentration" method (Scott, 1980). Electrolytes are supplied by the dissociation of magnesium chloride in sodium acetate buffer. The chloride ions compete with the CB and reduce its binding efficacy to the PGs. Thus, more CB molecules must bind to the PG in order to gain entropy, and the increase in staining enhances the contrast of the EM image.

Specimens were rinsed 3 times (each for 5 minutes) in the reduced pH sodium acetate buffer to remove the fixative and stain. Specimens were then washed 3 times (10 minutes each) in (aq) 0.5% sodium tungstate. Sodium tungstate has a dual purpose in EM preparation. Firstly, it is a heavy metal and binding creates electron scatter that enhances image quality. It also dissociates easily into its respective ions and so enhances contrast from the stains used, in a similar way to magnesium chloride.

Specimens were dehydrated for 15 minutes with a 1:1 mixture of 0.5% (aq) sodium tungstate and 50% ethanol, followed by increasing strengths of ethanol only (each for 15 minutes) 70%, 90%, 100% (x2).

Embedding

The removal of ethanol and subsequent embedding procedure is the same as for the '*Preparation for EM of collagen fibril architecture*' as detailed above.

5.2.4 Sectioning of samples for EM of collagen fibril architecture

Sections from the embedded samples were cut using a Reichert UCE ultramicrotome. Glass knives were used to produce 'semi-thin' sections of 2-3 μm for observation after staining with 1% Toluidine Blue in 1% sodium tetraborate using light microscopy in order to precisely locate the desired location of interest. Either glass or diamond knife was used to cut 'ultra-thin' sections of 90nm and floated onto G300 copper grids with or without plastic support films (1% pioloform in chloroform).

Grids were contrasted with saturated aqueous UA followed by lead citrate for 12 and 5 minutes respectively. Grids were then rinsed by floating on distilled water for 5 minutes each. Grids were then allowed to dry for 24 hours before being viewed using the electron microscope.

Electron Microscopy was performed with a Jeol 1010 transmission electron microscope equipped with a Gatan Orius SC1000 CCD (charge coupled device) camera.

5.2.5 Sectioning of samples for EM of collagen fibril architecture

Contrast enhancement with filtered uranyl acetate is used as a final step in order to stain the collagen background of the PGs that will be observed. As with the grids for

collagen observation, grids were then rinsed by floating on distilled water for 5 minutes each. Grids were then allowed to dry for 24 hours before being viewed on the electron microscope.

Quantification of fibril diameter

Cell Profiler (version 2.1.1, Cambridge, Massachusetts, US) was used to analyse collagen fibril diameter objectively (Lamprecht et al 2007), when measured at x25,000. Code was selected to invert the colours, smooth the edge profiles of the image, identify the fibrils and provide an analysis on various aspects of shape. The particular parameter of interest was 'minor axis length' to account for small errors in the plane of transverse imaging. The measurements were sampled in different areas across the available tissue image, and averaged to gain a representative result of collagen fibril diameter.

Dr Phil Lewis and Dr Rob Young (Cardiff University) kindly assisted in the embedding, sectioning and imaging of Tc1 and wild type samples.

Dr Neil Anthony (Emory University) kindly assisted in refining the 'Cell Profiler' code.

5.3 Results

Presented in figures 5.2a through 5.8b are a series of EM photos illustrating the collagen organisation from the mid-stroma (unless otherwise specified) of the central cornea in mutant and wild type mice.

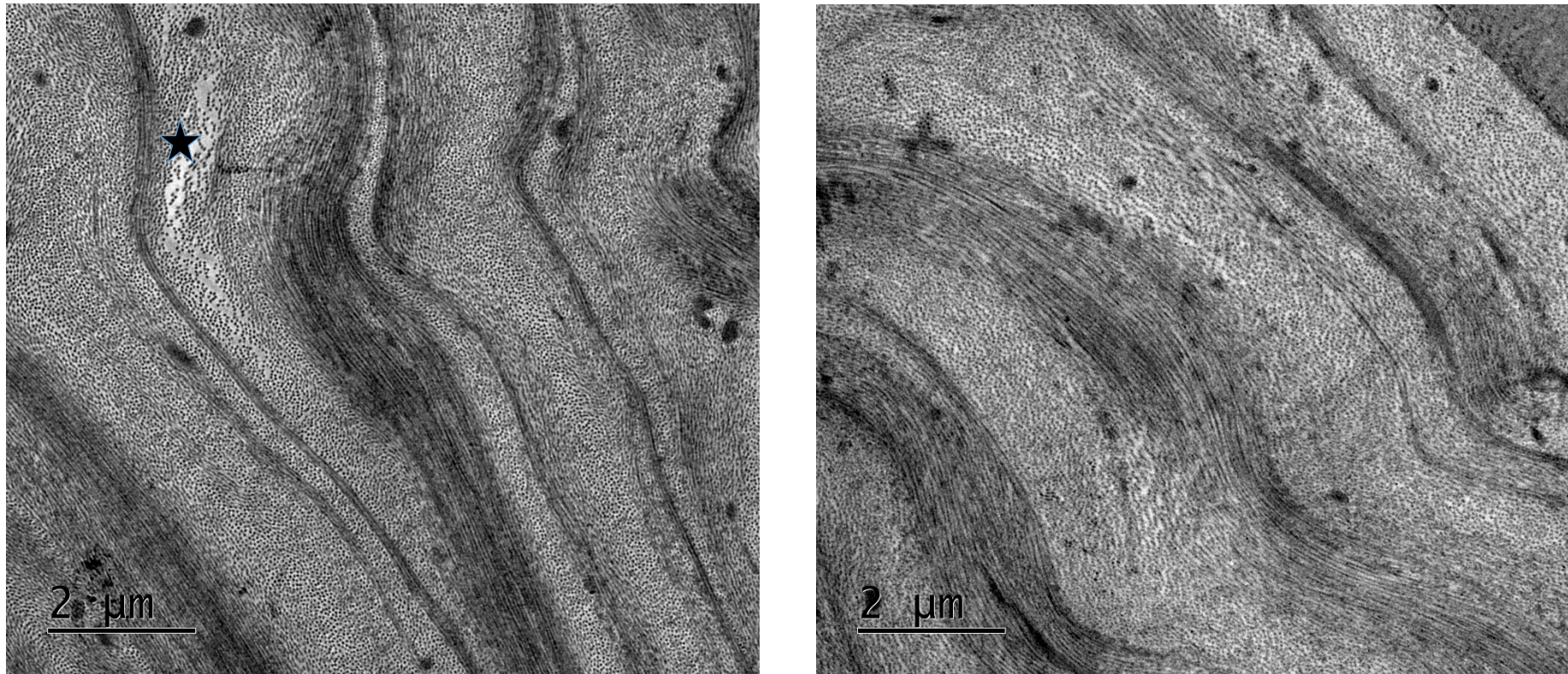


Figure 5. 2 a & b respectively. EM photographs of transverse and longitudinal collagen organisation in wild type mouse corneal stroma (left) and in Tc1 mutant mouse (right) at magnification x2000. Note the area of reduced collagen density (star). This is likely to be an artefact created during the dehydration process.

