The role of the Visual System Homeobox Gene 1 (*VSX1*) in keratoconus and mouse development

Thesis submitted to Cardiff University for the degree of Doctor of Philosophy in the disciplines of Molecular Biology and Biophysics

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To my darling Candy

All characters and events portrayed within this book are fictitious, and any resemblance to those living or otherwise is purely coincidental...

Better Than Life, RD

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Summary

The main aims of this thesis were to examine the role of the Visual System Homeobox gene 1 (*VSX1*) in keratoconus and development using molecular biology techniques and biophysics. The opportunity existed to examine a *Vsx1* knockout mouse using X-ray diffraction to see whether the structure of the cornea was altered due to the removal of this gene. Upon examination with X-ray diffraction further experiments were devised in order to clarify possible interactions of other genes that may play a part in keratoconus pathogenesis. The interfibrillar and intermolecular collagen spacings and the average collagen fibril diameters between *Vsx1* knockout mice and background matched littermate controls were compared. There were no statistical differences found in all cases. This is a similar finding to human keratoconic corneas when compared with normal controls. It was found that the *Vsx1* knockout mouse had significant alterations to the preferential alignment of collagen fibrils and altered corneal collagen mass distribution. This also is similar to human keratoconic corneas, strengthening the proposition that the *Vsx1* knockout mouse is a model system for keratoconus. Additionally, significantly altered expression levels of the genes *HSF1*, *Hsp47* and *Aqp5* were found along with no expression of *Col8a2*.

It was believed that the initial fault that allows keratoconus to develop occurs during development, so to explore this normal mouse development was explored using X-ray diffraction to map the collagen fibrils of the cornea as it develops in postnatal stages. This would provide a good baseline for future experiments. In addition to this, initial investigation of the expression of the *Vsx1* gene was undertaken to see whether it may play a role in development. It was found that the annulus of collagen fibrils that plays an important role in maintaining the structure of the cornea starts to develop at postnatal day 10 in the mouse and continues until maturity. It was also found that there is a significant alteration in expression of *Vsx1* which occurs in development between postnatal day 5 and day 12.

Lastly a population of keratoconus patients in South Wales were screened for mutations in the *Vsx1* gene. Mutations in *Vsx1* have been observed in other studies and this investigation was undertaken in order to clarify the ongoing debate into mutations in *Vsx1* and their link to keratoconus. In the study presented here a number of previously identified polymorphisms were discovered in out cohort of patients but no polymorphism was deemed to be a disease causing mutation.

Table Of Contents

List Of Figures

List Of Tables

Chapter 1: Introduction

Keratoconus is a bilateral corneal thinning disease that presents generally in the late teens and progresses until the third or fourth decade of life, when it usually arrests. There is no difference in the prevalence between males and females and it affects 1 in 2000 people (Claoue et al., 1990; Gorskova and Sevost'ianov, 1998; Haugen, 1992; Lindquist et al., 1991; Nielsen et al., 2007; Pearson et al., 2000; Rivera and Mendoza, 2004; Saini et al., 2004; Simmons et al., 1989; Yahalom et al., 2005). The disease causes the cornea to be misshapen and cone- like, protruding from the eye instead of having a uniformly curved surface. This changes the refractive properties of the cornea causing blurred vision. People with early keratoconus typically notice a minor blurring of their vision and initially seek corrective treatment. If keratoconus is suspected then slit lamp examination and corneal topography would reveal other signs associated with keratoconus such as distortions of the cornea and possible scarring. More advanced signs of keratoconus such as Fleischer ring, Vogt's striae and Munson's sign follow as the disease progresses (Castroviejo, 1949; Gorban, 1973; Green, 1947; Iwaszkiewicz et al., 1992; Knapp, 1929; Puchkowskaya and Titarenko, 1986; Tomalla and Cagnolati, 2007; Wygledowska-Promienska, 2005).

The classification of keratoconus has always posed a problem in that the symptoms of keratoconus have all the traditional hallmarks of being an inflammatory disease, except that in keratoconus the damage caused is non-inflammatory. It is probably better to think of keratoconus as an ectasia, which is a change in shape. However, for the purposes of this thesis it shall be herein described as a disease rather than an ectasia. It is a little-understood disease with an uncertain cause, and its progression following diagnosis is unpredictable. Keratoconus normally presents in one eye first, however, keratoconus is a bilateral disorder. In reported cases of monocular keratoconus the progression in the 'unaffected' eye is usually at a much lower level than that of the other eye (Mahon and Kent, 2004; Phillips, 2003). The deterioration in the patients' vision at later stages can be incredibly detrimental to their daily lives with some reaching a refractive error of 72 diopters. In most cases, and according to the severity, corrective contact lenses are effective enough to allow the patient to continue to function normally if they are able to wear them comfortably.

In early stages of the disease vision can be corrected with glasses, but there comes a point where the progression prevents any meaningful correction to be

achieved and contact lenses are the only solution prior to surgery. Further progression of the disease may require surgery such as corneal grafting and in these cases there are a number of surgical techniques that exist to try to correct the patients' vision. Despite its uncertainties, keratoconus can be managed with a variety of clinical and surgical techniques, but this is less than ideal and ultimately early identification and prevention of onset would be the best solution. To this end, many researchers have been looking into the potential causes of keratoconus with the aim of finding a single or collection of targets that may be used as early identifiers and treatment points.

Genetics is increasingly becoming more important in the study of diseases and is playing an important role in the research on keratoconus. Current studies of keratoconus have involved collecting cohorts of patients, either individuals or families that have one or more sufferers with the condition (Aldave et al., 2007a; Aldave et al., 2006; Bisceglia et al., 2005; Brancati et al., 2004; Heon et al., 2002; Hosseini et al., 2008; Liskova et al., 2007a; Nguyen et al., 2007; Tang et al., 2008; Tang et al., 2005; Udar et al., 2006; Udar et al., 2004). Studies on cohorts of families are especially useful as a first step in identifying possible genes that could be involved in the disease. These studies aim to identify genes based on their inheritance patterns through linkage analysis. Linkage analysis allows areas of chromosomes to be tracked to see if they segregate between affected and non-affected individuals. Once an area has been found, it can be cross examined with established databases of genes within that area and candidate genes selected based on their functionality. Once these genes have been selected they are screened within affected patients to see if there are any mutations within the coding sequence. This is compared to the coding sequences of unaffected individuals who act as controls to see if the mutations segregate with the disease. If they do then it can be said with some certainty that the gene is disease causing and can be studied further.

In addition to genetics the use of biophysical techniques such as X-ray diffraction has been employed to examine the ultrastructure of corneas of both keratoconus affected and non-affected individuals. These results have provided unique insights into the collagen structures that make up the cornea and demonstrate the way the corneal shape is maintained though its structural arrangement (Beecher et al., 2005; Beecher et al., 2006; Boote et al., 2005; Boote et al., 2004; Boote et al., 2003; Boote et al., 2006; Boote et al., 2008; Connon et al., 2003a; Connon and Meek, 2003; Connon et al., 2003b; Daxer and Fratzl, 1997; Fullwood and Meek, 1993;

Fullwood et al., 1990; Fullwood et al., 1993; Fullwood et al., 1992; Hayes et al., 2007a; Hayes et al., 2007b; Meek et al., 1987; Meek and Boote, 2004; Meek et al., 1982; Meek et al., 1986; Meek et al., 1981; Meek and Newton, 1999; Meek and Quantock, 2001; Meek et al., 2003b; Meek et al., 2005). This has allowed researchers to demonstrate that the structure in keratoconus affected corneas is different from unaffected controls in that the cornea is thinner and demonstrates an altered collagen fibril alignment. This indicates that there could be underlying defects within the cornea of keratoconus patients, which may be linked to altered or impaired gene expression.

This thesis aims to investigate one of the identified candidate genes for keratoconus, Visual System Homeobox 1 (*VSX1*), and explore a potential animal model of keratoconus by structural and genetic investigations. It also aims to investigate the possible causes and development of keratoconus by examining the structure of mouse corneas using X-ray diffraction in development. Lastly, both sets of information will be combined to examine what role *Vsx1* has to play in the pathogenesis of keratoconus and whether the possible defects that could occur within keratoconus can be linked back to any genetic anomalies that are found in the gene.

1.1 The Cornea

1.1.1 Corneal Development

The formation of the cornea occurs during the last series of major inducing events of the eye during embryogenesis. These inducing events allow the surface ectoderm at the front of the eye to become a transparent, multilayered structure with a complex extracellular matrix (Carlson, 2004). The basal ectodermal cells increase in height and begin to secrete epithelial derived collagen types I, II and IX to form the primary stroma. Neural crest cells around the lip of the optic cup migrate centrally between the primary stroma and the lens capsule. These cells form the corneal endothelium which is the posterior- most layer of the cornea. The endothelium is important for the remaining developmental process to occur. Once fully formed the endothelium secretes into the primary stroma a large amount of hyaluronic acid. Hyaluronic acid has great water binding properties and causes the primary stroma to swell (See fig 1.1).

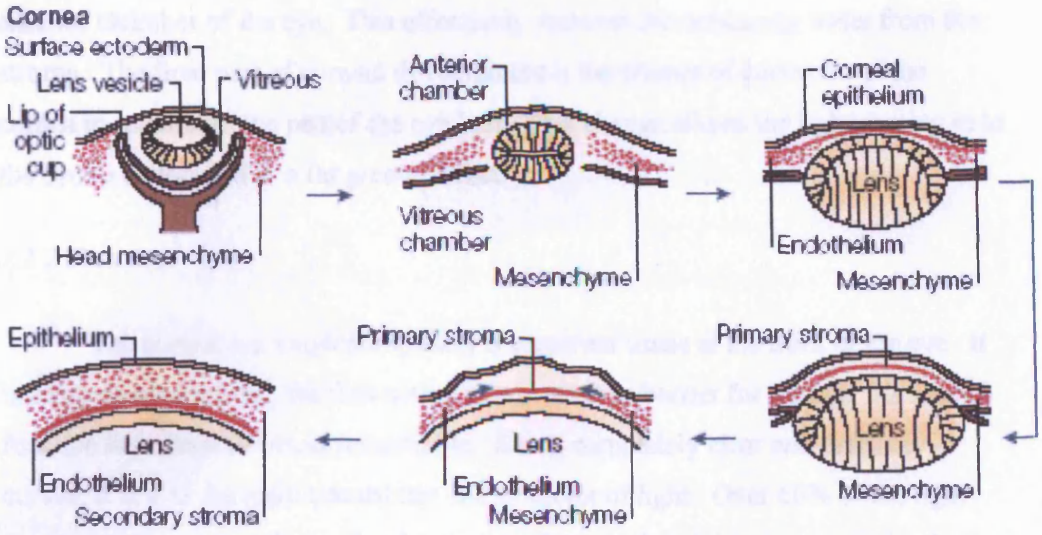


Fig 1.1. Flow diagram of corneal development. Figure taken from (Graw and Loster, 2003).

The stromal swelling provides a substrate for a second wave of cellular migration into the cornea. These cells are also derived from neural crest cells and are fibroblastic in nature. These cells migrate into the stroma between the layers of collagen and then secrete large amounts of hyaluronidase, which counteracts and breaks down the hyaluronic acid. This decreases the thickness of the cornea and the stroma is said to be in its secondary stage. The fibroblasts aid organisation of the collagen by secreting collagen into the stromal matrix. The endothelium and the epithelium continue to secrete acellular matrix constituents to form the rest of the cornea, the Descemet's membrane and Bowman's layer respectively (Graw, 2003). This makes up the mature cornea. It is worthwhile to note that the development of the cornea is not the same within all species. Avian corneas seem to have two distinct stromal stages of development, a primary stage and a secondary stage; although a process similar to this is found within murine and human corneas they do not have a primary stroma.

The last parts of corneal formation are the changes that allow a transparent pathway from the outside world to the retina without optical distortion. The amount of light that is transmitted increases from 40% to 100%. This is accomplished mainly in the stroma by the removal of water. This firstly occurs with the breakdown of hyaluronic acid, and secondly by thyroxine that is secreted from the thyroid.

Thyroxine acts on the endothelium to pump sodium from the secondary stroma to the anterior chamber of the eye. This effectively removes the remaining water from the stroma. The final part of corneal development is the change of curvature of the cornea in relation to the rest of the eye ball. This change allows the light coming in to the eye to be focused to a far greater effect.

1.1.2 Function

The cornea is a tough completely transparent tissue at the front of the eye. It has two main functions, the first acting as a protective barrier for the eye. Its second function is perhaps the most remarkable. Being completely clear and precisely curved, it acts as the main transmitter and refractor of light. Over 60% of the light that enters the eye is refracted by the cornea after which subtle adjustments by the lens acts to focus the light on the retina that sends an image to be interpreted by the brain.

1.1.3 Structure of a normal cornea

The cornea is composed of five layers each serving a different function to maintain the refractive surface allowing for normal vision (see fig 1.2). The outermost surface that is in contact with air is the epithelium that is coated with a glycocalyx tear film, which is supported underneath by the epithelial basement membrane. The largest layer of the cornea is the stroma that resides in the middle of the cornea and is the main structural component. Below the stroma is the Descemet's membrane and finally the endothelium that allows the cornea to take up nutrients and controls hydration.

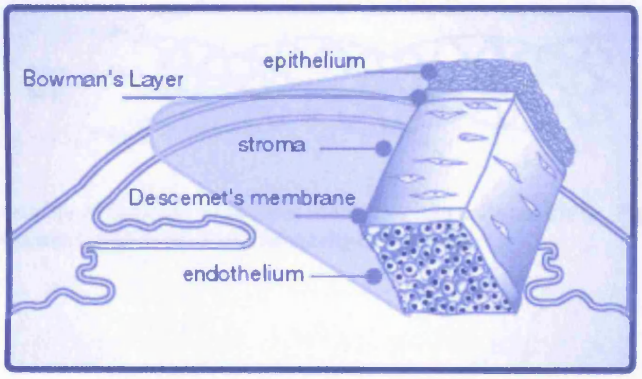


Fig 1.2. A schematic diagram of the different layers making up the cornea. Taken from the Ophthalmic Consultants of Boston website (www.eyeboston.com)

The bulk of the cornea is composed of the stroma which is where most of the corneal collagens in the cornea are located. The corneal stroma is based on a matrix of water, proteoglycans, glycoproteins, keratocytes, inorganic salts and soluble proteins into which fibril forming collagens are embedded. Collagens are insoluble extracellular glycoproteins that are found in all animals. They are the most abundant protein within the human body and account for 70% of the cornea's dry weight. They are essential structural components of all connective tissue and are an essential part of the cornea in terms of its structure and how it performs its function. So far 29 different types of collagen have been found in the body but the cornea is mainly composed of collagen type I, III, V, VI and XII. The basic unit of fibril forming collagen is a polypeptide consisting of a repeating sequence ((Gly)-X-Y)n where X is often a proline residue and Y is often a hydroxyproline. This results in the secondary structure adopting a left handed helix.

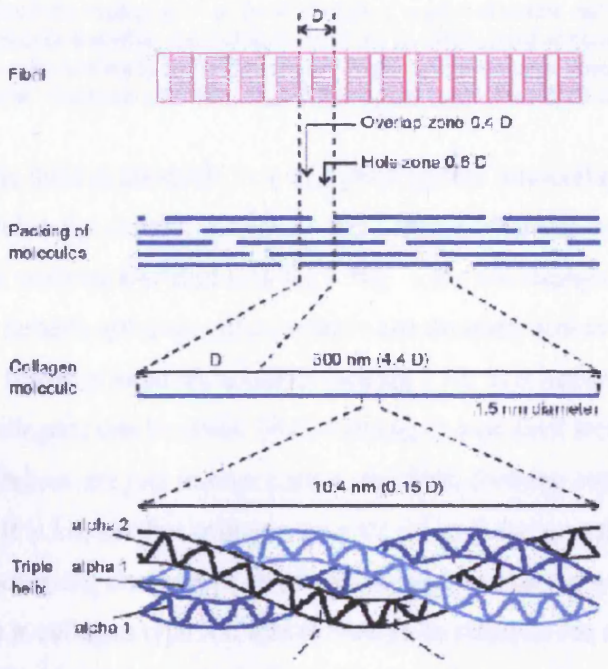


Fig 1.3 Assembly of collagen triple helix molecules into collagen fibrils. Figure taken from www.ccmbel.org/Chap5.html University of Brussels.

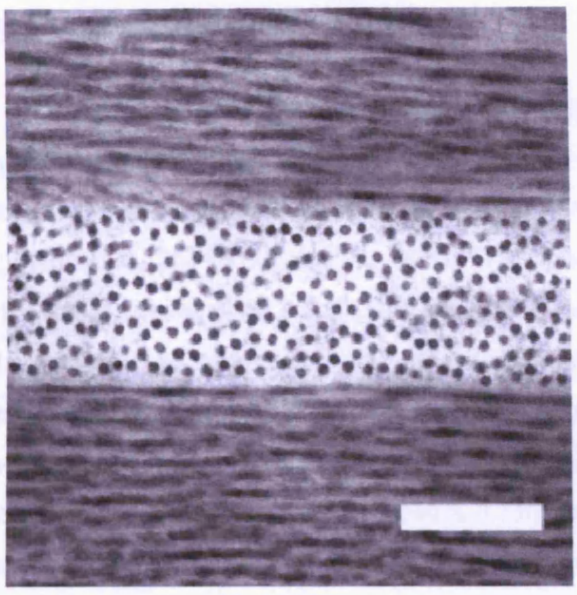


Fig 1.4. Transmission electron micrograph showing the orientation of collagen fibrils in adjacent lamellae in the corneal stroma. The micrograph shows three lamellae from the central stroma. In the middle lamella, the collagen fibres are in cross-section (running toward the reader) and can be seen to be of regular diameter and spacing. In the top and bottom lamellae, the collagen fibres are in longitudinal section (running from side to side) and are at approximate right angles to the collagen fibres in the middle lamella. Scale bar -200 nm. Figure and legend taken from (Fullwood, 2004).

These triple helices assemble in a staggered pattern into collagen fibrils. The collagen fibrils within the corneal stroma, unlike in most other tissues, are regularly spaced and have a uniform diameter (see fig 1.30). They are arranged in belts termed lamellae and in a lamella are arranged parallel to one another; however they form large angles with fibrils in adjacent lamellae (see fig 1.4). It is important to make the distinction that collagens can be either fibril forming or non fibril forming. The non fibril forming collagens are just as important as the fibril forming collagens physiologically. It is known that collagen type VI exists in the interfibrillar matrix and acts in the underlying framework by interweaving between the type I fibrils. Another example is collagen type XII that is thought to stabilize the collagen fibril arrangement by directly bridging the collagen fibrils. There are many examples of diseases that are caused by mutant collagen genes such as Osteogenesis imperfect (brittle bone disease) and Ehler-Danlos syndrome all caused by mutant collagen type I. The central cornea contains between 200 and 400 lamellae, and it is thought that lamellar organisation and distribution must control corneal shape and curvature (Fulwood 2004).

1.1.4 The structure of the cornea in humans

There have been many studies of the cornea on a variety of species (Hayes et al., 2007a); See appendix A) using X-ray diffraction and on humans (including patients) with keratoconus(Aghamohammadzadeh et al., 2004; Fullwood et al., 1992; Huang et al., 1996; Meek et al., 2003b; Meek et al., 2005). This has yielded information about the fine structure of the cornea and about the arrangement of collagen fibrils in both normal and keratoconus affected corneas. In February 2004 Aghamohammadzadeh et al. published the first X-ray diffraction map of the human cornea, which showed the preferred alignment of collagen fibrils (see fig 1.5). This map shows is that the collagen fibrils are highly ordered and demonstrate different preferred alignments at different parts of the cornea, most likely due to the different mechanical demands that are placed on the structure as a whole.