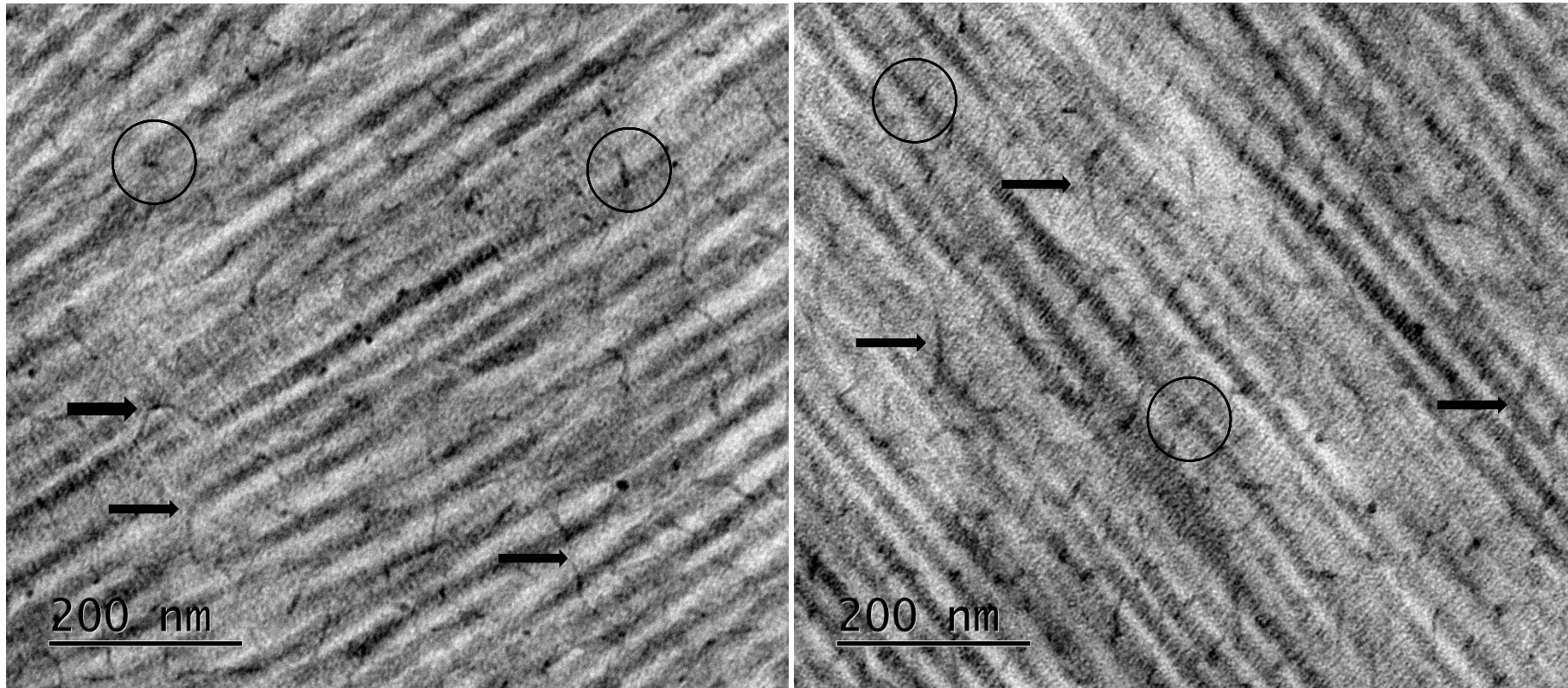


Figure 5.3 a & b, respectively. EM photographs of longitudinal collagen and associated proteoglycan organisation in wild type mouse corneal stroma (left) and in Tc1 mutant mouse (right) at magnification x25,000. Circles show the short, electron dense PG filaments associated with collagen fibrils, whilst the arrows show the large, elongated PG structures that run across fibrils, as described by Young et al. (2005). These are typical of mouse cornea, and appear to be similar between samples.

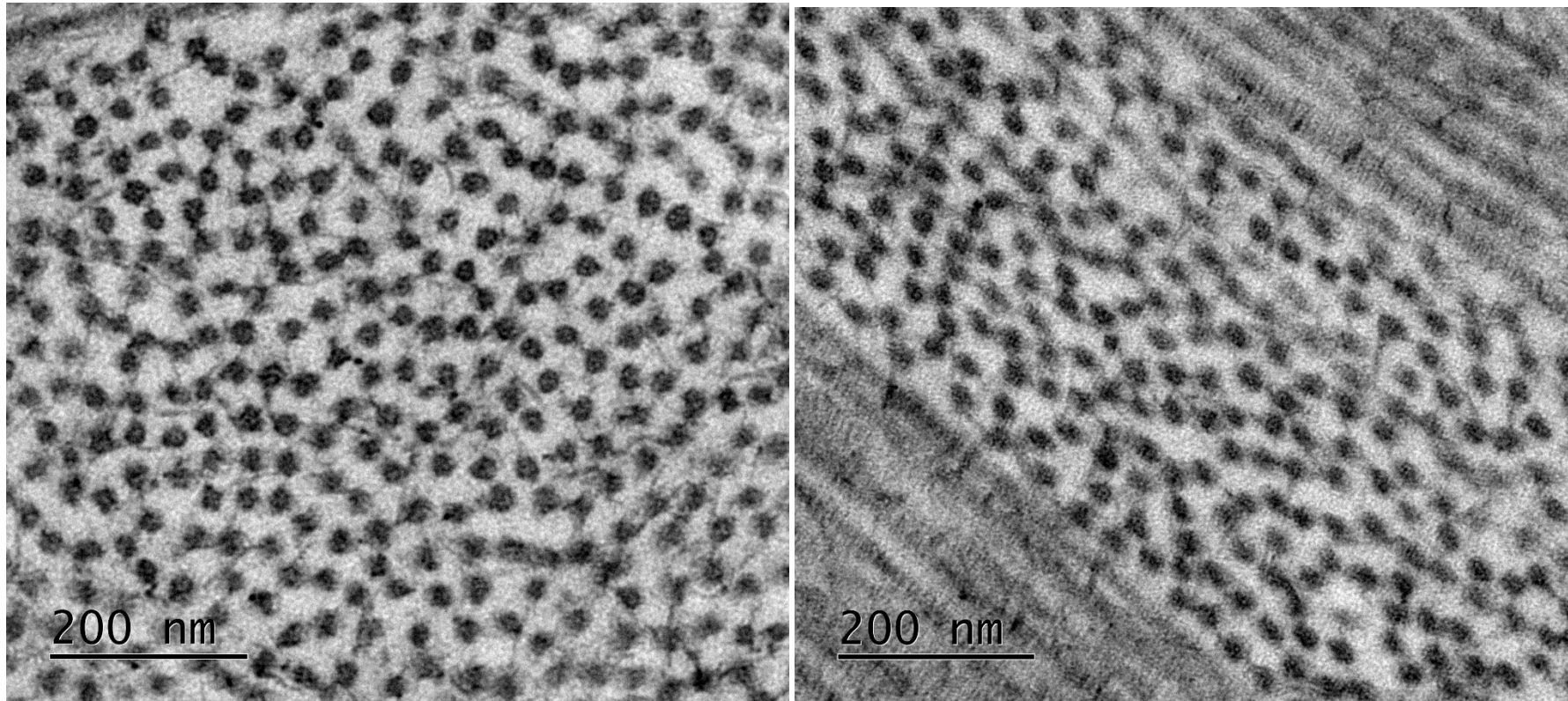


Figure 5.4 a & b, respectively. EM photographs of transverse collagen and associated proteoglycan organisation in wild type mouse corneal stroma (left) and in Tc1 mutant mouse (right) at magnification x25,000. Quantification of collagen fibril diameter in each of these samples is made later, but they appear largely similar in morphology and spacing. While 5.5a has been imaged in transverse plane, 5.5b appears slightly obliquely sectioned, with the fibrils appearing more oval in shape.

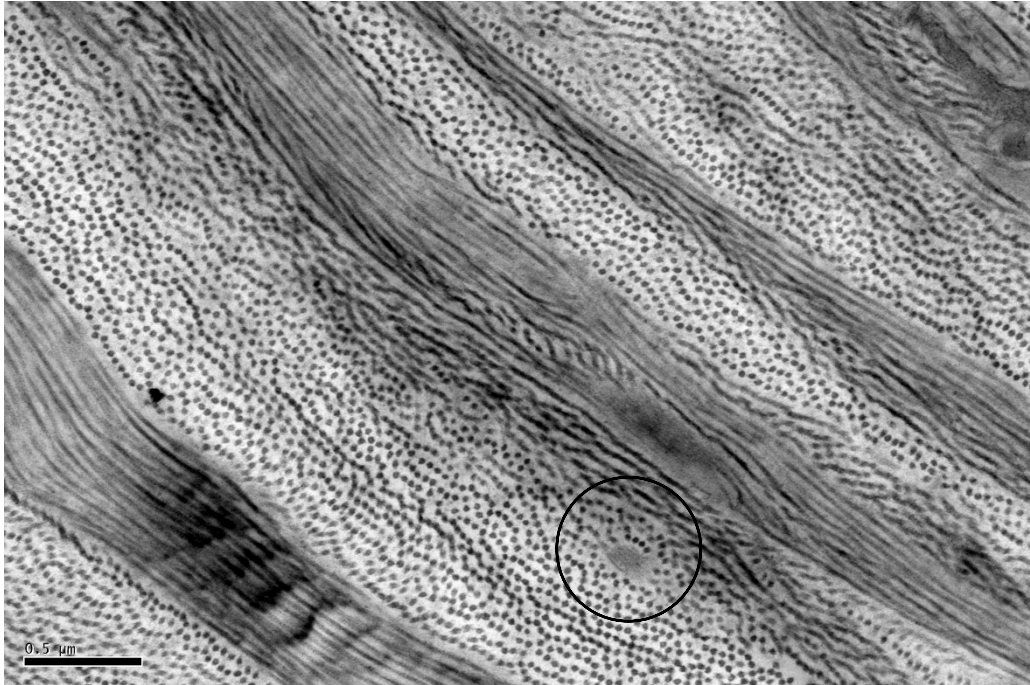


Figure 5. 5 *Tc1* mutant collagen lamellae and a microfibrillar bundle (circled) within a transverse section (x6,000).

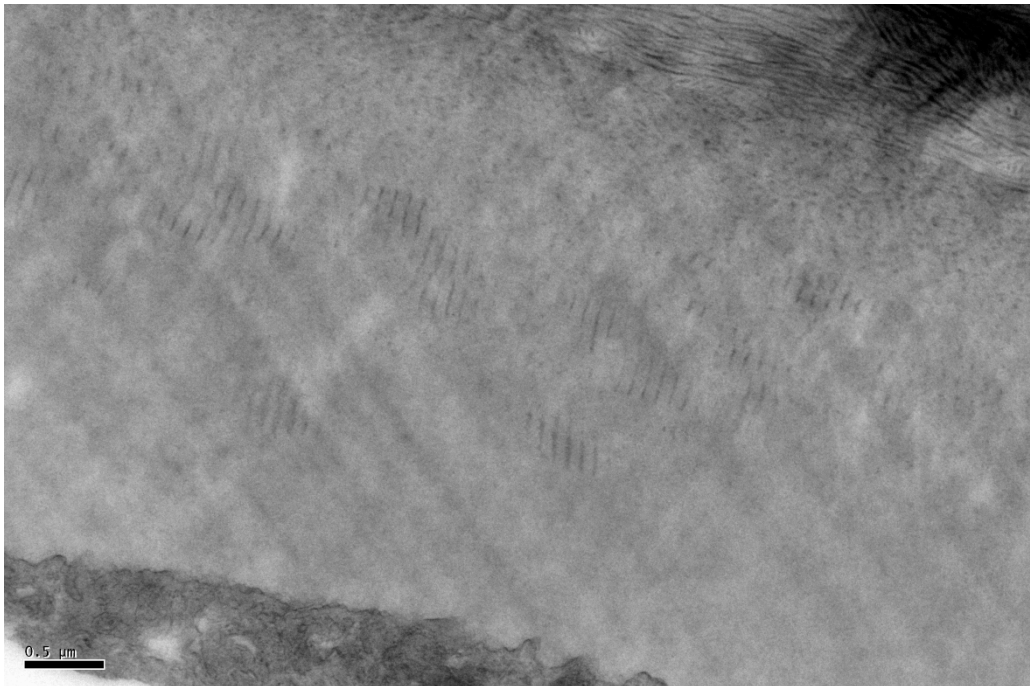


Figure 5. 6 Patches of banded collagen found in 'Descemet's membrane' of *Tc1* mutant collagen at magnification x6,000. The periodicity of this collagen was calculated to measure 83nm.

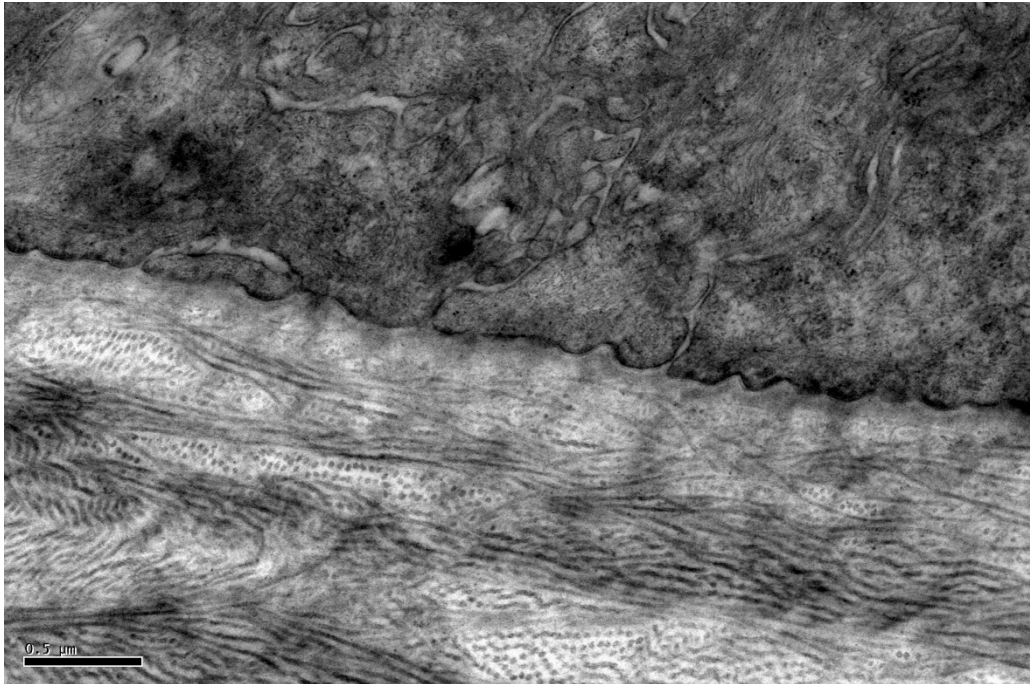


Figure 5. 7, 'Bowman's layer' (lower) junction with the posterior corneal epithelium (upper) in Tc1 mutant corneal stroma (x6,000). The boundary appears normal, with no epithelial invasion of Bowman's layer, as has been reported in some keratoconic corneae.

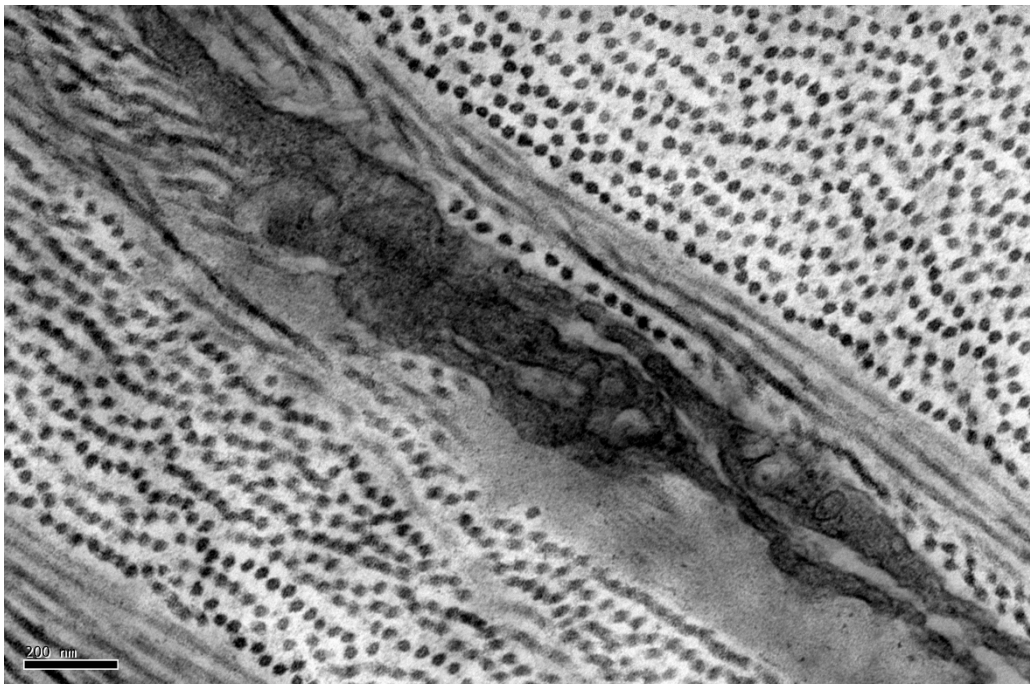


Figure 5. 8 A keratocyte imaged at x12,000 lying between collagen lamellae in Tc1 mutant corneal stroma. This keratocytes is well preserved, demonstrates a typical appearance, and has orderly collagen fibrils surrounding it, suggesting that it was functioning normally.