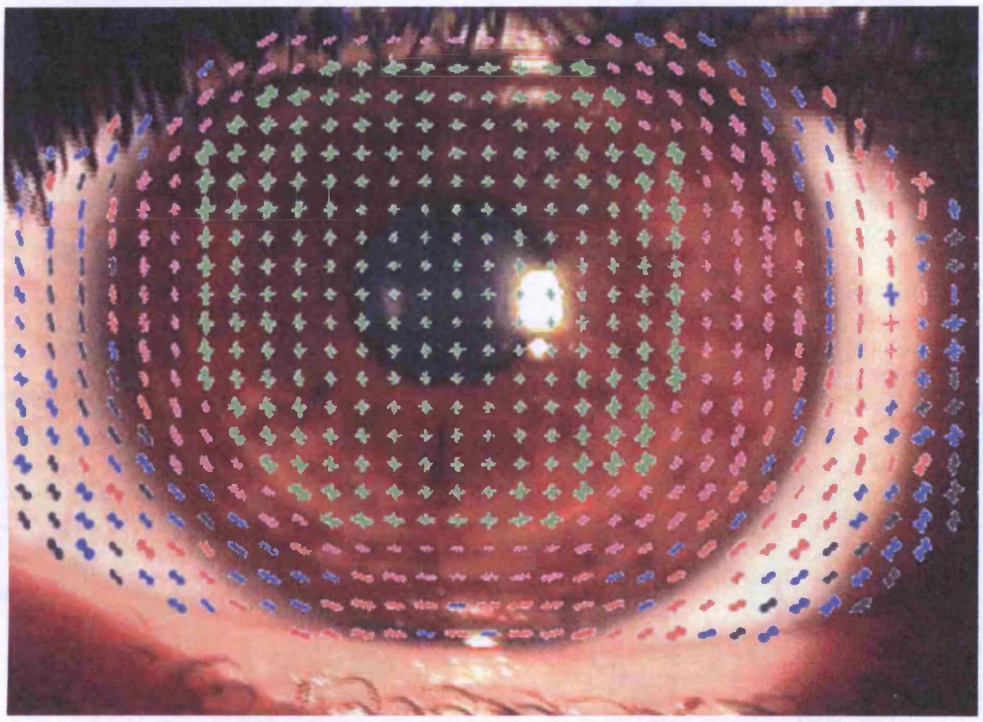


Fig 1.5. Collagen fibril orientation in a human cornea. Figure taken from (Aghamohammadzadeh et al., 2004).

In the human cornea the arrangement of fibrils is very ordered across the central region of the cornea with definite horizontal and vertical preferential alignment. This changes as you proceed to the peripheral areas of the cornea, where

the preferential alignment changes to a circumferential alignment. The alignment is thought to be due to an increase need for strength in the direction of the muscles that control ocular movement (Boote et al., 2006). The distribution of collagen within the cornea was elucidated via X- ray diffraction and shows that the central part of the cornea is fairly thin with an increase of collagen at the limbal regions that circles the entire cornea. This also showed that there is a horizontal symmetry between the left and right eyes demonstrating a rhombus shaped distribution of the aligned collagen. The corneal ultrastructure for the human cornea has also been measured; the interfibrillar spacing has been reported to be about 57nm in the centre of the cornea with this increasing to about 62nm in the periphery and an even greater increase at the limbus (Boote et al., 2003). The average fibril diameter in the cornea is 31nm which increases with age and is largely uniform across the cornea but increases at the limbus (Boote et al., 2003; Meek et al., 2003a).

In keratoconus patients the fibril organisational pattern differs from that of the normal human cornea. The vertical and horizontal preferred orientations are absent in the areas where a cone has manifested which is clearly visible from its orientation map. The map also demonstrates that the fibril pattern circles the cone and is itself much thinner than the surrounding tissue (see fig 1.6). The interfibrillar spacing and average fibril diameter remain unchanged.

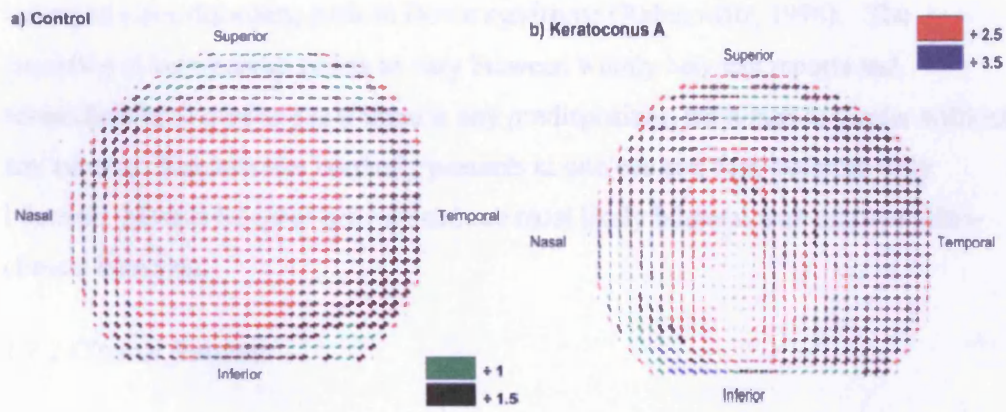


Fig 1.6. Fibrillar organisational patterns between a normal control corneal button and a keratoconic button. Figure taken from (Meek et al., 2005)

1.1.5 The structure of the cornea in mice

In mice the average collagen fibril diameter was reported to be 35.5nm in 3 month old balb/c mice and about 37nm in age matched SKC mice (Quantock et al., 2003). The interfibrillar spacing was stated to be at an average of 50nm. The arrangement of collagen fibrils within different species are different, which is suggested to be linked to the differences in visual acuity. Species with high visual acuity seem to have a more complicated fibril patterning (Hayes et al. 2007). In the mouse the fibril patterning appears to be circumferential around the centre of the cornea which extends all the way to the limbus.

1.2 Keratoconus

1.2.1 Epidemiology

The characteristic onset of keratoconus occurs in the second decade of life and continues to progress for two decades when it usually arrests. It is the most prevalent corneal disorder in the western world and is the leading cause for corneal surgery. The progression of the disease can vary from person to person and can also vary in severity. It is normally an isolated disease, but has been known to be linked to a variety of other disorders, such as Down syndrome (Rabinowitz, 1998). The incidence of keratoconus seems to vary between widely between reports and researchers have tried to see if there is any predisposition from race or gender without any success. Keratoconus normally presents in one eye and then becomes fully bilateral. Monocular cases are rare and are most likely bilateral with one eye below clinical detection.

1.2.2 Clinical features

The clinical features of KC are varied and depend on the stage of the disorder. In early stages patients could present with irregular astigmatism or a minor blurring of visions. In the moderate to late stages of keratoconus stromal thinning, conical protrusion, Fleischer's ring and fine vertical lines in the deep stroma and Descemet's

that are parallel to the axis of the cone (Vogts striae see fig 1.7) are visible via slit lamp examination. Other signs are epithelial nebulae, anterior stromal scars, enlarged corneal nerves and increased intensity of the corneal endothelial reflex and sub epithelial fibrillary lines (Rabinowitz, 1998). External signs of keratoconus are Munson's sign (see fig 1.7) and corneal hydrops. Patients with advanced keratoconus may present with hydrops, which is due to breaks in the Descemet's membrane that allow the aqueous to enter the stroma. This oedema may persist for a short duration but may eventually disappear, leaving scarring.

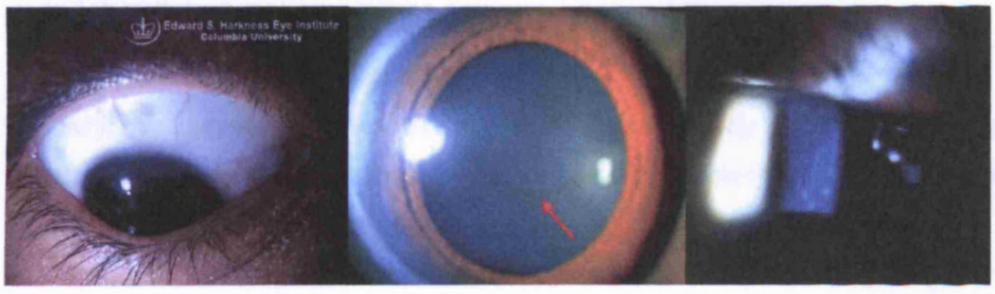


Fig 1.7. Examples of signs of keratoconus, A) External Munson's sign, B) Fleischer's ring, C) Vogt's striae. Pictures taken from Edward S Harkness Eye Institute, Columbia University.

Depending on the severity and stage of the disease every layer of the cornea can become involved. The epithelium may show basal cell degeneration, breaks in the Bowman's layer due to down growth of the epithelium and an accumulation of ferritin particles that build up within and between the epithelial cells. The Bowman's layer may show breaks that are filled by the underlying stromal collagen, periodic acid Schiff-positive nodules, and Z shaped interruptions possibly due to separation of collagen bundles and reticular scarring (Li et al., 2004).

1.3 Keratoconus and oxidative stress

There have been a number of theories proposed as to what causes keratoconus. They range from increased degradation of matrix metalloproteinases to links with magnesium deficiency (Thalasselis, 2005) and diabetes (Prakash et al., 2007). However, it appears that the theory that has been investigated above all others is the link between keratoconus and increased oxidative stress. Keratoconus, being a noninflammatory disease, shares many characteristics with inflammatory corneal

diseases and wound healing, which has led investigators to think that oxidative stress plays a large role in its onset and progression. There are also indications that in keratoconus there are corneal components that are altered that reduce the eye's capability to counteract generated free radicals and oxidative stress. ALDH3 and extracellular forms of superoxide dismutase (SOD) are reported to be reduced in keratoconus (Behndig et al., 2001; Behndig et al., 1998; Gondhowiardjo and van Haeringen, 1993; Gondhowiardjo et al., 1993). There has yet to be any evidence that demonstrates how these oxidative products accumulate in the cornea or how a detriment in these corneal components affects the cornea's susceptibility to UV damage. Apart from the damage induced by UV light there is also evidence from stress induced reactive oxygen species. It has been suggested that the thinning process that occurs in the cornea is related to an increase in degradative enzymes such as catalase (Kenney et al., 2005) and a decrease in degradative inhibitors (Opbroek et al., 1993; Sawaguchi et al., 1990), both of which are linked to oxidative stress.

The studies on oxidative stress have led to an identification of a candidate gene on chromosome 21, the superoxide dismutase 1 (SOD1) gene. Superoxide dismutase catalyses the dismutation of superoxide into oxygen and hydrogen and is an important antioxidant defence in nearly all cells. A unique genomic deletion within intron 2 close to the 5' splice junction of the SOD1 gene was identified in three patients with keratoconus and mRNA from 1 affected individual had two transcript splice variants (LE2 and LE2E3) that others have shown to code for proteins lacking the active site of the SOD1 enzyme (Udar et al., 2006). The reported deletion segregated with affected individuals and was not present in 312 control chromosomes indicating it is a good candidate for future study.

1.4 Genetic associations of keratoconus

1.4.1 Mapped loci of keratoconus

Keratoconus in the general population is sporadic, however positive family history has been reported in 6% to 8% of patients. In the families that have reported keratoconus it is mostly inherited in an autosomal dominant fashion, however there have been rare cases where autosomal recessive inheritance is the cause. It is also worth mentioning that rare sporadic cases have emerged where keratoconus has been associated with rare chromosomal translocations (Morrison et al., 2001). Several

linkage studies have been carried out on families that have one or more members affected by keratoconus in an attempt to identify candidate genes and loci for the disease. Tyynismaa et al (2002) performed a genome wide linkage study on 42 affected and 34 unaffected individuals in 20 Finnish families that were known to exhibit keratoconus. The family members affected presented with autosomal dominant keratoconus. The conclusion of the study was that a possible gene for the disease mapped to chromosome 16q23-q23.1. A possible gene family to emerge from this study is the ADAMT (a disintegrin and metalloproteinase with thrombospondin motif) gene family that does map to both the disease region in this study and in the study performed by (Tyynismaa et al., 2002). The linkage study ruled out loci for genes on 18p and 21q. This is interesting, as a study carried out by (Rabinowitz et al., 1992) reported a locus for keratoconus near the centromere of chromosome 21. The study that identified the locus for 18p used an association approach on eight unrelated individuals with keratoconus (Fullerton et al., 2002).

Another study performed by Hughes et al (2003) described a family of 30 individuals, 16 of which were affected. The affected individuals were assessed on two occasions with a five year interval. A genome wide linkage screen found a potential locus for the disease gene on chromosome 15q22.33-24.2. The disease interval mapped was 6.5Mb and four candidate genes were selected for screening; CTSH, CRABP1, IREB2 and RASGRF1. All were excluded through DNA sequencing as the cause of keratoconus in the family. Patients described in this study also presented with cataracts. The region that this gene maps to is exceedingly gene rich (Hughes et al., 2003) and so there are a number of possible candidate genes for cataracts and keratoconus. The disease interval was reduced further to 5.5Mb that excluded 28 positional candidates and a further 23 candidate genes were excluded through direct sequencing (Dash et al., 2006). This family of genes is interesting as it has both disintegrin and metalloproteinase activity which have both been reported to be altered in keratoconus (Hughes et al., 2003).

A fourth study identified an Italian pedigree with 11 members who had autosomal dominant keratoconus and identified a locus which mapped to chromosome 3p14-q13 (Brancati et al., 2004). Seven individuals of this study were diagnosed with definite keratoconus and the other four were identified as having "forme fruste" keratoconus, which is a term used to describe subtle topographic anomalies in people related to keratoconus patients. A candidate gene within this locus is COL8A1, which

codes for human α1(VIII) chain of type VIII collagen. The collagen encoding genes do make good candidates for keratoconus as other collagen genes have been implicated in two forms of corneal dystrophy Fuchs Endothelial Corneal Dystrophy (FECD) and Congenital Hereditary Corneal Dystrophy (CHED) (Biswas et al., 2001). However, mutation analysis of COL8A1 did not show any mutations that were considered to be disease causing, although a G to T change was detected in nine affected individuals. This was thought to be a polymorphic variant (Brancati et al., 2004). Linkage to all other suspected keratoconus loci in this study was excluded.

1.4.2 Genes linked with keratoconus

There are a number of other candidate genes that may, by virtue of their position within mapped genetic loci (2p24, 3p14-q13, 15q22, 16q23, 20p11 and 21q) (Biswas et al., 2001; Brancati et al., 2004; Hughes et al., 2003; Rabinowitz et al., 1992; Tyynismaa et al., 2002) or by their proposed function, be implicated in the genetic aetiology of keratoconus. A list of possible candidate genes based on genetic mapping and function is provided in Table 1.

Locus	Name	Known function	Ref.	GeneID
3p14–q13	*COL8A1*	Human alpha1(VIII) chain of type VIII collagen	(Brancati et al., 2004)	1295
15q22.33-24.2	*CTSH*	Cathepsin H gene, cysteine-dependent intracellular protease that is responsible for intracellular protein degradation and turnover	(Hughes et al., 2003)	1512
15q22.33-24.2	*CRABP1*	Cellular retinoic acid binding protein 1 gene, its product is one of a family of six vitamin A–binding proteins. Is thought to be involved in retinoic acid–mediated differentiation and proliferation processes	Hughes et al. 2003	1381
15q22.33-24.2	*IREB2*	iron-responsive element binding protein 2 gene	Hughes et al. 2003	3658
15q22.33-24.2	*RASGRF1*	Ras guanine nucleotide releasing factor gene, shares homology with the guanine nucleotide exchange activator son-of-sevenless in	Hughes et al. 2003	5923

		Drosophila melanogaster, which is involved in the sevenless signalling pathway in developing photoreceptors. It also shares similarity with mouse son-of-sevenless (*Sos1*), mutations of which dominantly enhance the weak allele of *Egfr* causing distinctive eye defects including lens opacity and eye dysmorphology		
15q22.33-24.2	*DKFZP434H204*	ADAMTS family,	Hughes et al. 2003	11173
15q22.33-24.2	*ADAMTS7*	ADAMTS family, disintegrin and metalloprotease activity	Hughes et al. 2003	11173
15q22.33-24.2	*ADAMTS18*	ADAMTS family, disintegrin and metalloprotease activity	Hughes et al. 2003	170692
16q22.3-q23.1	*LCAT*	Lecithin-cholesterol acyltransferase gene is responsible for fish-eye disease with corneal lesions	(Tyynismaa et al., 2002)	3931
16q22.3-q23.1	*TAT*	Tyrosine aminotransferase gene causes tyrosinemia, which is characterised by herpetiform corneal ulcers.	(Tyynismaa et al., 2002)	6898
16q22.3-q23.1	*CHST6*	Carbohydrate sulfotransferase 6 gene identified as the causative gene for macular corneal dystrophy types I and II	(Tyynismaa et al., 2002)	4166
2p24	*OSR1*	Encodes odd-skipped related 1, a putative transcription factor which possesses a homeobox domain	(Hutchings et al., 2005)	130497
2p24	*ARHB (RhoB)*	Actin reorganisation. Possible candidate gene since it stimulates actin stress fibre formation and focal adhesion points. Mutations in RhoB could explain structural defects observed in KC corneas. However, this gene is expressed in many other tissues where its functions are highly important, so it seems unlikely that mutations would lead to symptoms	(Hutchings et al., 2005)	11852

		restricted to the cornea		
2p24	*GDF-7*	Control of cell differentiation, GDF-7 (growth differentiation factor 7) is a good candidate gene due to the existence of TGF-ß domains in its sequence. TGF-ß and related proteins have already been investigated for their possible implication in KC because of their importance in the corneal epithelium but to no avail. However, probable roles for GDF-7 have been identified so far in the rhesus monkey neocortex, in seminal vesicle differentiation, and in commissural interneuron growth in the mouse spinal cord where dysfunction of this protein leads to aberrant development of the mouse embryo. Therefore, it seems improbable that a gene essential to nervous system development could confer only a corneal phenotype when mutated in man.	(Hutchings et al., 2005)	151449
6p25	*GMDS*	GDP-mannose 4,6-dehydratase, positional candidate	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	2762
6p25	*SERPINB6*	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	5269
6p25	*RIPK1*	receptor (TNFRSF)-interacting serine-threonine kinase 1	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	8737
6p25	*BPHL*	biphenyl hydrolase-like (serine hydrolase; breast epithelial mucin-associated antigen)	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	670
6p25	*CDYL*	chromodomain protein, Y-like	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	9425
6p25	*FARS2*	phenylalanine-tRNA synthetase 1 (mitochondrial)	Ferrini et el. 2005 ARVO abstract Id of a potential KC/*PPCD* locus on 6p25.	10667
6p25	*RREB1*	ras responsive element	Ferrini et el. 2005 ARVO	6239

housekeeping and inducible genes (Dynan and Tjian, 1983; Whitelock et al., 1997). *Sp1* expression is known to vary widely among different cell types and increased expression has been associated with late stage differentiation (Edmund, 1988; Saffer et al., 1991; Saffer et al., 1990). The increase of *Sp1* was mostly observed in the epithelium of the cornea. However the specific role of *Sp1* is unclear in keratoconus, but of interest is that the binding sites of *Sp1* have been shown to be present in the regulatory sequences of many enzyme and inhibitory genes that are affected in keratoconus (Whitelock et al., 1997). The lack of any differences in expression of the other transcription factors tested in this study is interesting as two of these, *AP1* and *NF-κB*, are known to be induced under stress conditions. Their lack of expression contests some theories that environmental factors such as eye rubbing play a role in the cause and progression of keratoconus (Sawaguchi et al., 1994; Whitelock et al., 1997), although there is a great amount of evidence for both sides of the argument.

The degradative enzymes that were found to be up regulated in keratoconus corneas include lysosomal acid phosphates (LAP) acid esterase, acid lipase and cathepsins B and G (Sawaguchi et al., 1989; Zhou et al., 1998). The reduced inhibitors are α1-proteinase inhibitor *(α1-PI)* and α2-macroglobulin. *Sp1* expression may be directly related to α1-PI down regulation in keratoconus corneas (Maruyama et al., 2001). It has been suggested that an increase in the net activities of serine and cysteine proteinases that are capable of digesting gelatin, casein and extracellular matrix elements may be responsible for the thinning and loss of protein in keratoconus corneas (Zhou et al., 1998).

1.5 *Vsx1/VSX1* a gene implicated in ocular disorders

1.5.1 Chx/Vsx family

These genes belong to the paired-like class of homeodomain transcription factors and contain a homeodomain and a CVC domain. Among this class are the genes *Chx10*, *ceh-10*, *Vsx1* and Vsx2. All are known to play a part in ocular development. As the CVC domain is highly conserved in its length and its position relative to the homeodomain, it has led these genes to be further categorised as Prd-L:CVC, to distinguish them from other Prd-L genes. Due to sequence homology *Chx10* is suggested to be the mouse homologue of Vsx2 (Levine et al., 1997). Homozygous expression of *Chx10* has been associated with micropthalmia in both

mouse and human (Burmeister et al., 1996; Percin, 2001). In the retina loss of *Chx10* impairs the proliferation of retinal progenitor cells and prevents the specification, differentiation or maintenance of rod and cone bi-polar cells (Bone-Larson et al., 2000; Burmeister et al., 1996). *Vsx1* was first discovered in the goldfish retina and was found to have similarities to the *ceh-10* gene of C.elegans in the homeodomain and the 55 amino acids that follow the homeodomain termed the CVC domain (Levine et al. 1997). The function of the CVC domain is unknown but it is thought that it might have DNA binding properties or be used in protein-protein interactions. It was found to be expressed in the inner nuclear layer of the goldfish retina. *Vsx1* expression was first found in a subset of progenitor cells that give rise to cells in the outer half of the retina (Levine et al. 1997).

1.5.2 Human homologue VSX1

Two independent studies have characterised the human homologue of *VSX1*. Semina et al (2000) used a degenerate PCR approach to isolate the *VSX1* gene from a human embryonic craniofacial library. Also in the same year Hayashi et al (2000) found the same gene using a yeast one-hybrid screen of a bovine retinal cDNA library to isolate the bovine *Vsx1* and termed it RINX for retinal inner nuclear layer homeobox. They then performed RT-PCR on the human cDNA homologue of RINX to determine the human sequence. Expression analysis was carried out in both studies producing rather different results. Semina et al (2000) found that *VSX1* transcripts were found in embryonic craniofacial, adult retinal and corneal cDNAs, which was thought accurate as it corresponds well with expression of other known CVC genes. However this contrasts with the expression analysis carried out by Hayashi et al (2000) who found that *VSX1* was exclusively only found in the inner nuclear layer of the retina and did not find any expression anywhere else. This seems to be supported by Chow et al. (2001) who also didn't find any expression of *VSX1* in the cornea but only in the retina, specifically within the cone bipolar cells of the inner nuclear layer. A possible explanation for the difference in expression could be that both the work by Hayashi et al. (2000) and Chow et al (2001) were carried out on different cell lines and cDNA libraries than to that used by Semina et al. (2000). Their studies involved animal models to derive the human *VSX1* sequence as well. This difference in expression seems to be controversial with no consensus in sight without additional

work. Interestingly though expression of mouse *Vsx1*, which is thought to be orthologous to *VSX1* (Chow et al., 2001), was found in many tissue types by RT-PCR during the late stages of embryonic development (Chow et al. 2001) although this study did not look at the cornea.

1.5.3 VSX1 structure

VSX1 encodes a 365 amino acid protein and the gene comprises 5 exons (see fig 1.8). The exon-intron boundaries indicate that *VSX1* is spliced by the GT/AG splicing mechanism (Hayashi et al., 2000; Mount, 1982). The initiation codon is found in the first exon and the homeodomain is found in the second, third and fourth exons. The CVC domain is found in the fourth and fifth exons (Semina et al., 2000).

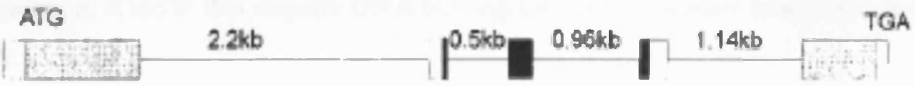


Fig 1.8. Genomic structure of the *VSX1* gene. The coding region is shown in grey, and the homeobox region is shown in black. The sizes of introns are indicated. Figure taken from Semina et al. (2000).

As with all homeodomain proteins, VSX1 contains a glutamine residue at position 50, which is characteristic of the aristaless-type paired-like homeobox proteins (Semina et al. 2000). As well as the homeodomain and CVC regions *VSX1* also contains a region of unknown function that is highly conserved between *VSX1* and goldfish *Vsx1*, termed the RV domain. There is also a proline-rich region and an acidic region that have been shown to have activation properties in the transcription factors AP-2 and CTF/NF-1. *VSX1* also seems to have a possible nuclear localisation signal (Hayashi et al., 2000). There is also a small domain that in other studies has been called the octapeptide sequence. This function of this sequence has now been elucidated as a Crm1-dependent nuclear export sequence that aids the export of the *VSX1* gene outside of the cell (Knauer et al., 2005).

1.5.4 Known functions of VSX1

Due to the expression of *Vsx1* in differentiating and mature cone bipolar cells (Chow et al., 2001) the role of *Vsx1* in visual signalling was investigated. To this end *Vsx1* knockout mice were produced in a loss of function study (Chow et al., 2004).

They found that *Vsx1* was essential for late differentiation and function of OFF-cone bipolar cells by regulating, either directly or indirectly 4 proteins expressed by OFF-cone bipolar cells, recoverin, NK3R, Netol and CaB5. *Vsx1* is critical in the function of the OFF visual pathway, however not all OFF-cone bipolar function is dependent on *Vsx1* and there is a possibility that other molecules are responsible. There is evidence to suggest there is an intrinsic regulatory feedback loop for the generation of cone bipolar cells (Ohtoshi et al., 2004). There has been speculation and some evidence that *VSX1* in humans works as a gene suppresser (Dorval et al., 2005). This study was conducted to investigate the similar transcription factor *CHX10* and found that when tethered to a promoter by its HD it was able to repress multiple classes of activators in different immortalised cell lines. As the *VSX1* gene contains closely related domains it was also able to repress transcription. It is interesting to find that the mutation R166W that impairs DNA binding hindered repressor function in this study.

1.5.5 VSX1 mapping in humans

The human homologue of *VSX1* has been mapped to chromosome 20p11-q11 by radiation hybrid mapping (Semina et al 2000). There can be no doubt as to its location as it was also mapped to 20p11.2 by Hayashi et al (2000) (See fig 1.9). Some other genes in this interval are thrombomodulin (Jackman et al., 1987) and cystatin 3 & 5 (Abalain et al., 2000; Aghamohammadzadeh et al., 2004; Freije et al., 1993). The two other genes, *VSX1* and *Tcf8* are known to be linked to ocular diseases, congenital hereditary endothelial dystrophy (CHED) (Toma et al., 1995) and *PPCD* (Heon et al., 1995).

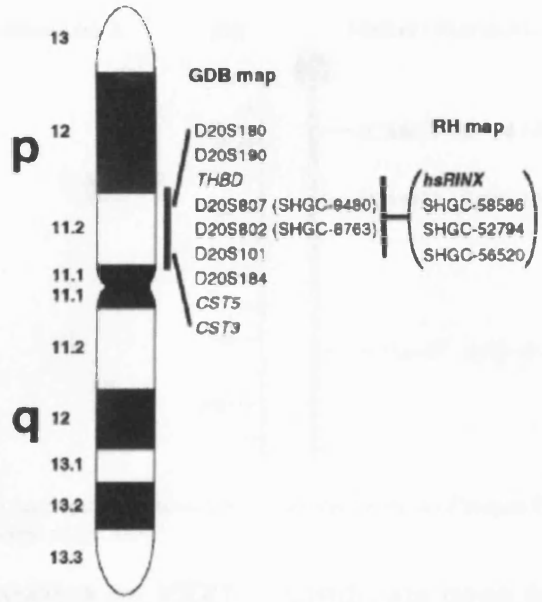


Fig 1.9. Chromosomal localization of the human *VSX1* gene. Diagram of human chromosome 20 showing location of the *VSX1* gene relative to other markers of the Stanford Human Genome Centre (SHGC) used in radiation hybrid mapping and to markers of the Human Genome Database (GDB). *VSX1* is closest to markers SHGC-58586 (9 cR ;315 kb), SHGC-52794 (11 cR), and SHGC-56520 (12cR). SHGC-58586 is located (unknown whether it is centromeric or telomeric) 35 cR from GDB markers D20S807 (SHGC-9480) and D20S802 (SHGC-8763), which have been mapped to 20p11.2. hsRINX = *VSX1*. Figure adapted from Hayashi et al (2000).

1.5.6 Vsx1 mapping in mouse

Using degenerate PCR on cDNA isolated from embryonic 7.5 day old mice *Vsx1* was mapped to the distal region on chromosome 2 of the mouse genome (see fig 1.10) (Ohtoshi et al., 2001). Mouse *Vsx1* consists of 363 amino acid residues and contains a homeodomain and a CVC domain. The gene consists of 5 coding exons that span an approximately 6.3 kb region of genomic DNA, similar to human *VSX1*. The intron-exon boundaries of mouse *Vsx1* are conserved between the mouse and human and the expression profile of mouse *Vsx1* is similar to the other homologues of *Vsx1* discovered in other species in that they are all expressed in the retina.

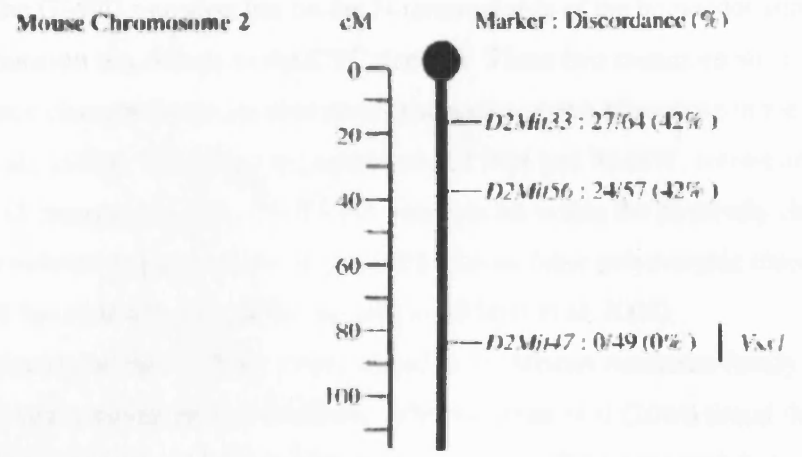


Fig 1.10. Distal region of chromosome 2 showing location of mouse *Vsx1*. Graphic taken from Ohtoshi et al. 2001

1.6 Mutational studies on *VSX1*, a candidate gene for keratoconus

1.6.1 Mutations identified within Vsx1

Linkage analysis has been performed in several studies on families with posterior polymorphous corneal dystrophy (*PPCD*), keratoconus or both, and the results of these studies are a number of loci of possible candidate genes, which are listed in Table 1. However, a link was made between keratoconus, *PPCD* and *VSX1*. Héon et al (2002) identified the first mutations in patients with *PPCD*/keratoconus by conducting linkage experiments on a cohort of families and then screening the coding sequences of *VSX1* in these patients (4 Canadian families and 2 unrelated individuals). They identified at least 4 mutations that were considered to be directly disease causing (see fig 1.11).

Case	Mutation	Phenotype	Familial disease? (no. of affected tested)	Control individuals tested (no. positive)
1	G160D[a]	PPD, ERG abnormal	Yes (5)	277 (0)
2	R166W	Keratoconus	No	277 (0)
3	L159 M	Keratoconus	Yes (4)	277 (0)
4	D144E	PPD and keratoconus	Yes (2)	277 (0[b])
5	H244R	Keratoconus	Yes (3)	277 (2)
6	P247R[a]	No clinical PPD or keratoconus, ERG abnormal	No	196 (0)

[a]Cases 1 and 6 are the mother and father of the proband of family A (Fig. 1A).
[b]The D144E was seen in one glaucoma patient (1/90).
PPD, posterior polymorphous dystrophy; ERG, electroretinogram.

Fig 1.11. Phenotype/Genotype Correlation. Table taken from Héon et al (2002).

The G160D mutation lies on the N-terminal side of the homeodomain and the P247R mutation is a change to the CVC domain. These two mutations were found to be sequence changes that in combination produced a severe phenotype in the patient (Heon et al., 2002). The other two mutations, L159M and R166W, correspond to amino acid changes and with the G160D mutation lie within the positively charged nuclear localisation signal (Héon et al. 2002). Seven other polymorphic changes were identified but were not specific for any disease (Heon et al. 2002).

New mutations in *VSX1* were isolated in an African American family who also demonstrated craniofacial abnormalities. Mintz-Hittner et al (2004) found through sequencing of patients DNA two new variations in the *VSX1* gene and characterised them to be disease causing, however this is a little speculative. For a mutation to be disease causing, the mutation has to be fully characterised and functional studies have to be performed. This was the first time that *VSX1* has been demonstrated to have a mutation in conjunction with visible craniofacial abnormalities (Mintz-Hittner et al., 2004). Clinical assessment showed that phenotypic variation between the patients varied greatly with a range of different changes: wide interpupillary distance, unusual pinnate, empty sella turcica, hypertelorism and other anterior segment disorders (Mintz-Hittner et al 2004). The patients were also found to have abnormal retinal and auditory bipolar cells, which in the case of retinal bi-polar cells are known to be reliant on correct *VSX1* expression (Chow et al., 2004). The family in the study had both keratoconus and *PPCD*. The new mutation found was A256S and it occurs in the CVC domain of *VSX1*. The other mutation found was considered a variation as it does not occur in a critical region and was seen in a few control subjects (Mintz-Hittner et al., 2004).

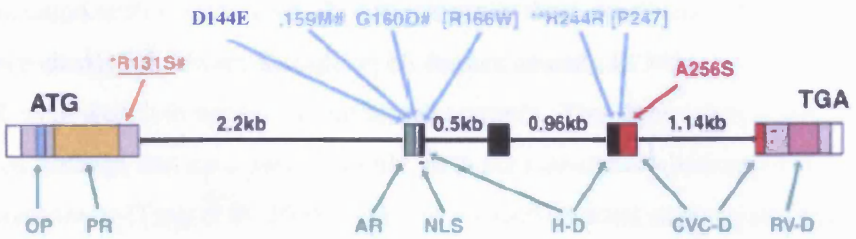


Fig 1.12. Structure of the *VSX1* gene and location of known mutations. *VSX1* (RINX) gene and mutations detected with posterior polymorphous corneal dystrophy, keratoconus, abnormal electroretinograms, or a combination thereof. Composite summary of the genomic structure of the *VSX1* gene with 5 exons. The coding region is shown in grey, and the following significant 7 regions are delineated by green letters and arrows (the amino acid numbers in parenthesis): the octapeptide sequence (OP; 31–38) is blue; the proline rich region (PR; 44–127) is yellow; the acidic region (AR; 142–152) is green; the nuclear localization signal (NLS; 162–167) is underlined; the homeodomain (H-D; 164–223) is black; the CVC-domain (CVC-D; 224–277) is red; and the RV-domain (RV-D; 322 336) is magenta. The sites of the mutations reported by Héon et al (2002) are indicated by blue letters and arrows. The variation identified by Mintz-Hittner et al (2004), is indicated by orange letters and arrow, and the site of the mutation is indicated by red letters and arrow. Mutations in brackets were isolated cases. Underlined mutations were not in critical regions. Mutations with number signs were amino acids that were not well conserved in the evolution of the documented homologous sequences. Mutations with asterisks were detected in families but also in controls (*5 of 624, **2 of 277). Note that A256S is the only familial mutation that is in a critical region, is highly conserved in evolution, and is not found in any controls. Figure modified from Mintz-Hittner et al (2004).

Another recent study has mapped more mutations to the *VSX1* gene, this time in a series of Italian families that have keratoconus. They confirmed the presence of three already described changes at D144E, G160D and P247R. They found a new mutation in 7 out of 80 unrelated patients at L17P (Bisceglia et al., 2005). This mutation is outside all critical domains. This brings the total known mutations in the *VSX1* gene thought to be directly linked to keratoconus to six, three occurring in the CVC domain (see fig 1.12).

1.6.2. Studies that have not found mutations within the Vsx1 gene

In recent years there have been a growing number of studies that have screened populations of patients for mutations in *Vsx1*. These studies however have not shown mutations in *Vsx1* leading to a certain amount of controversy as to whether *Vsx1* has been correctly associated with keratoconus and posterior polymorphous corneal dystrophy. There is mounting evidence that *Vsx1* is not associated with *PPCD* (Aldave et al., 2005; Gwilliam et al., 2005; Hosseini et al., 2008; Liskova et al., 2007a), yet the same for *Vsx1* cannot be said as there are still good arguments for

its association with keratoconus. In a recent study three previously described sequence changes that were thought to be disease causing L159M, R166W and H244R were shown to appear in unaffected controls. This does not confirm the previous findings and casts serious doubt as to the associations between these changes and keratoconus (Tang et al. 2008). Also in a recent familial study where a large family with keratoconus affected individuals were screened no causative mutations were found when compared with non affected controls (Liskova et al. 2007). A similar experiment in 100 unrelated patients failed to find any associated sequence changes and only found one previously mentioned polymorphism (Aldave et al., 2006). All the published material so far that casts doubt on *Vsx1*'s involvement suggest that other genetic factors are most likely involved but stop short at stating that *VSX1* is not associated with keratoconus.

1.7 Current treatments

The current outlook for treatments for keratoconus is promising and can be broken down into two stages; refractive management and surgery. Keratoconus cannot be cured but it can be managed up to a point before its severity forces surgery to be the only option to maintain vision for the patient. When the progression becomes too severe the only option is surgical treatment. Currently there are many options depending on the severity of the disease and they all involve strengthening the weakened areas of the cornea and slowing or halting its progression further. The standard technique is surgery that looks to replace part or the whole of the cornea although this method has its disadvantages. The newest technique that has been developed involves enhancing the rigidity of the cornea in the stroma by inducing cross links to form between the collagen fibrils. This locks the cornea in place preventing further progression. For corneal collagen cross linking the technique that has received most study is the use of ultraviolet A and riboflavin although this technique is in its infancy and is not in mainstream use.