Measurement of fibril diameter was evaluated objectively using quantification software, that over many images, useful averages could be gained. When calibrated for pixel size in nm, this resulted in a measurement of fibril diameter in control mice of $18.5\pm 2.1\text{nm}$ and mutant mice of $19.5\pm 2.4\text{nm}$ and when these averages were compared by the student's t-test, the difference was not significant ($p=0.06$). An example of an automated edge profiling using the Cell Profile software, is given in figure 5.9.

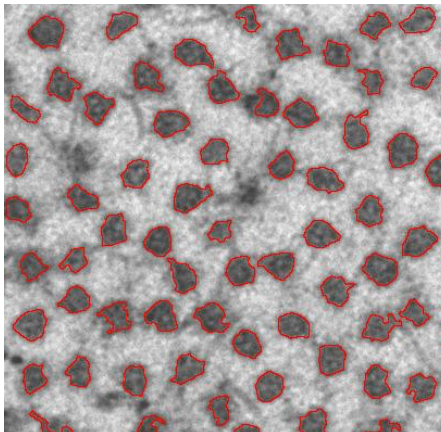


Figure 5.9 A figure extracted from Cell Profiler software detailing the automated edge detection of the fibrillar collagen from a control mouse. While the shapes do not approximate a circle, they are defined via a set threshold, minimising bias and providing a comparison between Tc1 and control tissue.

5.4 Discussion

The architecture of the corneal stroma is governed by several factors:

- I. Lamellar organisation
- II. Collagen types present
- III. In fibrillar collagens: fibril diameter, spacing, organisation
- IV. Proteoglycan type present and the sulphation of their GAGs
- V. Cellular arrangement

For the current study, each of these features will be discussed in turn.

The corneal stroma in mouse is fundamentally different from that of human, with murine stromal tissue carrying some unique features:

- Significantly thinner stromal thickness - 200 μ m rather than 550 μ m
- A predominance of CS/DS PGs, in contrast to the highly sulphated KS type that predominate in the human cornea
- PG complexes are much larger in mouse than in humans, draped along or wrapped along collagen fibrils in longitudinal section (up to 600nm)
- Collagen lamellae are thinner and appear less stratified than in humans, with a more 'wavy' appearance after EM processing.

It is therefore the comparison between the mutant mouse and the corresponding wild-type mouse that is of paramount importance rather than the absolute appearance of the mutant mouse in isolation.

5.4.1 *Lamellar organisation*

Figures 5.2a and 5.2b are low magnification (x2000) EM photographs of the mid corneal stroma. Twisting of the lamellae layers in each sample are likely to be an unavoidable product of the fixing and dehydrating process, as the parallel aspect of adjacent lamellae is maintained throughout the sample. Lamellae are seen in both transverse and longitudinal and transverse orientation, and on each sample there appears an area with a low density of fibrils (marked star). This is likely to be an artefact of the dehydration and embedding process, and does not appear large enough to be a true collagen-absent 'lake' as discussed by (Meek et al. 2003).

Both samples feature electron dense oval areas that are microfibrillar bundles and condensed lead carbonate as a contaminant product from the staining process. Upon gross examination, there does not appear to be any differences in the lamellar features between the control and the mutant mouse cornea.

5.4.2 *Collagen types present*

True identification of collagen type is provided by immunolabelling using relevant antibodies. The extensive study of corneal collagen, the availability of a wild-type control mouse and the exploratory nature of this work negates the need for this procedure in the current study, and instead fibrillar collagen typing was assessed by examination of:

- Shape and level of fibril organisation
- Fibril diameter, interfibrillar spacing and periodicity
- Interaction and proximity to neighbouring cells and lamina

Fibrillar collagen I was found in the corneal stroma of all specimens, the vast majority arranged in a lattice fashion and regularly spaced. Portions of a long-spacing structure similar to that found in other studies (Bruns 1984) was noted in the Descemet's membrane area of the posterior stroma in a particular Tc1 mouse (figure

5.6). The banding pattern had $\geq 83\text{nm}$ periodicity. Since it is obliquely orientated in the section and being viewed from a non-parallel reference plane, it was considered that this measurement likely underestimates the true periodicity. Further, the shrinkage observed in EM with respect to fresh tissue state likely further underestimates the periodicity.

Collagen VI is known to be abundant in Descemet's membrane, and was considered as a candidate for the observed wide-spaced structure in figure 5.6. Collagen VI is typically observed in a beaded appearance (figure 5.10) but has been found to aggregate into banded patterns of 100nm periodicity (Bruns et al. 1986) (see figure 5.11). This latter image of collagen VI in rat tail tendon carries a striking resemblance to that found in the current study, and has been verified by staining with collagen VI antibody, but has not yet been found in Descemet's membrane.



Figure 5. 10 Micrograph of collagen VI from synovial joint fluid with a tetramer unit length of 102nm (Kielty et al. 1993). This structure denotes the typical beaded appearance of collagen VI discussed in scientific literature.

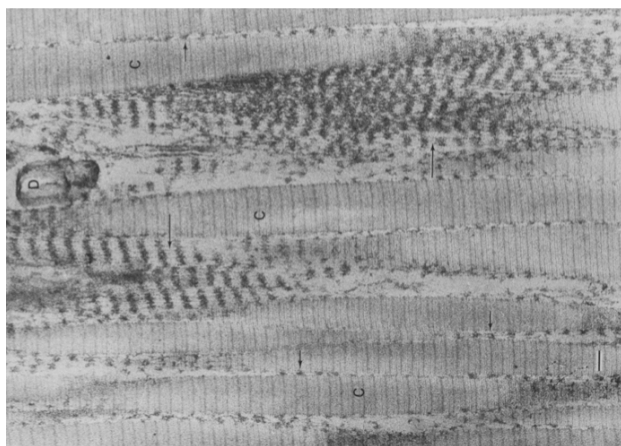


Figure 5. 11 : Longitudinal section of rat tail tendon showing 100nm periodic fibrils (long arrows) distributed among type I fibrils (Bruns et al. 1986). This is an atypical presentation of collagen VI

Collagen VIII is a likely candidate collagen for the observed wide-spaced structure, since the side view of collagen VIII modelled polygonal structure is a viable explanation for the pattern observed in the mouse tissue. When viewed en face using EM, the globular ends of collagen VIII create a hexagonal structure and banded appearance when viewed parallel to the structures side (figure 5.12).

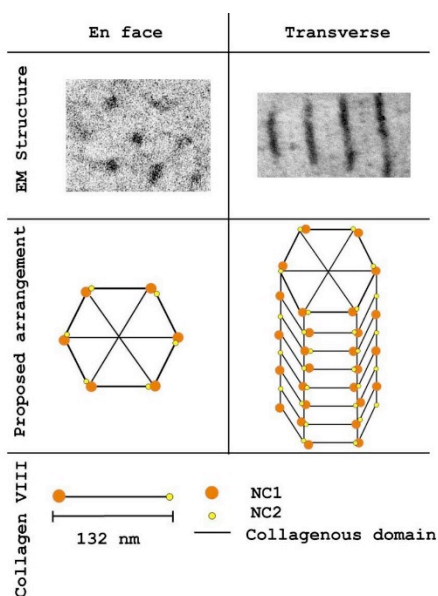


Figure 5. 12 Schematic reconstruction of the polygonal lattice arrangement observed in collagen VIII, from Jones (2013)

Akimoto et al (2008) found similar long-spaced structures in the rat cornea that increased in prevalence with maturity (fig 5.13), in line with work from (Jun et al. 2006). Akimoto et al confirmed the banded collagen in Descemet's membrane as type VIII with antibody staining and immunoelectron microscopy. On balance, it is highly likely that the Descemet's membrane collagen found in the current study is therefore also collagen VIII.