The basic principle of the technique is to induce cross links to form between the collagen fibrils that act to harden and stabilize the tissue so that the overall shape of the cornea is prevented from degenerating further. Cross linking in this way is a widespread method that sees applications in a range of disciplines from materials

science to dentistry. The treatment works by generating reactive oxygen species (ROS) using UVA at 370nm and the photosensitiser riboflavin that induce covalent bonds bridging amino groups of fibrils (Wollensak, 2006). Clinical studies of this procedure are positive with studies encompassing over 200 patients to date indicating a halt and sometimes slight reversal of progression (Wollensak et al., 2003). In one study it was demonstrated *in vivo* that there was an increase in rigidity (Braun et al. 2005). As well as clinical studies the biochemical, mechanical and morphological effects have also been examined although more testing is probably needed. This method of treatment is the most promising to date but does require long term evaluation to see if it is going to become the treatment of choice for keratoconus patients at least until a method of reversal or identification and prevention can be found.

The largest drawback with this technique is that it works by generating ROS and the effects of this have already started to be investigated. Keratocyte apoptosis has been observed down to 300μm 1 day postoperatively and this is sometimes seen clinically by a mild cornea edema (Wollensak et al., 2004). Also the location of the induced crosslinks plays a factor in this treatment, as it is suggested that the crosslinking effect is mainly seen in the anterior part of the stroma. Although this does not pose that much of a problem in the general population of patients the treatment may not work for acute keratoconus patients as the crosslinking effect does not happen at a sufficient enough depth as was observed in single patient (Wollensak et al., 2004). As with all treatments it is not without its side effects and risks especially to the eye with the involvement of UV light. The phototoxic damage of UV light is well known however the majority of it is filtered out, with nearly all UVB light absorbed by the epithelium. As the treatment uses UVA light this risk is removed and the damage posed by UVA itself is drastically minimised in the crosslinking process with the use of riboflavin. This results in only 7% of UVA transmission across the cornea (Wollensak 2006). Experiments on rabbit corneas have demonstrated that any subsequent damage can be avoided if the stromal thickness of the cornea is greater that 400μm thick. This can be achieved by performing pachymetry on the patient before each operation.

More established operations exist although they are more invasive than collagen crosslinking. The techniques that exist all aim to either enhance/correct rigidity or involve replacing the cornea altogether. To enhance the rigidity of the

cornea intracorneal ring segments (Intacs IRCS) can be placed into the cornea. They work on the basis of acting like a splint for the cornea increasing the mechanical strength and increasing the corneal symmetry (depending on their placement) to correct the curvature of the cornea and restore vision. This method of treatment is useful for patients that are contact lens intolerant and this method of correction may delay or remove the need for a full penetrating corneal operation. This method of correcting disease is still being improved on and may take several iterations in order to get right for each patient, depending on the segment size, placement or number of segments to be used. The effect of IRCS appears to be effective through at least 2 years following their insertion (Colin et al., 2000) however there is evidence that central region thinning continues, either due to the natural progression of the disease or due to corneal stretching from segment insertion.

Once the progression of keratoconus is beyond the use of IRCS then corneal augmentation or replacement is the next solution. These methods provide a permanent replacement on the weakened corneal tissue which can be generally divided into two categories, lamellar keratoplasty or penetrating keratoplasty. Full penetrating keratoplasty is associated with good long-term outcomes with graft survival of over 90% (Pramanik et al., 2006). However the underlying reasons for the remaining 10% of graft rejection are due to removal of the corneal endothelium, which is generally unnecessary as keratoconus is a stromal disorder. An anterior lamellar keratoplasty that only replaces the front of the cornea is ideal. Currently full penetrating keratoplasty produces better post operative vision but the use of automated surgical techniques and the development and refinement of lamellar keratoplasty is making the gap smaller. It is thought that lamellar keratoplasty will eventually become the method of choice for treating the disease at later stages with the exception of corneas that have previously developed hydrops.

1.8 A summary of methods used in disease investigation

1.8.1 The use of animal models to study human diseases

One of the best (if not only) way to study genetic changes is through the use of an animal model of a disease. There are many other techniques that exist looking often at the simple interactions between two molecules, but these interactions do not take into account the environment in which they exist. It is for this reason that even the use of cell and organ cultures are not useful as cell cultures lack the complex interactions across cell types and tissues and organ cultures cannot be sustained for the long periods of time often necessary to study disease. Therefore it is left to generated animal models to provide the conditions and interactions needed to test hypotheses about disease. The generation of a specific animal model for a particular disease is very time consuming and needs thorough investigations to firstly determine whether it is an accurate model for a specific disease; then secondly to see what changes are present when matched to a healthy wild type of the same genetic backgrounds. Currently many different animals have been used as models for disease, but the main one of interest when connected with keratoconus is the mouse.

Mouse models have been developed since the early 1980's and have always been one of the favoured animal species to be used as disease models mainly due to their gestation time, size, lifespan and constant use in scientific experimentation. From a genetic standpoint, the mouse has major advantages over other experimental animals as its genome is better known than that of other species and has been fully mapped and sequenced in recent years. It can be manipulated by the modern techniques of genetic engineering and is easy, quick and relatively cheap to produce large quantities of mice for systematic studies. Thus, transgenic techniques have made it possible to study consequences of specific mutations in genes coding for structural components of ocular connective tissues in mice. As these changes in mice have been shown to resemble those in human diseases, mouse models are likely to provide efficient tools for pathogenetic studies on human disorders affecting the extracellular matrix. Despite this there are drawbacks in using mice to study corneal diseases in humans.

Some species of mice have some inbred abnormalities themselves that must be bred out in order to provide a clean background to work with, so it is much easier to

explore any new characteristics that manifest after any changes have been made to its genome. Two strains of note are the C57/black 6 strain and the 129/s1 strain (Jackson Laboratories ME). The genetic background of the mice being used is often important as they do differ slightly and some are prone to inherent conditions such as blindness.

1.8.2 Generation of mutant mouse models for disease investigation

The generation of knockout mice is important for the analysis of the function of many unknown genes. A knockout mouse or transgenic animal is generated by replacing both alleles of a particular gene with an inactive allele. This is done by using homologous recombination to replace one allele which is then followed by two or more generations of selective breeding until a breeding pair is isolated that has both alleles of the targeted gene knocked out. These knockout mice allow researchers to determine the role of a particular gene by observing the phenotype of individuals that lack the gene completely.

In many cases, the functions of a particular gene can be fully understood only if a mutant animal that does not express the gene can be obtained. Whereas genes used to be discovered through identification of mutant phenotypes, it is now far more common to discover and isolate the normal gene and then determine its function by replacing it in vivo with a defective copy. This procedure is has been made possible by two fairly recent developments: a powerful strategy to select for targeted mutation by homologous recombination, and the development of continuously growing lines of embryonic stem cells (ES cells) (Evans and Kaufman, 1981). These are embryonic cells which, on implantation into a blastocyst, can give rise to all cell lineages in a chimeric mouse.

The technique of gene targeting takes advantage of homologous recombination. Cloned copies of the target gene are altered to make them nonfunctional, for example by the introduction of a stop codon that truncates the protein making it inactive, and are then introduced into the ES cell where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a nonfunctional copy. Homologous recombination is a rare event in mammalian cells, and thus a powerful selection strategy is required to detect those cells in which it has occurred. Most commonly, the introduced gene construct has its sequence disrupted by an inserted antibiotic resistance gene such as that for neomycin

resistance. If this construct undergoes homologous recombination with the endogenous copy of the gene, the endogenous gene is disrupted but the antibiotic resistance gene remains functional, allowing cells that have incorporated the gene to be selected in culture for resistance to the neomycin like drug G418. However, antibiotic resistance on its own shows only that the cells have taken up and integrated the neomycin-resistance gene. To be able to select for those cells in which homologous recombination has occurred, the ends of the construct usually carry the thymidine kinase gene from the herpes simplex virus (HSV-tk). Cells that incorporate DNA randomly usually retain the entire DNA construct including HSV-tk, whereas homologous recombination between the construct and cellular DNA, the desired result, involves the exchange of homologous DNA sequences so that the non-homologous HSV-tk genes at the ends of the construct are eliminated. Cells carrying HSV-tk are killed by the antiviral drug gancyclovir, and so cells that have undergone homologous recombination have the unique feature of being resistant to both neomycin and gancyclovir, allowing them to be selected efficiently when these drugs are added to the cultures.

This technique can be used to produce homozygous mutant cells in which the effects of knocking-out a specific gene can be analyzed. Diploid cells in which both copies of a gene have been mutated by homologous recombination can be selected after transfection with a mixture of constructs in which the gene to be targeted has been disrupted by one or other of two different antibiotic-resistance genes. Having obtained a mutant cell with a functional defect, the defect can be ascribed definitively to the mutated gene if the mutant phenotype can be reverted with a copy of the normal gene transfected into the mutant cell. Restoration of function means that the defect in the mutant gene has been complemented by the normal gene's function. This technique is very powerful as it allows the gene that is being transferred to be mutated in precise ways to determine which parts of the protein are required for function.

To knock out a gene *in vivo*, it is only necessary to disrupt one copy of the cellular gene in an ES cell. ES cells carrying the mutant gene are produced by targeted mutation, and injected into a blastocyst which is incorporated into the developing embryo and contribute to all tissues of the resulting chimeric offspring, including those of the germline. The mutated gene can therefore be transmitted to some of the offspring of the original chimera, and further breeding of the mutant gene to produce homozygous mice that completely lack the expression of that particular

gene product. The effects of the absence of the gene's function can then be studied. In addition, the parts of the gene which are essential for its function can be identified by determining whether function can be restored by introducing different mutated copies of the gene back into the genome by transgenesis.

A second powerful technique achieves tissue-specific or developmentally regulated gene deletion by employing the DNA sequences and enzymes used by bacteriophage P1 to excise itself from a host cell's genome. Integrated bacteriophage P1 DNA is flanked by recombination signal sequences called loxP sites. A recombinase, Cre, recognizes these sites, cuts the DNA and joins the two ends, thus excising the intervening DNA in the form of a circle. This mechanism can be adapted to allow the deletion of specific genes in a transgenic animal only in certain tissues or at certain times development. First, loxP sites flanking a gene, or perhaps just a single exon, are introduced by homologous recombination. Usually, the introduction of these sequences into flanking or intronic DNA does not disrupt the normal function of the gene. Mice containing such loxP mutant genes are then mated with mice made transgenic for the Cre recombinase, under the control of a tissue specific or inducible promoter. When the Cre recombinase is active, either in the appropriate tissue or when induced, it excises the DNA between the inserted loxP sites, thus inactivating the gene or exon. Thus, for example, using a T-cell specific promoter to drive expression of the Cre recombinase, a gene can be deleted only in T cells, while remaining functional in all other cells of the animal.

1.8.3 Generation of a knockout model for keratoconus

It is easier to create a mouse model for a disease when that particular disease has a well characterised mode of inheritance established via family tree studies and linkage analysis has established a link with a causative gene. It is much more difficult to assess the potential value of a created model for a disease if the cause of the disease is complex or if you are trying to study a collection of changes that present themselves as a wrapped up syndrome or disorder. As keratoconus is such a disease creating a model for it in the past has been quite difficult. Currently, a single mouse model has been proposed for keratoconus based on the resulting mouse's clinical features (Tachibana et al., 2002) and 2 models have the potential to be disease models based

on the virtue of the gene that was knocked out in order to create them (Chow et al., 2004; Ohtoshi et al., 2004; Ramalho et al., 2004).

In 2002 a mouse model was derived for keratoconus that used an inbred line of spontaneous mutant mice with keratoconus-like corneas as progenitors for an eventual use as a keratoconus model; this was achieved through repeated sibling mating (Tachibana et al. 2002). Morphology, cell growth, apoptosis of keratocytes and protein expression was examined in the corneas of these mice. Androgen dependency was also examined in this model as it was observed that the keratoconus phenotype was observed almost exclusively in sexually mature male mice. The phenotype in these mice is inherited in an autosomal recessive manner and the link with androgen dependency was investigated. In order to test their hypothesis male mice underwent castration and female mice were injected with testosterone at approximately 4 weeks of age. This resulted in female mice displaying the keratoconus phenotype at a much weaker level then in their male counterparts. However keratoconus did not develop in the castrated males. Macroscopically corneas were graded for their appearance and to the extent that a cone formed. It was found that at the early stages the corneas became cloudy and was then followed by protrusion. The changes seen appeared to be only occurring in the males with few female mice developing cones. Histological examinations revealed that the central regions of the cornea in the males were often thinned although they ranged and could also be edematous and thick. Where thinning was observed it was mainly due to a decrease of thickness in the stroma. Expression of c-fos was enhanced in these corneas mirroring the findings in human keratoconic patients. Collagen fibril diameters and interfibrillar spacings were examined in these mice and found to be slightly larger than wild type controls (Quantock et al. 2003).

1.8.4 Vsx1 knock-out mouse model

Recently two knock out mouse models for *Vsx1* have been created to study the effects of silencing the expression of the *Vsx1* gene (Chow et al., 2004; Ohtoshi et al., 2004). The mouse model created by Chow et al. was initially created to determine the function of *Vsx1* in the formation of the retina as previous expression studies indicated that it was expressed in the inner nuclear layer. Although this model was created with the retina in mind it is a valuable tool in assisting any investigation where this gene is concerned. However at present no corneal defects have been reported in the *Vsx1* null

mice (Chow et al. 2004) but no thorough examinations or studies were carried out. This leaves a great opportunity to further study this model.

In generation of the null mice gene targeting by homologous recombination was performed in R1 embryonic stem cells using established techniques (Chow et al. 2004). Targeting constructs were derived from the pPNT vector containing a neomycin resistance cassette for G418-mediated positive selection and a thymidine kinase cassette for gancyclovir-mediated negative selection of embryonic stem cell homologous recombinants. After homologous recombination a mutated *Vsx1* allele lacking 359bp from exon 1 and all of exons 2-4 was created. This completely eliminates the homeodomain and the first 46 amino acids of the CVC domain rendering the gene a complete null (Chow et al. 2004). Chimeric mice were generated by blastocyst injection of homologous recombinant embryonic stem cells and the resulting mice were bred with either the 129/SvImJ mouse strain or the outbred Black Swiss mice (Chow et al. 2004).

The *Vsx1* null mice produced demonstrated no behavioural or neurological defects and in contrast with human missense mutations the null mice had no corneal abnormalities grossly or at the histological level using light microscopy and EM (Chow et al. 2004). Corneal tissues from two month old *Vsx1* KO and WT controls were examined by haematoxylin/eosin staining and transmission electron microscopy. No significant changes were observed in the corneal epithelium, stroma or endothelium by light microscopy and no changes were observed under electron microscopy. However it should be noted that the areas examined by electron microscopy was the centre of the cornea (personal communication, Dr Chow) and this is not the site where one would expect any structural defects to occur in keratoconus as they are mostly seen in the paracentral regions in human keratoconics. Also with the inherent thinness of murine corneas the thinning that is observed with keratoconus corneas in humans may also not be apparent. Publications on the other mouse model were released a month after the KO model produced by Chow et al. This mouse model was also created to look at *Vsx1* in retinal expression and two mouse mutants were created; the first replacing the homeodomain and CVC domain with a loxP sequence. The second mutant was created by replacing part of the homeodomain with a lacZ expression cassette. They too observed that *Vsx1* is necessary for late stage differentiation of cone bipolar cells (Ohtoshi et al. 2004). The mechanisms offered to explain the regulation of off cone bipolar cells is that *Vsx1* could be part of a negative

feedback loop acting as a self regulator. Examination of the cornea in this model was not included in the phenotyping

1.8.5 Studies on the cornea using X-ray diffraction

X-ray diffraction is a technique that allows a detailed description of the macromolecular structure of a substance if it has structural regularity. If X-rays are directed against a regular array of scattering units then diffraction occurs. The diffracted X-rays can then be used to determine information about the scattering unit. For example if the scattering unit was cylindrical then the scattering pattern obtained can be used to tell the orientation of that unit (See fig 1.13).

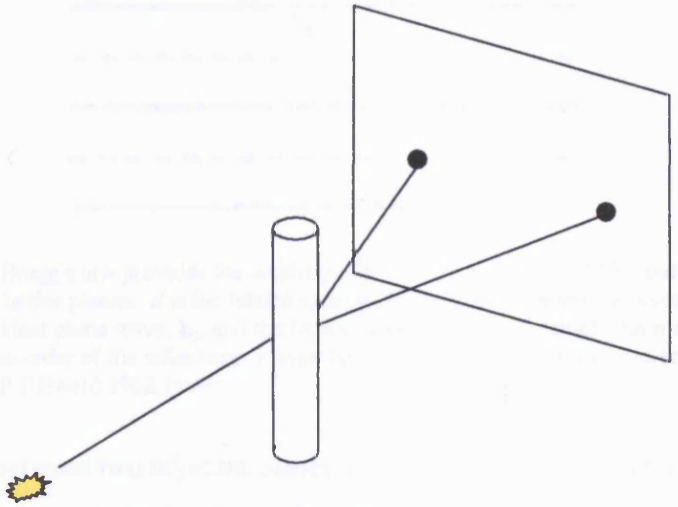


Fig 1.13. A theoretical diffraction pattern from a single cylindrical scattering unit. The yellow star represents a source of electromagnetic radiation. The cylinder the scattering object and the 2 black dots on the square the diffraction pattern on a detector.

This diffraction pattern would occur if the distance between the scattering units in a structure (e.g. collagen tissue) is the same order of magnitude as the wavelength of the waves. As X-rays are small enough they can be used to resolve structures at the nanometre scale. This method has many applications especially in finding out crystal structures as crystals are regular arrays, or the arrangement of molecules on less well ordered fibrous structures. In this report this method is used to examine the fibril structures in the corneal stroma. Figure 1.12 is an example of a diffraction pattern obtained if a single diffracting structure (collagen fibril) were being examined. If the cylinder in fig 1.12 were rotated in a plane at right angles to the x-

ray beam, the positions of the diffraction spots (reflections) would rotate similarly. If there were cylinders at different angles each would diffract independently, so the diffraction spots would be smeared into arcs, the degree of arcing representing the angular distribution of the cylinders.

Incoming X-rays make an angle θ with the diffracting planes and are reflected at an equal angle θ (see fig 1.14).

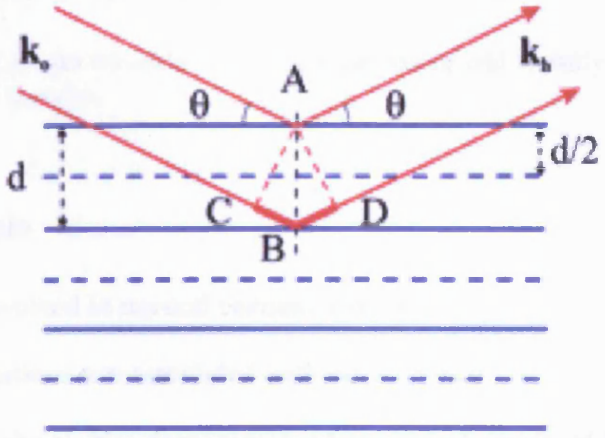


Fig 1.14. Bragg's law provides the condition for a plane wave to be diffracted by a family of lattice planes. *d* is the **lattice** spacing, θ the angle between the wave vector of the incident plane wave, $\mathbf{k_o}$, and the lattice planes, λ its wave length and *n* is an integer, the order of the reflection. Figure taken from www.wikipedia.com/braggs law and (P.P Ewald 1962,1999)

If the distance between two adjacent planes is d, constructive interference occurs when the difference in optical path length is an integer multiple of the wavelength.