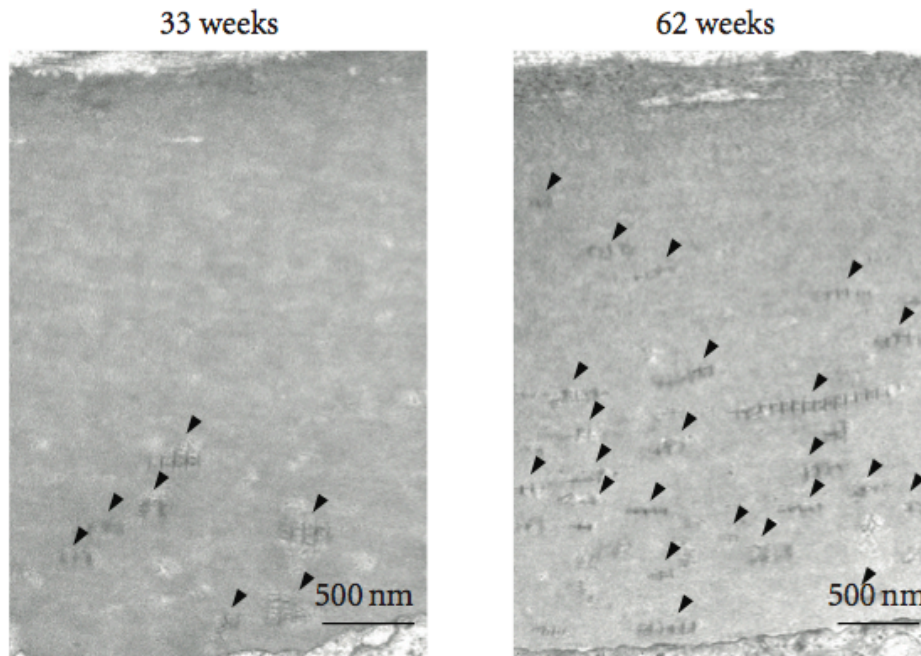


Figure 5.13 Increasing deposition of long spaced collagen (arrows) increased in prevalence with age in Descemet's membrane of rat. Adapted from Akimoto and Sawada (2008).

5.4.3 Fibril diameter, spacing and organisation.

Transverse imaging of collagen type I viewed at x25,000 (figures 5.4a and 5.4b) demonstrate comparable fibril organisation between control and wild type mice. Specifically, there appears to be no difference in either fibril diameter or interfibrillar spacing between the two groups. The diameter of the fibrils was $18.5 \pm 2.1 \text{ nm}$ in control mice $19.5 \pm 2.4 \text{ nm}$ in mutant mice, the difference was not significant ($p=0.06$). This appears to be slightly smaller than the 25-30nm typical fibril diameter expected of corneal collagen I when viewed with electron microscopy (Chakravarti et al. 2000, Hassell and Birk 2010). Unlike the skin in DS (Brand-Saberi et al. 1994), abnormal hydration was not found in Tc1 tissue. This corresponds to the clinical observation that Tc1 mouse cornea appeared clear when viewed using light microscopy and appeared to have regular curvature. Disorganised collagen type I fibrils are a hallmark of KC, of systemic collagen disorders and of many genetically engineered mice. Comparison of the above EM photographs with such affected collagen reveals no similarities and thus it is reasonable to determine that with the imaging techniques employed in the current study, collagen fibril arrangement is normal in the Tc1 mouse.

5.4.4 *Proteoglycans*

Despite the fact that proteoglycans in mouse cornea are fundamentally different from those found in human tissue, comparison with the wild type strain is still useful, and cupromoronic blue staining clearly defined electron dense filaments presumed to be PGs in both specimen groups (fig 5.3a and 5.3b). Short PG chains (circled) are observed perpendicular to collagen fibril length in both control and mutant cornea, whilst longer filaments are seen to drape over collagen fibrils (arrows). These findings are in line with PG observations discussed by (Young et al. 2005). The increased GAG staining seen in DS nuchal tissue (Brand-Saberi et al. 1994) was not mirrored in the current study.

5.4.5 *Cellular arrangement*

EM photographs of epithelium, keratocytes, and endothelium were obtained. The keratocyte in figure 5.8 is flat in appearance and seen to lie between lamellae, as expected. There exists an area of homogenous material inferior to the cell, which may represent the remodelling of collagen or fibrillogenesis – a normal, albeit limited function in adult tissue. The epithelium (figure 5.7) and endothelium (Fig 5.6) of Tc1 mice both demonstrate intact borders with their respective lamina, and do not show signs of discontinuity, or ‘breaks’ which is sometimes referred to in keratoconus, especially in Bowman’s layer (Sykakis et al. 2012).

5.4.6 *Limitations of mouse model*

Tc1 is currently the most complete model for DS in terms of genetic representation although it does have several limitations, primarily that the trisomy is lost from some cells during growth, resulting in mosaicism to some degree (Reeves 2006). Clearly, there are significant differences between mice and humans. The mouse genome is 14% smaller than that of humans and contains just 20 pairs of chromosomes. The humanised mouse requires the alteration of the mouse immune system in order to prevent rejection of the new genetic material. As a result, the T-cell system is known to respond at a lower level than what would be ordinarily expected, while other immunological aspects such as cytokines and adhesion molecules are known to be different in mouse (Shultz et al. 2007). Thus, using mouse as a model for the compromised cornea carries limitations, particularly given the new view of keratoconus as an inflammatory disease, and the complex interplay between biochemical signalling and the structure of the ECM.

There is an experimental risk that human DNA is misread when in a mouse environment (Reeves 2006). An understanding of the basis of species differences, in combination with comparative studies between wild type and mutants, allow observations that may be relevant for human tissue (Chinwalla et al. 2002). Thus, any differences seen between wild type and mutant corneae might be *indirectly*, rather than *directly* relevant in human.

5.4.7 *Summary of findings*

Results from the current study examined the suitability of the Tc1 mouse model as a model for corneal ectasia in DS. Primarily, the stromal collagen from the central cornea was examined for differences between the mutant and wild type mice. No differences were found in the samples examined, stromal collagen and PGs of Tc1 mice were within the normal limits with respect to the wild type littermate mice. The current study was limited in power by its sample size. Whilst 9 pairs of wild type

eyes and 9 pairs of control eyes were obtained for experimentation, good-quality samples were lost throughout the dissection phase, embedding phase and particularly at the sectioning phase. This resulted in 3 wild-type samples (each for collagen and PG analysis), and 4 Tc1 samples (each for collagen and PG analysis) producing results of which a sample is shown above.

Owing to stochastic loss of the human chromosome post fertilisation, mosaicism is present in the Tc1 mouse. Thus, some cells are affected by the trisomy and others are not. Resulting tissues therefore have a variable degree of mosaicism (Wiseman et al. 2010), which is not predictable. An example of this is the Tc1 cerebellum, which expresses the DS phenotype in between 7% and 77% of cells (O'Doherty et al. 2005). It is highly possible that while the heart and the cognitive system of the Tc1 mouse exhibit the DS phenotype, that the trisomic penetrance does not reach a critical phenotypic threshold to produce structural defects in mouse, the Tc1 model may simply not reflect that of human eyes. In order to quantify the proportion of trisomic tissue with the Tc1 mouse eye, quantitative polymerase chain reaction (PCR) could be used on ocular tissue. Likewise, due to the small sample size employed, it is unlikely but scientifically possible that each Tc1 sampled successfully with EM had coincidentally exhibited non-DS ocular tissue due to mosaicism.

Alternatively, it is possible that the Tc1 mouse and DS eyes in general both carry normal corneal ultrastructure, and that the increased prevalence of keratoconus does not stem from altered collagen or proteoglycan constituents.

It is possible that ultrastructural properties exist that were not picked up during the analysis in the current study. The current study aimed to evaluate the corneal ultrastructure of the Tc1 mouse for gross structural abnormalities that might give rise to KC, specifically in collagen fibril arrangement. There are possible ultrastructural differences that could be investigated that were beyond the scope of this work, but which could provide a useful insight into further aspects of the Tc1 cornea. Immunohistochemical staining using relevant antibodies (eg collagen VI, collagen

VIII) would identify differences between collagens present in the two groups. Further, Western blot analysis could be used to quantify the proportions of collagen types present.

Individuals with DS are known to have an unusual immune system, becoming prone to infections and resistant to particular cancers. As discussed in Chapter 1, the regulation of the immune system is heavily implicated in keratoconus. While atopy and eye rubbing were not associated with the development of KC, it is possible that other inflammatory processes (governing collagen turnover, activity of proteases, or the ECM environment) could give rise to collagen degeneration that is not a function of the collagenous ultrastructure. Further study surrounding the digestion of Tc1 corneae compared to the wild type counterparts could provide useful information on the relative degree of cross-linking across the two corneal types.

Chapter 6

General discussion and future work

This study was the first to prospectively explore the metrics of the cornea in Down's syndrome eyes with an established diagnosis of keratoconus and compare with those that were demonstrably healthy. The early identification of KC in a learning-disabled population is of particular importance given the necessity of a prompt referral for consideration for corneal cross-linking in order to halt disease progression.

The clinical aspects of this study confirmed that the use of retinoscopy was an effective screening tool for the identification of KC and KC suspect eyes; and that VA, contrast sensitivity and spectacle astigmatism alone provided insufficient data to monitor for the onset of KC. Optometrists in the community are therefore in an excellent position to take up regular screening of these individuals and should use retinoscopy as opposed to autorefraction. Further, slit lamp biomicroscopy was of limited use in detecting early KC (as is frequently the case in non-DS eyes) and so it is important to reiterate the importance of optometrists referring suspect KC even in the absence of slit lamp signs of the disease. This also has implications for secondary care (hospital environments) - sometimes it is very difficult to obtain an accurate topography on someone with learning disabilities and it is the author's experience that in such cases, a clinical slit lamp observation is often relied upon by the observing clinician. The results of the current study reinforce the need for topography examination 'teaching and training' for those individuals with DS who have an abnormal retinoscopy reflex, so that they can participate in a rigorous diagnosis. The current study is in line with the work of others indicating that the absence of slit lamp signs is not an absence of disease (Zadnik et al. 1996).

Topographic examination of healthy DS eyes revealed a greater magnitude of high order aberrations (HOAs) and ocular surface irregularity than expected for a healthy typical (non-DS) population. In some eyes, the 'abnormal' topography had been stable for many years. Previous literature documents that DS eyes have reduced optical quality (Little et al. 2007) and increased whole-eye HOAs than non-DS eyes. Results from the current study indicate that the cornea, at least in part, may contribute to these HOAs. It would be necessary to combine whole-eye

aberrometry with corneal tomography to investigate this further. It is known that children with DS fail to emmetropise concerning whole-eye refractive error, but the current study gives an indication that the typical DS cornea does not reach typical curvature or regularity by adolescence and therefore may contribute to the poor visual function seen in the DS eye as a whole. This ‘abnormal’ topography in demonstrably stable and healthy eyes provides some understanding as to why previous authors have reported a high incidence of early KC in DS when using topography in DS (Aslankurt et al. 2013). Vincent et al. (2005) studied non-KC eyes and suggests that children with DS may exhibit abnormalities of corneal shape in the absence of clinical keratoconus. The current study confirms this suspicion and indicates some topographical parameters that may be useful in differentiating healthy DS eyes from KC eyes. However, the current sample was small and requires validation by using a ‘test’ dataset of further healthy and KC DS eyes as part of a longitudinal study that has the capacity to monitor the ‘suspect’ eyes until they are ultimately deemed stable or ectatic.

Eye rubbing was not associated with KC in the current study. Although the study had a limited size, it was significantly larger than those in the current literature, and the only one to investigate the impact of atopy on the results, a known confounding factor. The predominance of scientific literature associating eye rubbing and DS does not remark on or investigate the altered corneal shape that exists naturally in DS eyes; until Haugen (1992), there was little consideration that people with DS might be predisposed to KC in some way rather than KC being the result of self-induced corneal degradation. The current study is the first to examine corneal biomechanics in DS, the results indicating that the DS cornea exhibits greater deformation ability attributable to its reduced corneal thickness.

A necessity of the current study was to investigate if the ectasia in DS was phenotypically comparable to that of non-DS eyes. No significant differences in cone morphology were evident, suggesting that it is likely that DS ectasia is representative of KC in the wider population. Clinical KC is generally considered to be a common final pathway of a multifactorial disease. This means that the

underlying aetiology of KC in DS may or may not be the same as that of the wider population. Even in the wider population, there may be more than one ‘category’ of KC. It was first suggested by Nottingham (1854) that more than one aetiology of KC may exist, “If conical cornea be met with as an error of development of the eye, it is obvious that cases of it, viewed in connection of their source, must be more or less of the same nature, whether they occur before or after birth, while those cases of this malady which present themselves as direct or indirect consequences of inflammatory action, or of any ordinary form of ocular disease, must be regarded as belonging to another variety. It is evident that this distinction may have its use in a therapeutic as well as in a pathologic point of view...”. Much more recently, McMonnies and Boneham (2003) state, “...it appears that itch and atopy are neither sufficient nor necessary conditions for the development of keratoconus with the association between atopy, ocular itch and rubbing being found only in a subset of the keratoconic population”. Contrary to Grewal et al. (1991), the current study indicates that atopy and KC, and eye-rubbing and KC, are not associated with the atopic, inflammatory type of keratoconus. Rather, KC in DS may be the result of biomechanical changes secondary to an underlying genetic abnormality that results in abnormal corneal ECM, such as that seen in DS skin (Brand-Saberi et al. 1994). With recent advances in genetic testing, it is now possible to provide ‘whole genome screening’ for a group of individuals to elicit genetic correlations in phenotypic expression. Although the current sample size was limited, the dataset represents high-quality topography and clinical data with extensive optometric records. An analysis of regular and irregular astigmatism in the healthy and affected corneas of the current study should be explored in line with genetic information from each individual. Since the cones in DS and non-DS KC are morphologically similar, a basis is provided for using KC in DS as a model for non-atopic KC in the wider population.

The Tc1 mouse model of DS revealed no ultrastructural abnormalities when compared to wild type controls. For reasons outlined in Chapter 5, this does not mean that the DS cornea will exhibit a normal corneal ultrastructure. It is possible

that the Tc1 mouse exhibited a significant proportion of ocular mosaicism and that a much larger sample size would have resulted in some abnormal tissue being picked up in a small number of samples. It is possible that staining for particular collagens or particular genetic expression would have revealed abnormalities of the corneal ultrastructure not visible with the methods employed in the current study. Alternatively, the DS expression may not affect the eye in Tc1 mice.

In the thinner human DS cornea, it is not known if the collagen fibrils are compacted, or if the collagen mass is reduced overall. It would be advantageous to examine healthy human DS tissue from donor corneae, and examine using TEM with immunohistochemical analysis. Western blot analysis or immunolabelling could provide data on the differences in the proportions of collagens I, V and VI and examine the expression of relevant genes such as VSX1, LOX or SOD1.

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Appendices

Study Information sheet

Title: Characterising the cornea in young people with Down's syndrome

Principle researchers: Miss Stephanie Campbell, Dr Margaret Woodhouse, Prof Keith Meek, Miss Valdeflors Vinuela Navarro.



Before any consent is given to participate in this study, it is important to understand what the reasons are that have led to the study and what being a participant will involve. I am more than happy to answer any questions that you have, and my contact address is listed below.

Introduction to study:

People with Down's Syndrome are known to be predisposed to many eye problems. One particular condition is that of 'keratoconus', where the outer coat of the eye, the cornea, becomes thinner, and protrudes as a result. While sometimes mild, this often results in a marked reduction in visual ability and can be very threatening to the communication ability and the independence of a young person with Down's syndrome. There are new treatments becoming available that can halt the progression of keratoconus but which are only applicable at the very early stages, meaning that the early diagnosis is now a priority.

This project aims to understand the typical Down's syndrome cornea and gain a bank of information to establish what is 'normal', so that in the future, people who do not have normal corneas can be easily picked up and referred for treatment, from this point of view it's very exciting indeed!

In order for this to be a success, it requires the eyes of many young volunteers (both with and without Down's syndrome) to be measured with a variety of equipment that opticians, optometrists and ophthalmologists (eye doctors) use on a daily basis.