The maxima (spots) are observed in the direction θ given by:
$2d \sin\theta = n\lambda$
λ – wavelength of the X-ray beam
d – the plane distances

This is the Bragg relationship. It shows that for a fixed wavelength, there is an inverse relationship between spacing and diffraction angle. Thus structures with larger spacings (such as collagen fibrils) diffract at small angles and structures with small spacings (such as collagen molecules) diffract at wide angles. In this report, X-ray diffraction is used to measure the values of (d) corresponding to the intermolecular separation within collagen fibrils, the centre-to-centre distance between collagen fibrils, the average fibril diameter and orientation.

1.9 Aims of thesis

- To provide data on the development of the mouse cornea using various investigative techniques with focus on a few key genes of interest.
- To test the idea that keratoconus has a genetic cause through use of existing animal models and investigative techniques.
- To screen a local population of keratoconus patients to investigate whether mutations in *Vsx1* can be found.
- To expand on the possible causes of keratoconus and identify any potential targets for therapy.

1.10 Hypothesis

1. ***Vsx1* is involved in normal corneal formation**
2. ***Vsx1* mutations are associated with keratoconus**
3. ***Vsx1* Knockout mice demonstrate phenotypic features of keratoconus**

Chapter 2: General materials and methods

2.1 Semi-quantitative PCR

PCR was carried out using 12.5µl of PCR BioMix™ Red kit (Bioline, UK). The reaction kit comprises BIOTAQ™ DNA Polymerase, 2mM dNTPs, 32mM (NH4) 2SO, 125mM Tris-HCl (pH 8.8 at 25°C), 0.02% Tween 20, 4mM MgCl2, Stabilizer and an inert dye. To this was added 2µl of DNA and 1.5µl 20µM of both forward and reverse primers. The total reaction volume was brought up to 25µl with the addition of 8µl of sdH2O and then the samples were vortexed and spun down. They were then placed in a thermocycler for 35 cycles of denaturing at 97°C, annealing and strand extension at 72°C. Annealing temperatures as well as sequence for primers are given separately in each chapter. PCR products were then stored in the fridge or held at 4°C until separated via agarose gel electrophoresis.

2.2 Agarose gel electrophoresis

PCR product samples were separated on either a 2% agarose gel prepared by adding 4g of agarose to 200ml of 1xTBE buffer. Products were stained by ethidium bromide (2 µl, 10mg/ml) and separated in TBE buffer at 150 volts for ~ 40 minutes. Quantification of bands and size estimates were made possible by running out 7µl of DNA Hyperladder™ IV (Bioline, UK) on each gel.

2.3 Quantitative RT-PCR

Quantitative PCR was carried out on the Roto-Gene™ (Corbett Research) in a similar fashion to semi-quantitative PCR described above. The difference lies in the detection method and incorporation of a fluorescent dye that monitors the level of product in real time as the reaction is running. In all reactions cDNA was amplified using Sigma SYBR® Green Jumpstart™ Taq ReadyMix™ (20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl2, 0.002% gelatin, 0.4 mM of each dNTP, stabilizers, 0.06 unit/ml *Taq* DNA Polymerase, JumpStart *Taq* antibody). The reaction mixes consisted of 2µl of cDNA, 1.25µl 20µM of the forward and reverse primers respectively, 12.5µl of the ready mix and 8µl of dd H_20 to bring the reaction to a total

volume of 25µl, which is half of the recommended manufacturer's instructions. The most important part of quantitative PCR is in the analysis of the product. There is an initial hold of 95°C for 2 minutes followed by denaturation at 95°C, annealing and extension at 72°C. Annealing temperatures are dependent on primer sequences and are given in the relevant methods sections in subsequent chapters.

2.3.1 Establishing which method to use for analysis of amplified products

The quantification of gene-specific mRNA expression is one of the major issues in life sciences research. Quantitative real-time RT-PCR (qPCR) has been widely used, allowing fast, accurate and sensitive mRNA quantification with a high throughput of samples. Unfortunately, the issue of real-time PCR data analysis is often underestimated by researchers leading to inaccurate expression levels. The two most commonly used methods of data analysis for the expression of genes are the standard curve method and the comparative C_T cycle threshold ($2^{-\Delta\Delta C_T}$) method. Both of these methods have their advantages and disadvantages, which is why other researchers have tried to look at better methods for data analysis. A third method called the comparative quantification method (Ramakers et al., 2003) was also considered for the data analysis.

The standard curve method of qPCR data analysis uses a standard curve of known concentrations for each gene that is being examined and the housekeeping gene. This absolute method of quantification looks to establish the copy number of an amplicon when compared to the standard curve. The disadvantages of this method is that it is very resource consuming as standard curves must be used for each time the target sample is amplified. There is also a need to have consistent and repeatable standards that do not reflect the true nature of sample amplification (see fig 2.1).

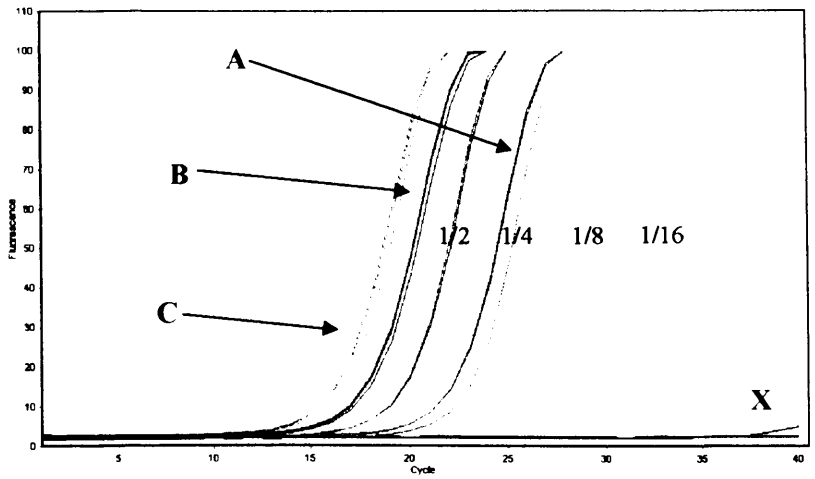


Fig 2.1. Raw expression curves of *Aqp5* at postnatal days 5, 12 and 17 in murine corneas. A - expression at p5, B - expression at p12, C - expression at p17. Numbers indicate dilution factors of the standard curve. X – NTCs and no RT controls.

The expression curves from each sample are then used to form a standard curve in order to determine the concentration of the unknown samples being tested. It is important for this do have a low standard deviation in order to produce a standard curve with good efficiency (see fig 2.2).

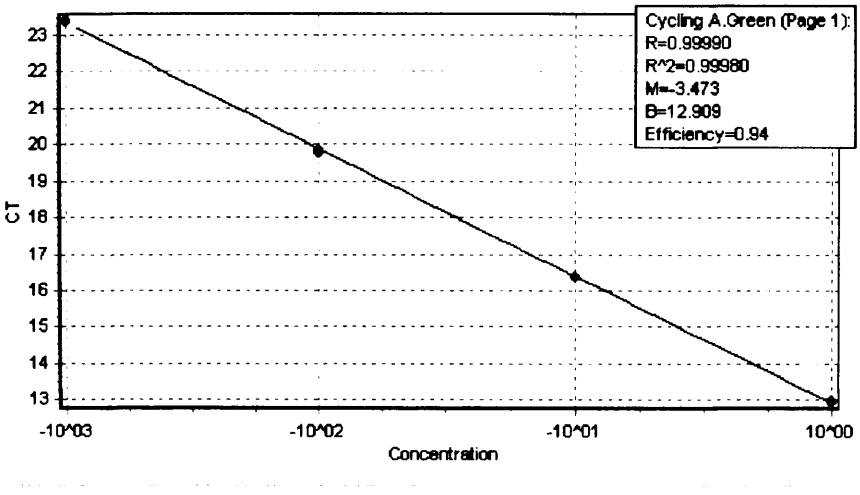
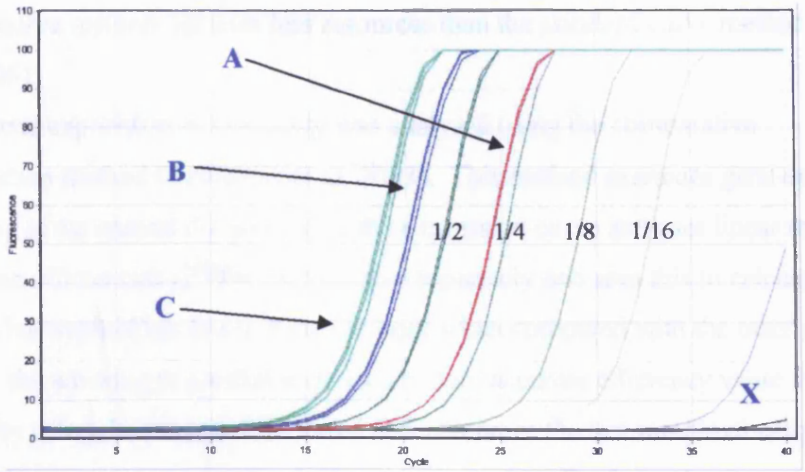


Fig 2.2. Standard curve formed from the raw expression curves of *Aqp5*

The second method is the $2^{-\Delta\Delta C_T}$ method. The main advantage of this method is that only one set of standard curves needs to be prepared and can be used for multiple amplifications. This method always assumes perfect PCR conditions and the efficiency to always be 100%, which is not the case. This assumption of efficiency leads to an over estimation of expression and is therefore less accurate than the



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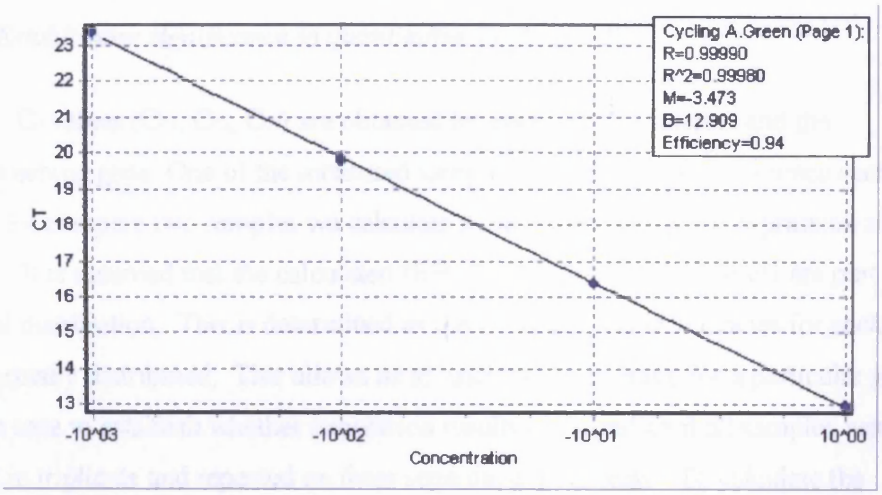

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standard curve method but uses less resources than the standard curve method (Schefe et al., 2006).

Gene expression in this study was analysed using the comparative quantification method (Ramakers et al. 2003). This method examines gene expression by looking at the second differential of the expression curve and uses linear regression to establish efficiencies (E) for each sample separately and uses this to calculate the C_t values. This method has fewer disadvantages when compared with the other two methods; the advantages are that it obtains a more accurate efficiency value for each reaction by using the second differential and compares the test sample directly to the control. There is no need for standard curves and take off values are more consistent. This means that less tissue is needed, which is a major advantage in this study as the amount of tissue is very limited. It should be noted that in all RT-PCR reactions three time points are used: postnatal days 5, 12 and 17. For each experiment data is firstly normalised to the housekeeping gene then calibrated to the lowest time point, which in all cases is postnatal day 5. This time point is assigned a value of 1 and all subsequent reaction values for other time points are ratios to it.

2.3.2 Establishing significance in quantitative PCR (qPCR)

C_T values (C_{T1}, C_{T2}, C_{T3}) are obtained for each gene of interest and the housekeeping gene. One of the measured samples is defined as the reference sample (ref). To compare two samples we calculate three different relative expression ratios rERs. It is assumed that the calculated rERs for one sample-of-interest are part of a normal distribution. This is determined as the C_T values and efficiencies for each gene are normally distributed. This allows us to calculate an average for a particular gene. In this case to establish whether expression results are significant all samples were tested in triplicate and repeated on three separate experiments. To calculate the significance of expression an unpaired 2-tail *t test* was applied. Significance was established to be $P<0.05$.

2.4 Solution preparation

2.4.1 4% paraformaldehyde

8g of solid PFA was added to 100ml of 1xPBS in a conical flask. This was then placed on a heated stirrer for 2 hours making sure the heat does not exceed 65°C. If the PFA has not entered the solution then sodium hydroxide was added drop-wise to the solution until it became clear. This was then filtered into a fresh conical flask. pH was adjusted to 7.4 and the solution was placed into aliquots and frozen at -20°C for future use. This method generates 8% paraformaldehyde so to use at its final concentration of 4% it is diluted with fresh 1xPBS on a heater stirrer as before.

2.4.2 Phosphate buffered solution

PBS was made from the tablet form available from according to the manufacturer's instructions.

2.5 Small angle X-Ray Data Collection

Small-angle X-ray diffraction (SAXS) patterns were collected on Station 2.1 at the UK Synchrotron Source (Daresbury, UK), using a 9m-long camera equipped with a multiwire gas detector. The X-ray beam had a wavelength of 0.154 nm and a cross section at the specimen measuring 0.5 mm vertically and 1 mm horizontally. Specimens were weighed before and after measurement to determine the difference in wet weight and ensure that samples did not become dehydrated during X-ray exposure. Samples were placed in airtight Perspex (Databank, UK) chambers with Mylar (DuPont-Teijin, UK) windows to further minimize tissue dehydration and keep the sample in a fixed position. Incident X-rays were passed through the anterior corneal face parallel to the optical axis. Single SAXS patterns were gained from the centre of each sample. If there was any ambiguity in position due to the size of the sample a small 0.1mm correction was made and a second SAXS pattern gained. The pattern that measured the largest interfibrillar reflection value was then used. Exposure time per data point was 3 minutes.

2.6 Small angle X-Ray Data Analysis

X-ray patterns were gained from the centre of each of the samples. X-ray intensity profiles were measured along a vertical transect through the centre of each pattern because this was the direction in which the x-ray beam was most finely focused. Processing of samples was then carried out in 3 steps. Firstly a square-power law background curve was fitted to and subtracted from the experimental data. This removed scattering from stromal matrix components other than fibrillar collagen.

Secondly assuming cylindrical collagen fibrils, small-angle equatorial X-ray diffraction from a corneal lamella can be approximated as the fibril transform (i.e., scattering from a single fibril), multiplied by the interference function (deriving from the ordered arrangement of the cylinders). A theoretical fibril transform (Bessel function) was fitted to the experimental data by varying two parameters: the fibril radius and an arbitrary scaling factor.

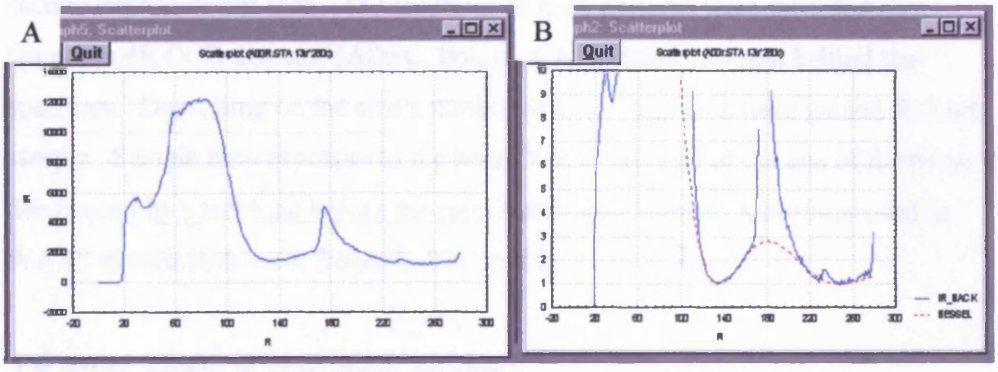


Fig 2.2. A) Linear profile of scattering from an X-ray pattern. B) Bessel function applied to remove the background scatter.

Fitting was performed on the first subsidiary maximum of the experimental data, because this peak derives entirely from the fibril transform, with no significant contribution from the interference function (Worthington and Inouye, 1982). The experimental data was then divided point-for-point by the calculated fibril transform to leave the interference function, whose peak position is determined by the average interfibrillar Bragg spacing.

Thirdly the interfibrillar Bragg spacing and fibril radius were calibrated from the position of the 67-nm meridional reflection from a diffraction pattern of hydrated rat tail tendon and finally the average center-to-center interfibrillar spacing (i) was calculated from the interfibrillar Bragg spacing (p) with $i = 1.12p$, where 1.12 is a

packing factor that assumes that the arrangement of fibrils in a lamella approximates the short-range order of a liquid (Boote et al. 2003).

2.7 Wide angle X-Ray Data collection

Wide angle X-ray diffraction (WAXS) data was collected at the UK Synchrotron Radiation Source (Daresbury, UK) at the 14.1 station using a 200μm x 200μm X-ray beam of wavelength 0.1488 nm at right angles to the cornea. Rasta scans were made of the entire surface of the cornea at 0.2mm intervals. In order to further minimise tissue dehydration during X-ray exposure, wrapped corneas were positioned in an airtight Perspex (Databank, UK) chambers with Mylar (Dupont-Teijin, UK) windows. X-ray beam position was determined before samples were examined. Rasta scans of the entire cornea were performed using a specimen translation stage (Newport, UK), interfaced with the X-ray camera shutter for 60 second exposures per scan. The resulting WAXS patterns were recorded on a Quantum 4R CCD detector (ADSC, Poway, CA) located 150 mm behind the specimen. Depending on the size a minimum of 100 patterns were gained for each sample. Sample time exposure to the beam was dependent of the age of the tissue and determined by a test scan before the rasta scans were begun. All tissues used in WAXS experiments were frozen before testing.

2.8 Wide angle X-Ray data analysis

A typical WAXS pattern from a mouse cornea is shown in fig 2.3. It features lobes of intensity that are X-ray reflections formed by interference between X-rays scattered by the regularly arranged collagen molecules lying near-axially within the stromal fibrils (Meek and Quantock, 2001). The X-rays used penetrates the entire thickness of the cornea so the scattering that is detected is the sum is from fibrils from each lamella in the path of the beam. Scattering of X-rays occurs at 90° to the direction of the fibril so each single stromal lamella will produce a pair of diffraction spots either side of a line representing the long axis of its constituent fibrils. If the stacked lamellae in the stromal had a homogenous or unordered arrangement, a circular X-ray scattering pattern of uniform intensity would be produced at the

appropriate angle defined by Bragg's Law. If lamellae have preferred orientations, as in figure 2.3, the intensity around the scatter pattern will vary.