What will happen to my young person if they take part?

Firstly, I will measure the vision of each eye, and look at how the eyes work together. It is possible that we may need to take a photograph of the eyes in order to do this, and if this is the case then we will ask your verbal consent and keep you in the room at all times. These will only be stored for as long as necessary for the project and will be done so securely.

Next, I will use lenses in front of your young persons eyes in order to work out an up to date glasses prescription for the purposes of the study. This means that I will record the values, but it does not mean that you will have to buy any new lenses, and it may be that no glasses are required to be worn anyway!

I will look at the front of each eye using a microscope which opticians use every day, in order to see the 'cornea' in fine detail and do a health check of the front of the eye only. We may use a drop of orange water to check for any dryness in the eye (this does not sting, and is not necessary if your young person would prefer us not to-most children, however, love the colour!)

A scan of the cornea is one of the main aspects of this study and involves resting a chin on a rest and concentration on a spot for 30 seconds or so, to let the machine take photographs of the eye's shape and form an image. This is repeated several times on each eye if possible and does not come into contact with the face at all.

A little puff of air will be directed to the front of each eye, slightly less forceful than that which is used to test pressure in adults in many high street optician's practices. If possible, up to 5 measurements will be taken in each eye.

Is it safe?

All equipment used is already used extensively in eye centers across the world, and rigorously tested for safety for many years. There is no association with pain with any of the tasks that your young person will be offered to take part in, and if worried, they can change their mind at any time.

How will my young person benefit?

Your young person will benefit from full screening of the front area of the eye, the 'cornea', and ongoing monitoring, if necessary. Moreover, we find that our subjects enjoy taking part and having a go on the very exciting equipment which may not be part of their usual eye examination. Since we are geared up for seeing young people with additional needs and children, the environment is very inviting and we all tend to have a great time!

How will my young person's information be stored?

Just like information from any eye examination at Cardiff University, all information is stored securely and for no longer than necessary. For information storage on our equipment, all participants details are made anonymous by the use of participant numbers where possible, and only essential data is stored where relevant in full compliance with the Data Protection Act.

How can I find out the results of the study?

If there are any concerns about your young person's eyes, we will inform both of you straight away. In terms of the study as a whole, we would delight in sharing the research outcomes with you when these are made available at the end of the study, and invite you to provide details on the consent form so that we may contact you.

Please do not hesitate to direct any further questions to myself, using the details below.

Many thanks,

Stephanie Campbell

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Title: Characterising the cornea in young people with Down's syndrome



Principle researchers: Miss Stephanie Campbell, Dr Margaret Woodhouse, Prof Keith Meek, Miss Valdeflors Vinuela Navarro.

Please read the instruction leaflet and feel do ask any questions that you have about the study, relating to your young person's involvement, the equipment, the use of results, or anything else that you wish. Please check that the information below is correct, and if so, tick the boxes and sign below.

- I have been given a written explanation of the purpose and the content of the study by one of the researchers named on this form and have had the opportunity to read it and ask any questions raised.
- I understand that it is up to my young person to decide to partake in the study or not, and that they, or I, may decide to change our minds at any point and without reason.
- I understand that the personal information taken for this study will remain confidential and that this and the results stored will be stored accordingly.
- I understand that, through the nature of the study, it is likely that the knowledge gained will be presented and/or published for the benefit of our young people, and that all personal details will be removed, unless I give permission for a special case study to be made of my young person at a later date.
- I agree for my young person to participate in the study, with their consent.
- I would like to receive the results of the study and attach my details below:

Name _____

E-mail address _____

Postal address _____

.....
 Name of participant Name of parent/carer Signature of parent/carer Date

.....
 Name of researcher Signature of researcher Date

Dear Parent,

Thank you so much for agreeing to take part in our study on behalf of your young person.

As we have discussed, certain factors lead some people to be at a higher risk of developing keratoconus than others. One of these is widely considered to be Down's syndrome, however two others which are possibly connected are eye rubbing and the presence of atopy (which includes dermatitis and acne, asthma, eczema, hay fever and food or other allergies). Whilst these risk factors are thought to be connected, they may not be connected at all, and so it's important that you consider carefully if each of these factors are currently a problem, or if they have been in the past, or if there has never been a concern over that particular factor. It is as important for us to know if there is no connection with a condition as it is for us to know if there is one.

In terms of eye rubbing, I would be very grateful if, over the next couple of weeks, you would monitor how much your young person tends to rub their eyes. It's vital that you don't tell them that you are looking out for this! If there is someone else who spends time with your young person regularly, it might be useful to ask them to record the same. If something causes a disruption to the normal eye rubbing (eg an eye infection such as conjunctivitis or having dust in the eye or an eye injury), please do not include this in your 'average' results.

It would be very useful to know how often that your young person tends to 'itch' at something, and if they are the kind of person who likes to scratch and rub a lot, even if they do or don't complain much about the itch itself. It is normal to itch occasionally during the day, but it is not as common to scratch and rub in the same locations over and over again, or regularly throughout the day. We appreciate that this symptom of 'itchiness' is very hard to define, so please take time to compare the 'itch behaviour' your young person with Down's syndrome to that of other members of the family and to yourself. For the purposes of this study, please keep recording of 'eye rubbing' separate from 'itch behaviour'.

If you do have any questions at all about the survey below, please do not hesitate to call me on 07849540455 or drop me an email at campbells@cardiff.ac.uk

If you could post the form back addressed to myself at the School of Optometry and Vision Sciences, Cardiff University, Maindy Road, Cardiff, CF24 4LU, or using the envelope provided, within a month of your corneal screening, I would be very grateful indeed.

Many thanks,

Stephanie Campbell

| | Has been diagnosed with this | Has been a problem in the past but since recovered | No history of the condition |
|---|------------------------------|--|-----------------------------|
| inflamed, itchy skin (dermatitis/eczema) | | | |
| acne | | | |
| rashes or dry/inflamed skin at skin creases (inside elbows, behind knees, around ankles or neck) | | | |
| dry skin | | | |
| asthma | | | |
| food allergy | | | |

| | Eye rubbing tendency |
|---|----------------------|
| Not noticed at all | |
| little: once or twice daily | |
| moderate: three to five times daily | |
| regularly: six to ten times | |
| frequently: eleven to twenty times daily | |
| excessively: more than twenty times daily | |

Has 'eye rubbing' been consistent over the two-week period? Yes / No

| | itch' tendency |
|---|----------------|
| Not noticed at all | |
| little: once or twice daily | |
| moderate: three to five times daily | |
| regularly: six to ten times | |
| frequently: eleven to twenty times daily | |
| excessively: more than twenty times daily | |

Has 'itching' been consistent over the two-week period? Yes / No

Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
Yn rhan o seilwaith ymchwil Cymru a ariannir gan y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd, Llywodraeth Cymru



Research Ethics Committee (REC) for Wales
Sixth Floor, Churchill House
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Fax : 029 2037 6824

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22 October 2012

Dr J Margaret Woodhouse
School of Optometry and Vision Science
Cardiff University,
Maindy Road
Cardiff CF24 4LU

Dear Dr Woodhouse

Study title: Visual Development and Visual Defects in Children with Down's Syndrome
REC reference: 08/MRE09/46
Protocol number: SPON 536-08
Amendment number: 2
Amendment date: 25 September 2012

The above amendment was reviewed at the meeting of the Sub-Committee held on 19 October 2012.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

| Document | Version | Date |
|--|----------------------------|-------------------|
| Investigator CV | Stephanie Alayne Campbell | |
| Notice of Substantial Amendment (non-CTIMPs) | 2 | 25 September 2012 |
| Investigator CV | Valdeflors Vinuela-Navarro | |

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

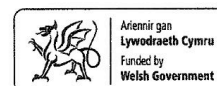
All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics



Cynhelir Cydweithrediad Gwyddor Iechyd Academaidd y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd gan Fwrdd Addysgu Iechyd Powys
The National Institute for Social Care and Health Research Academic Health Science
Collaboration is hosted by Powys Teaching Health Board



NOTICE OF SUBSTANTIAL AMENDMENT (non-CTIMP)

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) available in the Integrated Research Application System (IRAS) at <http://www.myresearchproject.org.uk> or on the EudraCT website at <https://eudract.ema.europa.eu/document.html>.