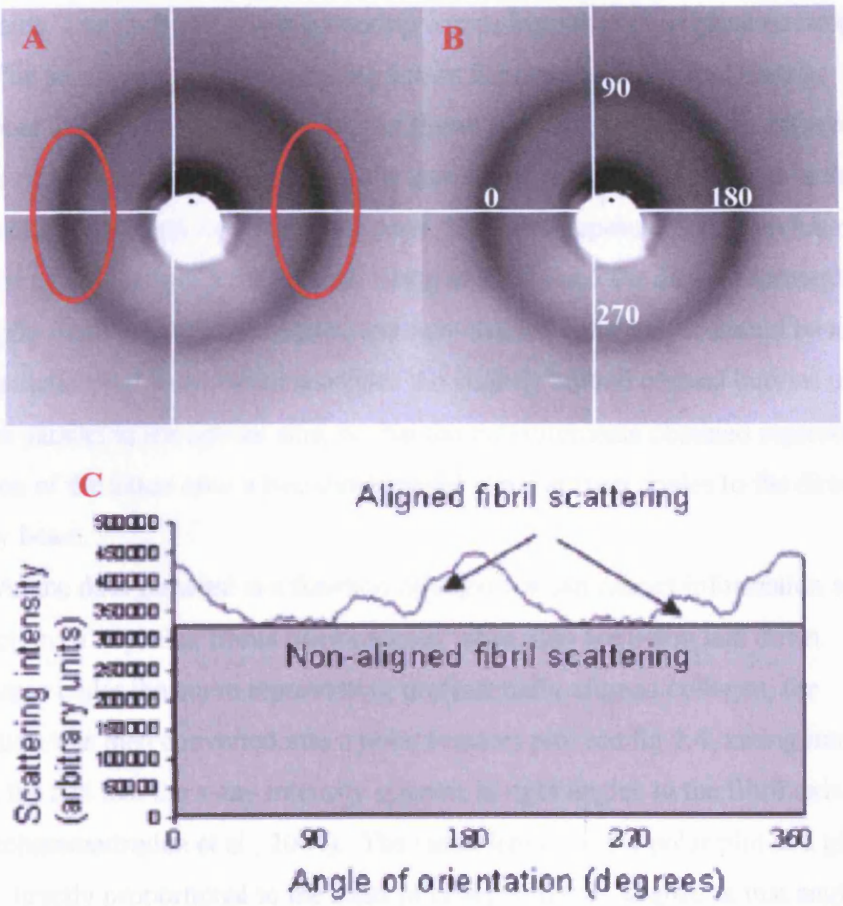


Fig 2.3. A and B). A typical WAXS pattern from a mouse cornea. The lobes of intensity arising from X-ray reflections perpendicular to the alignment of fibrils are outlined in A. C) The intensity as function of angle. Intensity profile from (Meek et al., 2003b)

By measuring the distribution of intensity of the scatter pattern around the detector as a function of angle we can quantify firstly the relative mass of collagen at that specific point in the tissue and secondly determine whether the fibrils at that point in the tissue show any degree of preferential fibril alignment. It has previously been shown that mouse corneas exhibit a circular pattern of arrangement although it appears to be quite variable. For each WAXS pattern, the normalised intensity profile was obtained using Optimas 6.5 (MediaCybernetics, UK) image analysis software and Excel (Microsoft, UK) spreadsheets. Starting at 0° at the left of the reflection, the intensity of the scattered x-rays was measured as a function of angle. The result is a

linear intensity profile of these reflections as shown in fig 2.3. The total area under the graph (the total scattered intensity) is proportional to the total mass of fibrillar collagen in the path of the X-ray beam, and this may be subdivided into two components. The first part of the scattering comes from the non-aligned (isotropic) fibrils. The second part of the scattering shows the mass of fibrils that have a preferential alignment of fibrils as seen in figure 2.3. By integrating the respective areas we can get a relative measure of the amount of collagen at that point in the tissue broken down into its constituent parts. This was repeated at 0.2mm intervals across the entire surface of the corneas being analysed and the data is represented as topography maps for both the aligned and non-aligned collagen. It should be noted that, in practice, the X-ray beam traverses the slightly curved corneal buttons in a direction parallel to the optical axis, so that the measurements obtained represent a projection of the tissue onto a two-dimensional plane at right angles to the direction of the x-ray beam.

As the data gathered is a function of angle we can extract information about the direction (if any) that fibrils have adopted when they are being laid down. Taking just the area under the curve representing preferentially aligned collagen, the distribution was then converted into a polar (vector) plot see fig 2.4, taking into account the fact that the x-ray intensity appears at right angles to the fibril axis (Aghamohammadzadeh et al., 2004). The radial length of the polar plot at a given angle is directly proportional to the mass of collagen fibrils aligned at that angle. The overall size of the plot is therefore related to the total mass of aligned collagen, and the asymmetry gives an indication of the preferential direction(s) of the collagen at that point in the tissue. Polar plots were produced from the scattering patterns obtained from each point of the cornea scanned, and were subsequently arranged according to their geometric positions on the cornea giving a directional map of the collagen across the entire corneal surface.

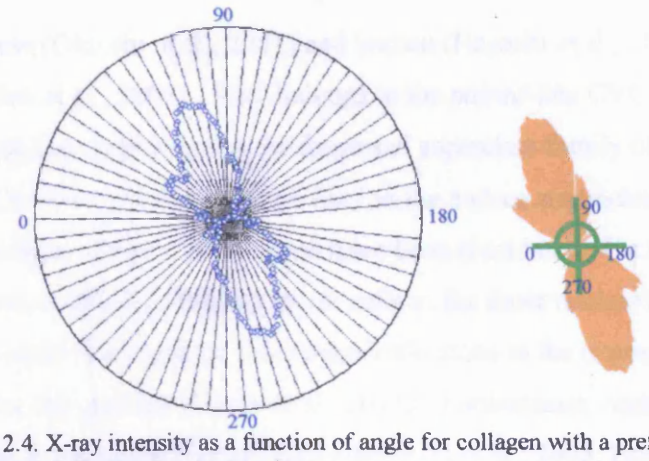


Fig 2.4. X-ray intensity as a function of angle for collagen with a preferential alignment displayed as vector plots

Chapter 3. Mouse corneal development

3.1 Aim

- To profile the expression changes of several genes over postnatal murine corneal development including the *paired-like*:CVC gene murine homologue *Vsx1* and the collagen gene *Col8A2*.

- To profile the changes in collagen mass, distribution and fibril alignment in developing murine corneas in order to provide the background necessary for future genetic studies.

3.2 Introduction

It is important to establish a baseline for any kind of study so that the effects caused by induced changes, if any, can be identified. The production of mouse models in studying disease is an invaluable tool for investigating disease and offers possibilities to discover the first signs of a disorder at a very early stage. One of the problems that exist with identifying and treating keratoconus is that by the time the symptoms appear, the progression of the ectasia is already at a fairly advanced stage. Therefore a study of the development of the cornea is necessary in categorising the changes that naturally occur so that, later, disease models can be examined for any anomalies.

Vsx1 is a gene that codes for a homeodomain transcription factor and was originally characterised in goldfish (Passini et al., 1997) and found to be expressed during retinogenesis. Other orthologous sequences have since been found in the zebrafish (Passini et al., 1998), chicken (Chen and Cepko, 2000), cow (Hayashi et al.,

2000), mouse (Ohtoshi et al., 2001) and human (Hayashi et al., 2000; Ohtoshi et al., 2001; Semina et al., 2000). *Vsx1* belongs to the *paired-like*:CVC class of homeobox genes, which in turn belongs to the dispersed superclass family of homeodomain proteins. Currently the functions of *Vsx1* in the cornea are unclear. However the known functions of *Vsx1* in the retina have been elucidated. *Vsx1* is crucial for the development of off-centre cone bipolar cells in the inner nuclear layer as the absence of expression of *Vsx1* leads to substantial reductions in the expression of at least 4 cone bipolar cell proteins (Chow et al., 2004). Furthermore, mutations in *VSX1* have been linked to both keratoconus (KC) (Bisceglia et al., 2005; Heon et al., 2002; Mintz-Hittner et al., 2004) and posterior polymorphous corneal dystrophy (*PPCD*) (Heon et al., 2002; Valleix et al., 2006). The expression profile of *Vsx1*has mainly been investigated in the murine eye; currently the earliest detection of *Vsx1* has been at embryonic (E) day E14. Current data mostly focuses on the expression of *Vsx1* in the retina (Chow et al., 2001; Hayashi et al., 2000; Ohtoshi et al., 2001) but it is also expressed in the cornea (Krafchak et al., 2005).

As described previously keratoconus is a major ocular disease in the western world and the main candidate for corneal surgery (Atilano et al., 2005). It is a non inflammatory, progressive corneal thinning disease leading to myopia and irregular astigmatism. As yet there is no known cure for keratoconus other than corneal grafting; however numerous studies are being carried out into the structure of keratoconic corneas and its developmental mechanisms. There is evidence that keratoconus is inherited in some patients and families and that the cause in all patients could have a genetic contribution. Current research suggests a number of possible genetic aetiologies for keratoconus caused by the interplay of environmental factors and different mutations in certain genes that lead to a predisposition for its development. Keratoconus probably develops in early life and is usually leads to clinical symptoms within the first few decades. This suggests that normal developmental mechanisms that stabilise the cornea within the first few years after birth, fail to occur in keratoconus. There is therefore an argument that studies need to be made in the developing stages of the cornea. Treating a cornea once the clinical symptoms have manifested can also be argued is too late in order to provide the best treatment. With the introduction of mouse models to disease it was thought necessary to establish a control characterising the structure of a normal developing cornea. This would allow any future mouse models for the disease to have a measure of what is

normal so that any changes noted could be better defined as having arisen from the mutation. With mouse models there is an advantage in that the latter stages of their corneal development happen postnatally and one of the most crucial times in mouse corneal development is during eye opening.

Eye opening in the mouse (P12-14) is preceded by large developmental changes in the cornea in preparation for interactions with the outer environment. Changes that have been characterised during this period include an increased proliferation of epithelial cell layers, a significant increase of stromal thickness and a decrease in stromal density (Song et al., 2003). The endothelium does not undergo as much change as the other layers. This critical time in development provides an excellent opportunity to examine gene expression and structural changes. The increase in activity would indicate that a number of genes are active and available for detection that would be otherwise difficult to do in an adult system. To investigate whether *Vsx1* and other genes have a role in the cornea during this period their expression profiles were examined using quantitative RT-PCR. X-ray diffraction was used to determine how the mature structure of the cornea develops and to look at how the collagen organises itself throughout the developmental process. The expression of *Vsx1* in other tissues was also investigated to confirm what had already been published.

3.3 Methods and materials

3.3.1 Tissue collection and preparation

For quantitative PCR 129/S1 wild type (WT) were raised within the lab with a 14 hour light cycle and standard diet. 129/S1 mice were chosen over other strains of mice as the *Vsx1* KO mouse produced my Chow et al. 2004 used this strain. As this KO model is examined later in this thesis developmental studies were conducted using this strain in order to make direct comparisons. Whole eyes, brain, liver, lung and heart from 4 mice were extracted and placed in RNAlater® (Ambion, Inc.) at postnatal (P) days P5, P12 and P17 of development. Corneas at each time point were dissected out and placed in RNAlater® and frozen for further isolation. For X-ray scattering experiments whole murine eyes of 4 wild type 129/S1 (Jackson Laboratories) mice were isolated and the cornea rapidly excised and wrapped in clingfilm at 10-28 postnatal days of development. Excised corneas had their

orientation maintained throughout removal and the 12 o'clock position and epithelial side were identified and marked using a surgical suture. Corneas upon removal were immediately wrapped in Clingfilm™ and frozen at -80°C for storage until future examination. Corneas were weighed before and after experimentation with no discernable decrease in weight. Mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.3.2 RNA isolation, primer sequences and product amplification by QPCR

Total RNA was isolated using the RNEasy® Mini kit (Qiagen) according to the manufacturer's protocol and extracted RNA was quantified using spectrophotometry. RNA was separated into aliquots to avoid the need of repeated freeze/thaw cycles and RNA was transcribed to cDNA using the Quantitect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. cDNA was amplified using Sigma SYBR® Green Jumpstart™ Taq ReadyMix™ for Quantitative PCR on the Roto-Gene™ (Corbett Research). Isolated RNA was pooled for each time point and three sets of pooled RNA were examined and repeated three times. In order to ensure similar starting product concentrations each RNA sample was measured and then aliquoted so that each subsequent reaction was optimised for an RNA concentration of 0.5µg. Genes were amplified using cDNA specific primers for the following genes:

Name	**Primer sequence 5' - 3'**	**tmp °C**
Vsx1 F	5'-ACTGCCCAGTTACCCACAAT-3'	55
Vsx1 R	5'-AAGTGGCGTAAGCGAGAGAA-3'	55
Aqp5 F	5'- ATCTACTTCACCGGCTGTTCC-3'	60
Aqp5 R	5'- GTCAGCTCGATGGTCTTCTTC-3'	60
Aqp1 F	5'-GAAGAAGCTCTTCTGGAGGGCTG-3'	60
Aqp1 R	5'-GGCTTCATCTCCACCCTGGAGTT-3'	60
Col8a2 F	5'-GGTAAAGTATGTGCAGCCCA-3'	60
Col8a2 R	5'-AGTAATACCTGAGGGACCAG-3'	60
RpL19 F	5'-GGGAAGAGGAAGGGTACTGC-3'	55
RpL19 R	5'-GGACGCTTCATTTCTTGGTC-3'	55

Table 2. Primer sequences used for amplification, F- forward, R – reverse, tmp – temperature. All primer sequences are 5' to 3'.

Rpl19 was used as a housekeeping gene for normalization and all primer sequences were designed to be intron spanning. Primers were designed using the Primer3 program (Rozen and Skaletsky, 2000) to the *Vsx1* sequence accession # NM_054068. As significance is being compared between three different time points a standard t-test does not apply so a one way analysis of variance (ANOVA) test was used. Significance was determined to be $P<0.05$.

3.3.3 Conditions for Semi quantitative PCR

Name	**Primer sequence 5' - 3'**	**tmp °C**
Vsx1 F	5'-ACTGCCCAGTTACCCACAAT-3'	55
Vsx1 R	5'-AAGTGGCGTAAGCGAGAGAA-3'	55

Table 3. Primer sequences used for amplification of *Vsx1* for Semi quantitative PCR, F- forward, R – reverse, tmp – temperature. All primer sequences are 5' to 3'.

All other conditions were the same as described in the methods chapter.

3.3.4 Band densitometry for semi quantitative PCR

Band densitometry was carried out using the Scion Image© program distributed by Scion. A mask of a fixed size was placed over a PCR fragment band of a digital picture of the gel taken using a UV gel dock. Once the mask was placed over the band an average intensity was measured. This measurement was then corrected by removing the background; this was achieved by taking a measurement of a blank part of the gel at an equivalent level of the band being measured. The subsequent value was then removed from the measurement of the PCR product band to give its final intensity. This was repeated for all the bands on the gel, and an average value for 3 repeats generated to give the overall value of intensity for that sample.

3.3.5 Histology

Mouse whole globes were mounted in wax as described above. For histology sections were de-waxed in xylene for 2 x 5 mins then re-hydrated through a graded series of alcohols until free of wax (100% ims 20s, 100% ims 20s, 90% ims 20s, 70% ims 20s, 50% ims 20s). After this sections were place under running water for 1 minute and immediately afterwards placed in haematoxylin for 5 mins. Sections were then rinsed in water until the water ran clear then immersed in eosin for 1 minute. Sections were rinsed again then quickly taken back through the alcohol gradient (50% ims 20s, 70% ims 20s, 90% ims 20s, 100% ims 20s, 100% ims 20s) until free of water. Finally the sections were immersed in xylene for 20s then mounted as described previously. Treated sections were then viewed under a light microscope. Sections were taken from the central region (0.5mm) of the cornea.

3.3.6 SAXS data collection

Data was analysed as described previously in the Methods chapter. Six corneas were isolated for each time point.

3.3.7 SAXS data analysis

Data was collected as described previously in the Methods chapter however due to the noisy nature of mouse data the theoretical fibril transform was not fitted. Instead, a secondary square power law background curve was fitted thus eliminating the background in 2 stages.

3.3.8 WAXS data collection

Data was collected as described previously in the Methods. Exposure time for each pattern was 60 seconds and a step size of 0.2mm was used. As the corneas are a fairly irregular shape an initial test pattern was gained from the centre of the cornea then the translation stage was moved until no scatter pattern was gained. In this way the edges were traced then rasta scans were made of the entire surface. Four corneas at each time point were used. No other changes were made from that of the general methods.

3.3.9 WAXS data analysis

Scattering patterns were analysed as described previously in the Methods chapter.

3.4 Results

3.4.1 Temporal expression profile during development

Two genes (*Vsx1, Aqp5*) were selected to be examined based either on their selection as possible candidate genes or as controls (*Aqp1, Rpl19*)as they are known to be expressed in the cornea. Changes in expression can be seen in figures 3.1-3.4 and table 4.

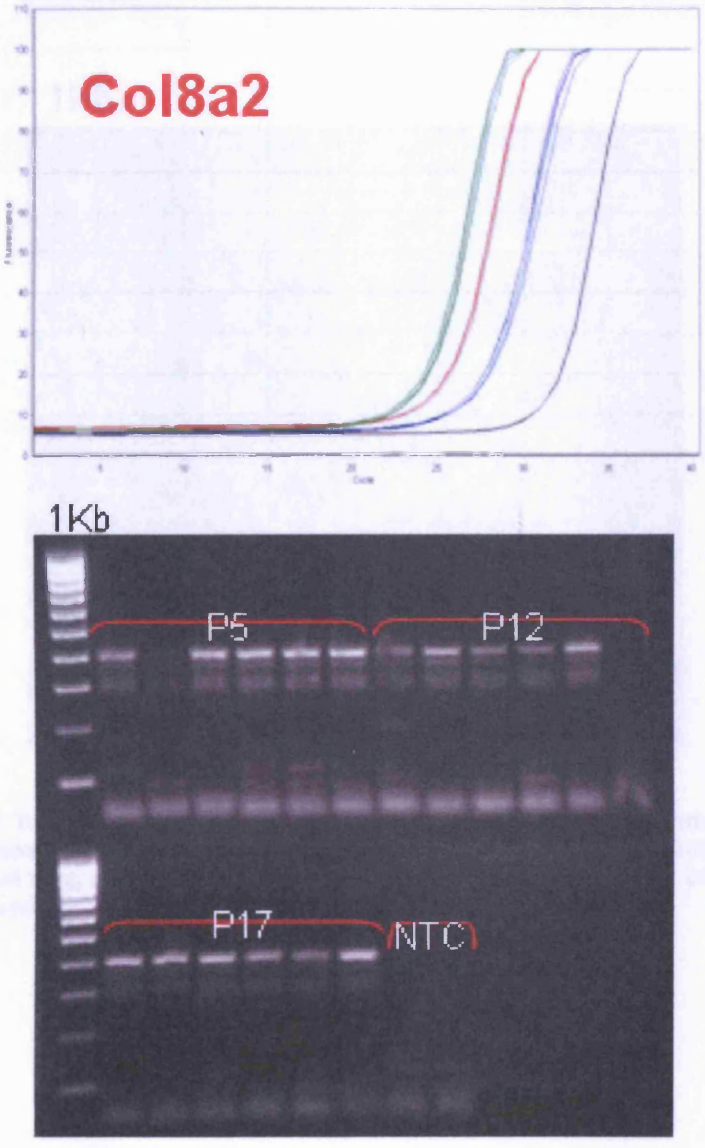


Fig 3.1. Temporal expression profile of *Col8A2*. P5 - red, P12 - blue P17 - green. Raw expression data is given on the right whilst on the left is a graph of the products separated using agarose gel electrophoresis. 1kb – 1 kilobase DNA marker ladder, P – postnatal age.

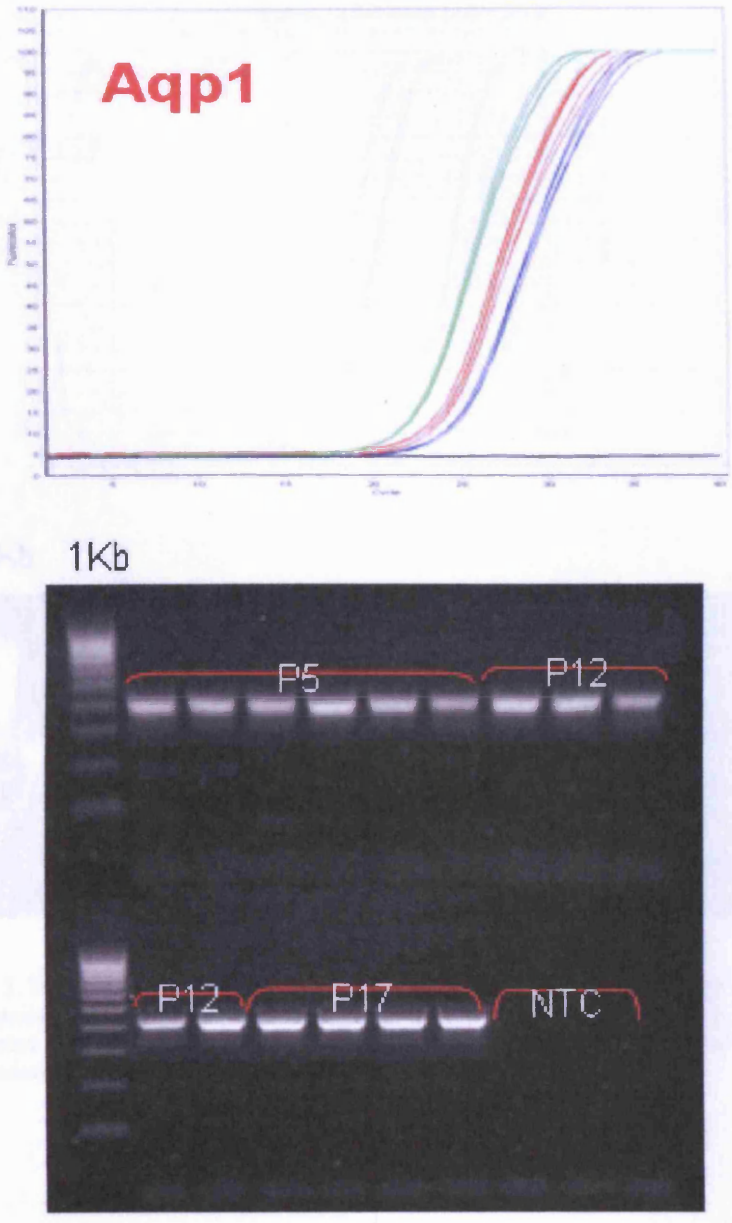


Fig 3.2. Temporal expression profile of *Aqp1*. P5 - red, P12 - blue P17 – green. Raw expression data is given on the right whilst on the left is a graph of the products separated using agarose gel electrophoresis. 1kb – 1 kilobase DNA marker ladder, P – postnatal age.

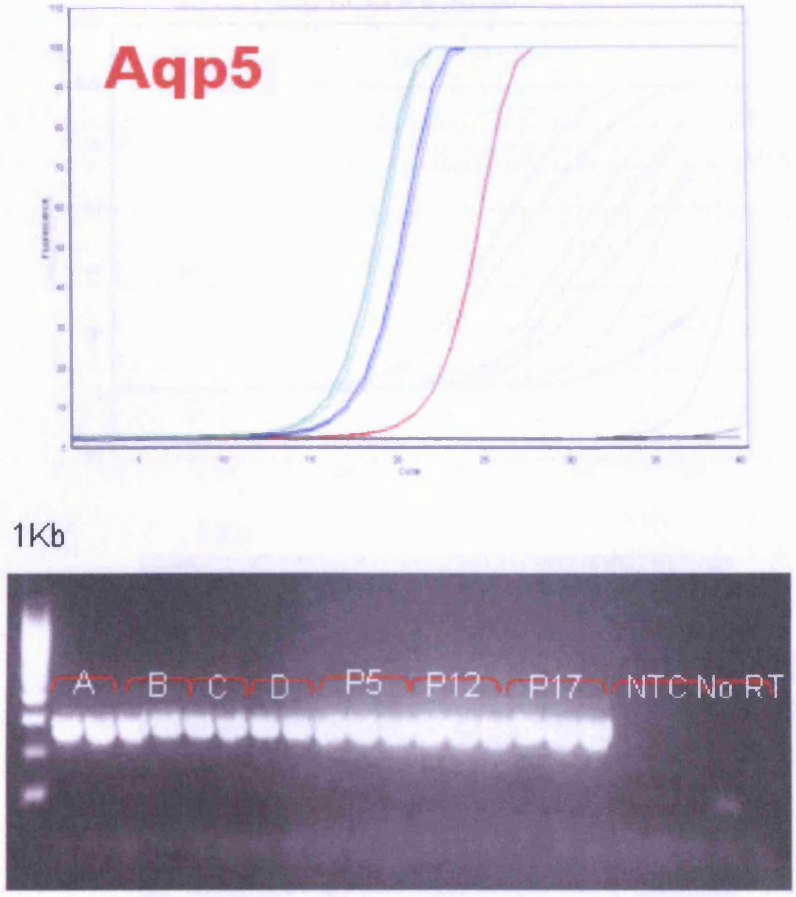


Fig 3.3. Temporal expression profile of *Aqp5*. P5 - red, P12 - blue P17 – green. Raw expression data is given on the right whilst on the left is a graph of the products separated using agarose gel electrophoresis. 1kb – 1 kilobase DNA marker ladder, P – postnatal age.

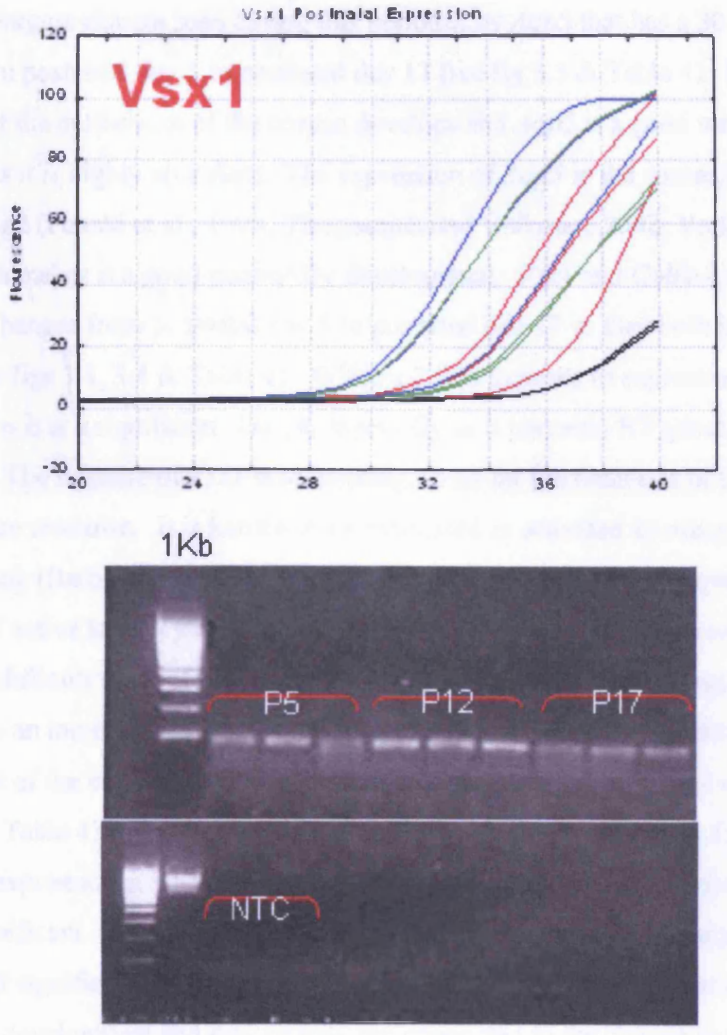


Fig 3.4. Temporal expression profile of *Vsx1*. P5 - red, P12 - blue P17 - green. Raw expression data is given on the right whilst on the left is a graph of the products separated using agarose gel electrophoresis. 1kb – 1 kilobase DNA marker ladder, P – postnatal age.

Gene	P5	P12	P17	Sig
Vsx1	1	2.43	2.02	P = 0.0001
Aqp1	1	0.662	1.96	P = 0.0001
Aqp5	1	13.1	30.4	P = 0.0000001
Col8a2	1	0.318	2.09	P = 0.001

Table 4. Expression levels of all genes normalised to the housekeeper and calibrated to the lowest time point.

The largest change seen during this period is by *Aqp5* that has a 30 fold increase from postnatal day 5 to postnatal day 17 (see fig 3.3 & Table 4). It is during this time that the epithelium of the cornea develops and *Aqp5* is a good marker for the epithelium as it is highly abundant. The expression of *Aqp5* in the cornea has been greatly studied (Funaki et al., 1998; Thiagarajah and Verkman, 2002; Verkman, 2003), which makes it a good control for development. *Vsx1* and *Col8a2* all have significant changes from postnatal day 5 to postnatal day 17 as they both have 2 fold changes (see figs 3.1, 3.4 & Table 4). While a 2 fold increase in expression is relatively low it is a significant change, especially as it concerns RT genetic expression. The increase of *Vsx1* is interesting, as so far the functions of the gene in the cornea are unknown. It is known to be expressed in activated keratocytes during wound healing (Barbaro et al., 2006) so it could be that the increased expression is indicative of active keratocytes at this time point. However it should be noted that at present it is difficult to say if the increase in expression is due to an up regulation of activity or to an increased number of keratocytes, which would be associated with the development of the cornea. There is also a significant change in the expression of *Col8a2* (see Table 4) which is a component of collagen. It is interesting that there is a decrease in expression at P12 which is not significant and then a 2 fold change at P17, which is significant. When dealing with relative expression a 2 fold change or more is considered significant. This could be due to an increased generation of collagen at this point in development and may or may not connected to the change in expression of *Vsx1*, but without examination of the *Col8a2* for possible *Vsx1* binding sites this connection at this point is speculative.

3.4.2 Genetic expression profile during development

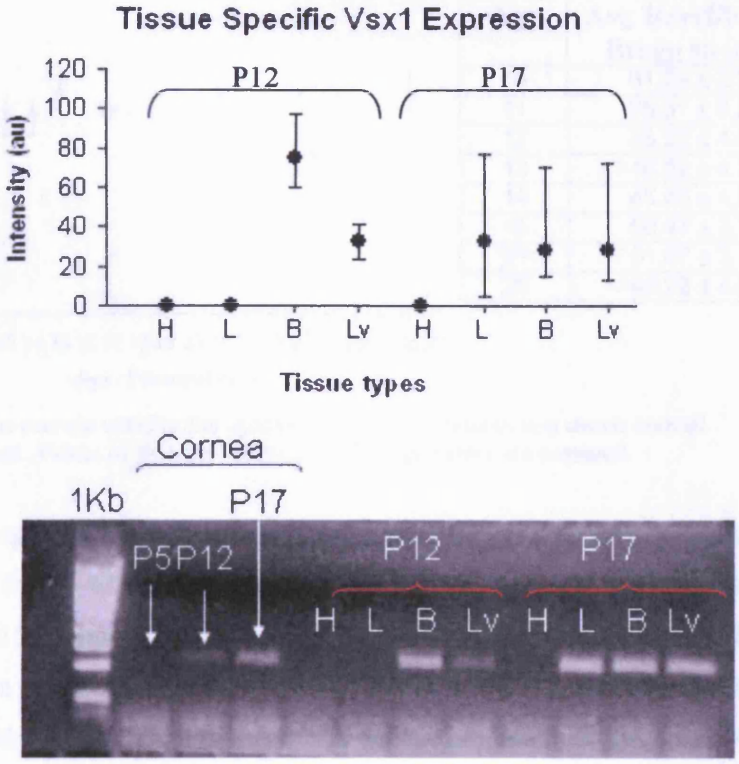
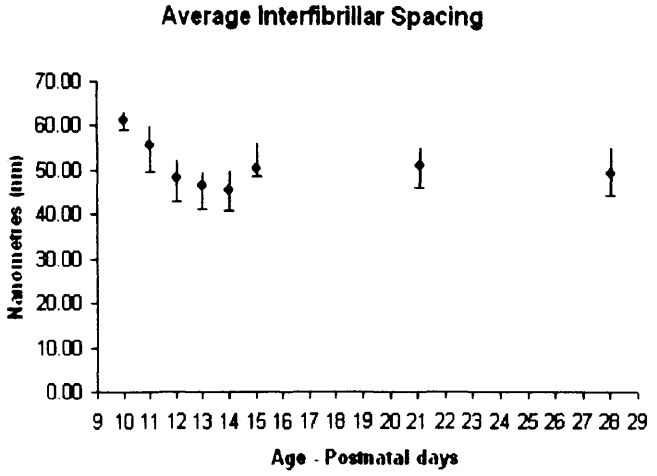


Fig 3.5. Semi quantitative expression of *Vsx1*. H - heart, L - Lung, B - Brain, Lv - Liver, P – postnatal, 1kb - size marker, normalised to *Rpl19* expression.

Semi quantitative PCR could not detect *Vsx1* within any tissue at postnatal day five, although this is most likely due to the lack of sensitivity inherent in this method, as expression has been found using quantitative RT-PCR (see fig 3.4). From figure 3.5 it can be seen that *Vsx1* is not expressed at all in the heart during the time points studied or within the lung at postnatal day 12 of development. According to the gel electrophoresis there appears to be a large difference in the expression of *Vsx1* in the liver from postnatal day 12 to day 17 although there is no statistical significance.

3.4.3 Interfibrillar spacing of collagen fibrils.



Age	Avg Interfibrillar Bragg sp (nm)
10	61.29 ± 2.93
11	55.51 ± 5.29
12	48.22 ± 4.02
13	46.52 ± 4.31
14	45.45 ± 4.53
15	50.47 ± 3.76
21	51.07 ± 3.19
28	49.23 ± 4.66

Fig 3.6. The average interfibrillar spacing of collagen fibrils during mouse corneal development. Values in the table are mean ± SD. Age values are postnatal.

The average interfibrillar spacing for the mice in this study over postnatal days 10-28 decreases from ~ 61nm to ~ 49nm (see fig 3.6). There is an apparent increase at postnatal day 15 although this is not significant. This gentle decline in interfibrillar spacing mirrors a previous study that looked at interfibrillar spacing over this time period although the values stated in this thesis are lower than previously described (Beecher et al., 2006). This can be explained as the samples used here were preserved by freezing instead chemical fixation. Freezing corneas and then thawing prior to experimentation has been shown to not affect the ultrastructure of the cornea (Fullwood and Meek, 1994).

3.4.4 Aligned Collagen distribution and collagen fibril patterning

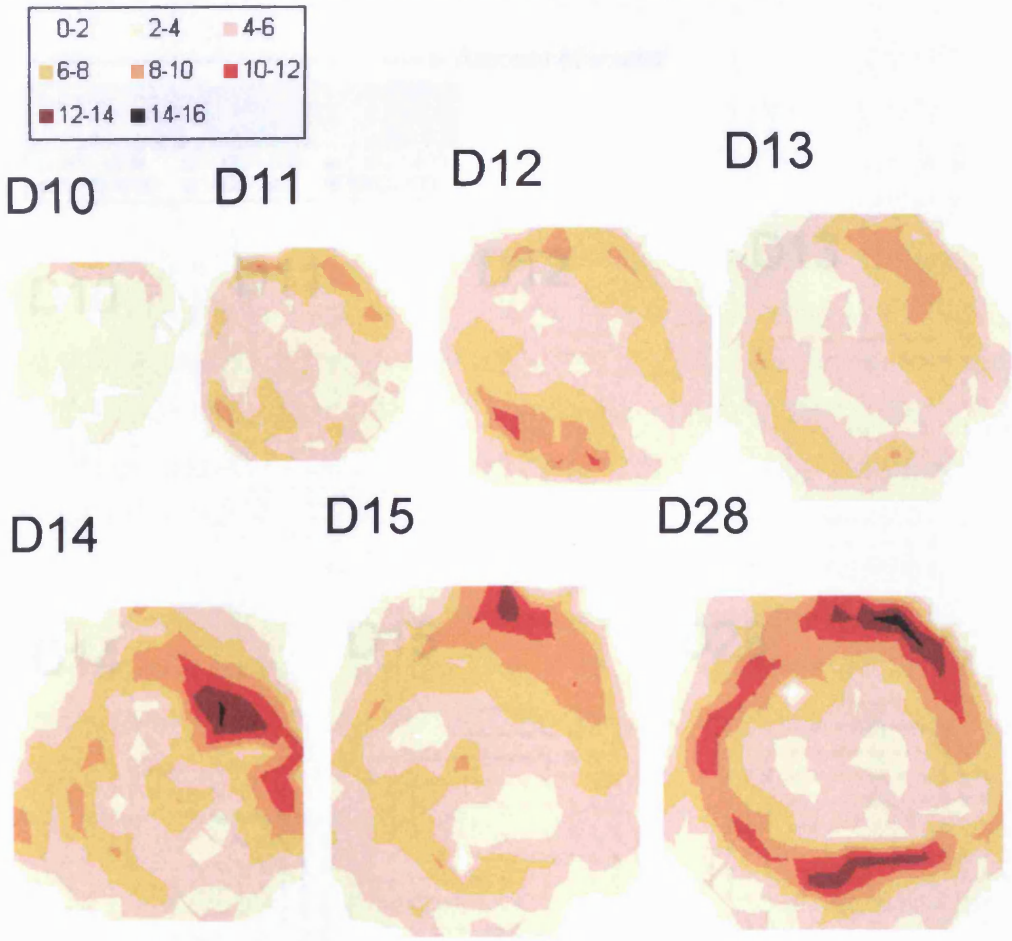


Fig 3.7. Relative mass distributions of preferentially aligned collagen in the developing cornea. Measurements are made in arbitrary units, the key indicates the darker the colour the greater the mass of collagen. D – Postnatal days.

Figure 3.7 shows the increase and distribution of aligned collagen as the mouse cornea develops. At postnatal day ten the increase in aligned collagen first starts to appear in the superior half of the cornea at the limbus. The increase in collagen appears to develop in opposite places to each other around the cornea in the limbus, as can be seen at P11 (Fig 3.7). This development continues until postnatal day 28 until an annulus of collagen fibrils at the limbus is fully formed; this preferential alignment at the limbus in mouse was first described by (Quantock et al., 2003). The increase in collagen around the centre of the cornea is most likely due to extra corneal fibrils being laid down to add to the structural strength at this region where the sclera meets the cornea. As collagen fibrils are strongest along their axis

you would expect a circular pattern for these fibrils to form, as is observed in human corneas (Aghamohammadzadeh et al. 2004) (See fig 3.8).