To be completed in typescript by the Chief Investigator in language comprehensible to a lay person and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available at <http://www.nres.nhs.uk/applications/after-ethical-review/notification-of-amendments/>.

| | |
|---------------------------------------|--|
| Details of Chief Investigator: | |
| Name: | Dr Joy Margaret Woodhouse |
| Address: | School of Optometry and Vision Sciences Cardiff University Mandy Road Cardiff |
| Postcode: | CF24 4LU |
| Telephone: | |
| Email: | woodhouse@cf.ac.uk |
| Fax: | 2920874859 |

| | |
|-------------------------------------|--|
| Full title of study: | Visual Development and Visual Defects in people with Down's syndrome |
| Lead sponsor: | Cardiff University |
| Name of REC: | Research Ethics Committee for Wales |
| REC reference number: | 08/MRE09/46 |
| Name of lead R&D office: | Cardiff University |
| Date study commenced: | 7th August 2008 |

| | |
|--|--|
| Protocol reference (if applicable), current version and date: | n/a |
| Amendment number and date: | Amendment 2, submitted 26th September 2012 |

| |
|---|
| <p>Type of amendment (indicate all that apply in bold)</p> <p>(a) <i>Amendment to information previously given on the REC Application Form</i></p> <p>Yes <i>No</i></p> <p><i>If yes, please refer to relevant sections of the REC application in the "summary of changes" below.</i></p> <p>(b) <i>Amendment to the protocol</i></p> <p><i>Yes</i> No</p> <p><i>If yes, please submit <u>either</u> the revised protocol with a new version number and date, highlighting changes in bold, <u>or</u> a document listing the changes and giving both the previous and revised text.</i></p> <p>(c) <i>Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study</i></p> <p><i>Yes</i> No</p> <p><i>If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold.</i></p> |
|---|

| |
|---|
| <p>Is this a modified version of an amendment previously notified to the REC and given an unfavourable opinion?</p> <p><i>Yes</i> No</p> |
|---|

Summary of changes

Briefly summarise the main changes proposed in this amendment using language comprehensible to a lay person. Explain the purpose of the changes and their significance for the study.

If this is a modified amendment, please explain how the modifications address concerns raised previously by the ethics committee.

If the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study, supporting scientific information should be given (or enclosed separately). Indicate whether or not additional scientific critique has been obtained.

People with Down's Syndrome are known to be predisposed to many eye abnormalities. One particular condition is that of 'keratoconus', where the outer coat of the eye, the cornea, becomes thinner, and protrudes as a result. While sometimes mild in form, this often results in a marked reduction in visual ability because of glare, blur and loss of transparency. Rabinowitz(1998). In very advanced cases, the cornea swells and nerve exposure results in pain and a further loss of vision. New therapies such as corneal cross linking are becoming available that can halt the progression of keratoconus but which are only applicable at the very early stages, Wollensak (2006). This means that the early diagnosis is now a priority.

Children with DS have corneas that are thinner and steeper (more protruding) than normal and therefore the onset of keratoconus can be difficult to differentiate before the condition has progressed beyond the stage that corneal cross linking can be applied.

As eyesight is particularly important for someone with special needs who relies on visual communication, the aim of the changes to our study is to fully understand the normal cornea in Down's syndrome in order to detect keratoconus at the earliest possible stage, where treatment is known to be most successful and where preventative treatments can be used.

Taking measurements to correlate and understand the biomechanical nature of Down's syndrome corneas both with and without suspect keratoconus is important in understanding the normal variations that occur in the cornea of such people. Using the Ocular Response Analyser and the aberrometer (widely used in eye clinics) is a key way to do this, and can be a clinical marker of corneal structure and stability. Using this equipment and also a very high magnification bio-microscope will help to identify key features which will differentiate 'normal' from 'suspect' and from 'keratoconus' and establish early referral protocols accordingly.

Many procedures required to investigate the presence of keratoconus have already been approved for our study (retinoscopy, topography, slit lamp biomicroscopy), but this amendment represents the addition of new technology and equipment available in optometry/ophthalmology, which are currently in use in practice and in hospitals to aid corneal diagnosis. These additional measurements will be taken when our subjects attend their existing appointments and will not affect existing eye examination protocol.

Addition of Miss Stephanie Alayne Campbell BSc MCOptom, (Optometrist) PhD student. Employer: Cardiff University, Address: School of Optometry and Vision Sciences, Maindy Road, Cardiff CF24 4LU +442920870247.

Addition of Miss Valdeflors Vinuela-Navarro BSc MCOptom, (Optometrist) PhD student. Employer: Cardiff University, Address: School of Optometry and Vision Sciences, Maindy Road, Cardiff CF24 4LU +442920870247.

Removal of Mr Mohammad Al-Bagdady who has successfully gained his PhD.

Philip Jones has now gained his PhD and taken up a position at Princess of Wales Hospital, Coity Road, Bridgend CF31 1RQ. He will remain as part of the research team and we would like to add his new place of work as an additional site.

Rabinowitz YS (1998), Keratoconus. *Survey of Ophthalmology*. v2 297-319

Wollensak G (2006), Crosslinking treatment of progressive Keratoconus: New Hope. *Curr Opin Ophthalmology* v17, 356-360

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

The Ocular Response Analyser (ORA) is a modification of the commonly used non-contact tonometer (NCT) which is in widespread use to measure the intra-ocular pressure. Like the NCT, the ORA utilises a small puff of air in order to gain a different type of reflection from which the pressure inside of the eye is deduced, this is a routine part of any eye exam and is commonly used for people with Down's syndrome. In particular, the ORA uses a smaller puff of air and a second reflection point to determine the timescale of the pressure change and provides data on the biomechanical properties of the cornea from this. There is no discomfort or damage to the ocular surface; Luce (2005).

An aberrometer measures the aberrations, or distortions, in the light reflected from the eye. It is a quantifiable method of measuring the 'distortion' seen during retinoscopy which is indicative of keratoconus. Abberometry is used in a non-contact fashion in eye clinics and operates in a similar way to the topographer currently in use by the Down's syndrome study. This has been used extensively in people with Down's syndrome in a research study in Northern Ireland, McCullough (2011).

The confocal microscope is similar to the slit lamp biomicroscope currently used in the study, but utilises a higher power magnification system in order to view the cornea in greater detail. This is used in ophthalmological practice to aid the diagnosis of anterior eye problems and monitor degeneration or healing, Ucakhan (2006)

The majority of our existing Down's Syndrome study cohort are now aged late teens/early twenties, the oldest being 25. This is the prime age at which keratoconus is likely to onset as now control subjects will be recruited from clinic patients as well as from schools and children of university staff. Families of children with Down's syndrome might form an interesting sub-group because of hereditary corneal characteristics. This change to our study is therefore in line with the age increase in our cohort, we would therefore amend the title of our study to reflect this, "Visual Development and Visual Defects in Children and Young Adults with Down's syndrome".

Luce DA (2005) Determining in vivo biomechanical properties of the cornea with an Ocular Response Analyzer, *J Cataract Refract Surg* v31 156-162.

McCullough , Sara J, Little, Julie-Anne and Saunders, Kathryn J (2011) *Anomalous Wavefront Aberrations Reduce Optical Quality In Down Syndrome*. In: Association for Research in Vision & Ophthalmology, United States of America. Lippincott Williams & Wilkins . Vol 52 (2810-D659) 1 pp. [Conference contribution]

Ucakhan OO, Kanpolat A, Yilmaz N, Ozkan M, (2006). In Vivo Confocal microscopy findings in Keratoconus, *Anterior Eye and Contact Lens*. 32 (4) 183-191.

Appendix E

A priori power calculation for the measurement of deformation amplitude using CorVis ST, using the technique for two independent study groups and the endpoint of a continuous variable. Alpha was set at 0.05 and the power at 80%, as standard. It was anticipated that a difference of 10% from the control group would be meaningful.

Anticipated mean (control group) = 1.07 ± 0.10 (from Hon and Lam 2013), with an enrolment ratio of 2:3 (control:DS).

Requires a sample size:

Control participants: 17

DS participants: 11

Total: 28