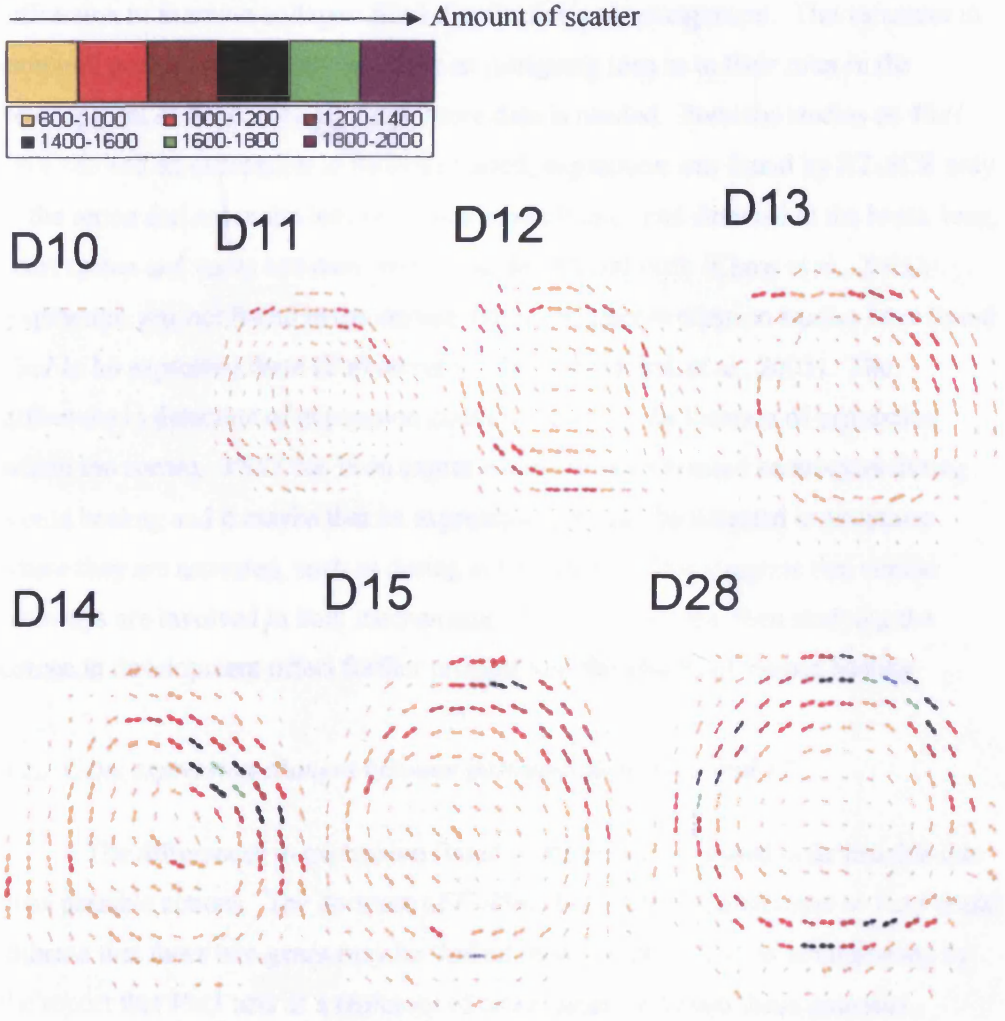


Fig 3.8. Preferential alignment of collagen during development. Measurements are in arbitrary units. Polar plots have been scaled to make them the same size for ease of viewing. The scaling factor is given by the legend with the higher numbers representing more collagen at that particular position. D – Postnatal days

Figure 3.8 shows that mouse corneas have a circumferential corneal fibril pattern throughout unlike the human cornea, which has a circular annulus of collagen fibrils that surround collagen fibrils that have a horizontal and vertical alignment in the central region of the cornea. This is most noticeable with an annulus of fibrils at the limbus.

3.5 Discussion

This is the first developmental study of mouse corneas that uses X-ray diffraction to examine collagen fibril distribution and arrangement. The variances in temporal gene expression shown offer an intriguing idea as to their roles in the development of the cornea although more data is needed. Previous studies on *Vsx1* have showed its expression to be quite varied; expression was found by RT-PCR only in the retina and not in the lens or cornea in adult mice and detected in the brain, lung, heart spleen and testes between embryonic day 14 and birth (Chow et al., 2001). Expression was not found in the cornea; however, later expression studies have found *Vsx1* to be expressed there (Barbaro et al., 2006; Krafchak et al., 2005). The difference in detection of expression could be down to the location of expression within the cornea. *VSX1* has been expressed in human activated keratocytes during would healing and it maybe that its expression can only be detected in situations where they are activated, such as during development. This suggests that similar pathways are involved in both mechanisms. If this is the case, then studying the cornea in development offers further insights into the effects of wound healing

3.5.1 Gene expression changes between postnatal days 5, 12 and 17.

The differences in expression found in the genes examined offer insights into their possible actions. The decrease of *Col8a2* that follows the increase in *Vsx1* could indicate that these two genes may be 'linked' and this possibility is strengthened by the report that *Vsx1* acts as a repressor of other genes, although these potential downstream targets are not known. A potential downstream target is the transcription factor *HSF1* that acts to regulate heat shock proteins under non stressed conditions. This gene is also found in the cornea and acts on the heat shock protein HSP47, which is a chaperone for collagen type I precursors. It could also act directly with a collagen gene itself, as *Vsx1* has many similarities to another important ocular gene, *Tcf8*. This gene has been previously linked to the disease posterior polymorphous corneal dystrophy and acts as a repressor of Col4a3 ((Krafchak et al., 2005). One could therefore hypothesise that *Vsx1* and *Col8a2* have the same interaction and are both important in tissue development and maintenance. Further testing of this hypothesis would be desirable. This could be achieved initially through bioinformatics to

identify any *Vsx1* binding sites in the promoter of the *Col8a2* gene. After evaluation of possible binding sites and if they match statistically significant criteria then further testing can be explored. The most logical course of action would be to evaluate activation via a CAT assay, using the promoter region of the *Col8a2*. The increase in *Col8a2* at postnatal day 17 is similar in the peak of collagen mass witnessed in developing mice (Song et al., 2003). This is yet a further indication of a possible interaction between the noted gene changes and a change in structure of the cornea.

There are many methods that have been used to quantify gene expression, all with their benefits and limitations. The comparative quantitative method used in this study is the most advantageous to use in this case as it is more accurate and uses less resources than the others. The disadvantages of using this method is that it relies on having a low standard deviation between samples but this only adds to strengthen the repeatability of the reactions as they would need to be of a higher standard. This is especially important as the changes expected were thought to be small as the experiment is examining a transcription factor. Both positive and negative controls were used throughout the reactions. No template controls were used to see whether the products that were being amplified were of the correct size and not due to non-specific binding of primers. Melt curves were used in all reactions to determine if products amplified were of the correct size. To prevent the amplification of genomic or erroneous DNA from being amplified no RT controls were used to optimise the RNA extraction and amplification to eliminate them from the reaction. No RT controls were then amplified along side and separated on an agarose gel to see if amplification had taken place.

All reactions were normalised to a housekeeping gene, which in these experiments was the ribosomal protein subunit 19 (*Rpl19*). This housekeeper was chosen over more traditionally used housekeepers such as *GAPDH* because there has been some ambiguity as to whether the expression of *GAPDH* fluctuates through development (Al-Bader and Al-Sarraf, 2005). *Rpl19* was demonstrated to have similar expression in the cornea throughout development and has previously been used in other published experiments involving the cornea (Krafchak et al 2005). Real-time quantitative PCR was chosen over semi quantitative because it is far more accurate for small expression changes and has better detection.

The changes noted here, while few, do indicate that the period just prior to eye opening of mice is a time of genetic activity and merits further exploration into which

genes are active. The flurry of activity that is concentrated into such a small time span offers good opportunities to study a great number of gene changes, which if significant, could be an alternative way in seeking candidate genes for diseases based not on their mapped loci but on their temporal functionality. It would be very difficult in looking for disease causing genes at the time of disease presentation, if the initial defect had already occurred previously during development. A proposed disease causing gene would be screened via conventional methods and report no changes at time of presentation but the defect could have been in its activation due to a dysfunctional activator, such as a transcription factor. This could explain why no mutations have been found in *Col8a2* in conjunction with keratoconus and its association has been dismissed (Aldave et al., 2007b). However if this was true then changes would have to be found in the transcription factor. This is the case with *Vsx1*.

Ectopic expression of transcription factors in these diseases could be the big factor in disease progression that may well relate back to earlier defects. At the very least this study proposes that microarray experiments be performed on mouse corneas and other tissues in the days prior to eye opening at postnatal day 12. The problem with this is that Microarrays do not detect the presence of transcription factors as the expression is too low but it is a good starting point in the continued research into development and its links with disease.

3.5.2 Spatial expression of Vsx1

From the semi quantitative data presented here *Vsx1* has differential expression in the lung and liver and is not expressed in the heart. It is expressed in the brain at both P12 and P17 with similar levels. This data while offering insights into the location of expression of *Vsx1* could be strengthened by repeating the experiment using real-time quantitative PCR as this would be much more accurate. Indeed expression in the heart cannot be ruled out as semi-quantitative PCR is most likely not sensitive enough to detect it. If expression is present then it can be thought that the expression level is similar to that of expression in the cornea at postnatal day 5, as this was also not detected using this method but found using real-time. However this data should not be discarded as it still shows changes in expression. For the purposes of this thesis it was thought that it did not need confirmation using real-time as the focus

of this thesis is on the cornea. It must be noted though that any future study on *Vsx1* in other tissues should confirm expression using a more sensitive method.

3.5.3 Collagen fibrillar arrangement

The circumcorneal annulus that had previously been alluded to by Quantock et al. (2003) has been unambiguously demonstrated by this study. This structure starts to develop in the mouse at postnatal day 10 and continues to develop until a fully mature annulus is formed at postnatal day 28 (see fig 26). Looking at the full pattern of preferential alignment it appears that the circular fibril patterning that can be seen in mice develops from opposite quadrants of the cornea and continues until the pattern is evident across the entire surface. As the corneas used are from the right eye of mice, the increase in preferentially aligned collagen fibrils appears to start from the superior-nasal and inferior temporal quadrants of the eye. Thus most of the increase in tissue mass at this stage is due to the increase of aligned fibrils rather than isotropic fibrils, indicating that the final fibril arrangement is built up in stages as new tissue is laid down and organised.

The difference in structure between the mouse and the human cornea has been thought to be linked to the differences in visual acuity between the two species, with greater visual acuity requiring a greater deal of collagen organisation (Hayes et al. 2007). It is also been suggested that the orthogonal arrangement of fibrils seen in humans is pointed towards the insertion of the rectus muscles on the globe to add additional strength against globe distortion during eye movement (Daxer and Fratzl, 1997; Hayes et al., 2007a). This would support the lack of orthogonal or any preferential pattern of alignment within the mouse cornea, as eye movement is often secondary if not absent completely in favour of head movement. The change in collagen organisation is also not related to size, radius of curvature or thickness of the cornea (Hayes et al. 2007).

The circumcorneal annulus appears to be a common feature among mammalian species being present in cows, rats, chickens, marmosets, rabbits and pigs. Despite the large differences in change in radii of curvature between the species the annulus appears to play the same function, which is to strengthen the cornea against the changes in curvature from the sclera to the cornea. Its development at p10 suggests that it may play additional strengthening roles as it is the predominant

developing feature and most of the collagen mass that is being laid down contributes to its development. This would also suggest that parallel functions are acting to organise whilst laying down the annular fibrillar collagen rather than the organisation occurring afterwards, which happens with cartilage formation (Williams et al., 2008). The fact that the development of the annulus continues during eye opening until its fully developed state at postnatal day 28 may suggest that the organisational processes require reactions with the outside environment. An obvious experiment to test this would be to prevent the eyes from opening and see if a mature annulus is developed although this is outside the scope of this study.

In conducting X-ray diffraction experiments with developmental samples several limitations must be overcome to ensure consistent and reliable results. Although measures can be taken to minimise and prevent these limitations some still persist. However when using this species for X-ray experiments the benefits of having the mouse as a model system far outweigh any difficulty in its use for these types of procedures. The mouse cornea has several drawbacks when compared with other species due to the small size of the cornea and its thickness and these must be taken into consideration when used. For WAXS experiments that look at the overall mass distribution in the cornea it is not possible to get strong enough diffraction patterns below the age of postnatal day 8. Even though you can get data for postnatal mice aged 7 to 9 the patterns gained are too noisy get any meaningful results (data not shown). Stronger patterns for X-ray diffraction can be gained by modifying the sample exposure time however care must be taken to ensure that the intensity of the beam does not destroy the tissue. The size of the cornea in relation to the size of the beam means that a higher resolution for WAXS experiments could not be reached although this would have given better determination of the location of the annulus. The beam size and the step size of the rasta scans were the smallest they could have been given the experimental setup. An alternative does exist at the ESRF synchrotron facility however extensive optimisation to the equipment is needed in order to recreate the study.

3.5.4 Interfibrillar spacing of the mouse cornea in development

The change in interfibrillar spacings from day to day is not significant and the conclusions do not differ from the lower resolution developmental study performed by Beecher et al. (2006). It is reassuring to see that there were no trends that were being masked by the resolution of the earlier study, especially around the time of eye opening, a critical developmental time point. However the difference in the values between this developmental study and the study performed by Beecher et al. is quite interesting. It emphasises the care needed when handling mouse corneas when performing X-ray diffraction experiments. In this study to measure the interfibrillar spacing an X-ray beam 1mm wide and 0.5mm in height was used. This would cover a large amount of the cornea as an adult mouse cornea has a diameter approximately of 2.2mm. It has been shown using fine focus X-ray beams that the interfibrillar spacing can vary remarkably across the surface of the cornea (Meek et. al private communication). This would mean that very precise positioning of the X-ray beam within the cornea is necessary in order to achieve an accurate result and difficult to achieve with any sort of reliable accuracy. What this also means is that any measurement of corneas with a diameter under the size of the beam would be pointless as the margins of error would be so large as to make any result gained meaningless. It is believed that the results here represent a truer reflection of the values of interfibrillar spacing for mouse corneas as they have been confirmed in additional studies (see chapter 4 and Meek et al. private communication).

3.6 Conclusion

In terms of progressive diseases such as keratoconus there is a case to begin looking for initial causes in developmental stages rather then when symptoms present, as the progression has got beyond the point the body can correct. Although this would be the time that the problem can be first identified it leaves diagnosis of progressive diseases very difficult. Nevertheless through identifying genes that are active in controlling and organising structures in development it may be possible at a later date to use them in treatment methods currently beyond technical expertise. A follow up study to this one to determine whether the expression changes found here in

Vsx1 and *Col8a2* would be to examine their expression and look at the changes in corneal ultrastructure in developmental knock out mouse models.

Corneal structure develops from postnatal day 10 and continues through eye opening until postnatal day 28. These structural changes should be taken into account when examining the development of the cornea and similar experiments in other species are encouraged. This would not only begin to form a good foundation for future research but perhaps offer explanations as to how the different orientation patterns in different species emerges. It is hoped that this may reveal some common mechanisms that govern the organisation of collagen fibrils. The current study conducted here does add the knowledge of how the corneal structure develops and this may provide future researchers with a good background in conducting their own experiments.

Chapter 4. Evaluation of *Vsx1* KO corneas

4.1 Aims

- To compare the average fibril diameter, interfibrillar and intermolecular spacings between *Vsx1* -/- mice and background-matched WT controls.
- To compare collagen fibril patterning, preferred orientations and collagen distribution between *Vsx1* -/- and background matched controls to see if similar differences to human keratoconic corneas can be found.
- To measure the expression levels of various genes including the heat shock protein *Hsp47*, the transcription factor *HSF1* and the collagen gene *Col8A2* in *Vsx1* -/- mice and to compare them with background-matched wild types by QPCR.
- To measure the amount of collagen breakdown products (crosslaps) in the tear film of *Vsx1* -/- mice and to compare them to background-matched controls.

4.2 Introduction

In 2004 Chow et al. generated a *Vsx1* -/- mouse that showed *Vsx1* was crucial for late stage differentiation of cone bipolar cells; however gross examination of the cornea revealed no obvious abnormalities. Nevertheless, the production of a *Vsx1* knock out provided an excellent opportunity to thoroughly examine the corneas of these mice with techniques and methods that have been previously employed to study corneas from keratoconic patients. If similar data could be gathered from the knock out corneas that is found in keratoconic human corneas it would be a very strong indication that we would have found the best model for keratoconus that has been produced so far and thus help to get closer to an understanding of the mechanisms behind the ectasia and eventually a cure or preventative measure. The *Vsx1* knock out model also affords the opportunity to study any possible pathway of how *Vsx1* could be involved with keratoconus. Existing evidence does not offer any possible explanations of the pathophysiology of *Vsx1* – associated keratoconus, and only makes associations.

In keratoconic patients there is a reported 2- to 5-fold increase of collagen type I breakdown products in the tear film compared to normal controls (Abalain et al., 2000). The experiment was initially carried out to see whether the breakdown of

collagen could be used as a diagnostic tool for keratoconus. There have been many studies of keratoconus that try to categorise the biological changes that occur in the cornea and these have led to numerous hypotheses on the causes of keratoconus. *Vsx1* has been linked to keratoconus and *PPCD* as mutational studies have found mutations in *Vsx1* segregating with individuals with these conditions. To date there are 8 published missense mutations (Bisceglia et al., 2005; Heon et al., 2002; Mintz-Hittner et al., 2004; Valleix et al., 2006). In some patients where these mutations have been found there have also been indications of decreased retinal function (Heon et al., 2002; Valleix et al., 2006).

The present study is the first to try and elucidate a possible mechanism for the involvement of *Vsx1* in keratoconus. *Vsx1* has been shown to be expressed in the mouse cornea (Krafchak et al., 2005), and *VSX1* has been shown to be expressed in activated keratocytes in human corneas that are undergoing wound healing (Barbaro et al., 2006). In a recent study looking at the transcriptional activity of *Chx10* and Vxs1 it was found that *Vsx1* acts in some part as a repressor of genes and has been shown to bind to and repress the activity of heat shock transcription factor 1 (*HSF1*) (Dorval et al., 2005). *HSF1* is a transcription factor that up-regulates heat shock proteins and, in the cornea as well as other tissues, up-regulates the heat shock protein Hsp47, a chaperone of collagen type I precursors. As well as its chaperone activities, *Hsp47* acts on keratocytes to up-regulate the production of collagen type I.

The above sequence of events prompts the hypothesis that the absence of functional *Vsx1* would lead to keratoconus through a deficient collagen production pathway in a subset of patients. This would not explain why keratoconus happens in all patients as it is a complex disease, but it offers an explanation as to why the mutations that were found in the *Vsx1* gene have been associated with keratoconus. As *Vsx1* is nonfunctional, it is unable to repress the function of *HSF1*, which in turn increases *Hsp47* that leads to an increase of collagen type I synthesis. In any system that acts to maintain a controlled level of its constituent parts when it needs an increase of collagen production, it will activate measures to decrease production to normal homeostatic levels. In this case, as there is more collagen, there will be an increase of matrix metalloproteinases that act to breakdown the collagen thus leading to an increase of collagen breakdown products that accumulate in the tear film (see fig 4.1).

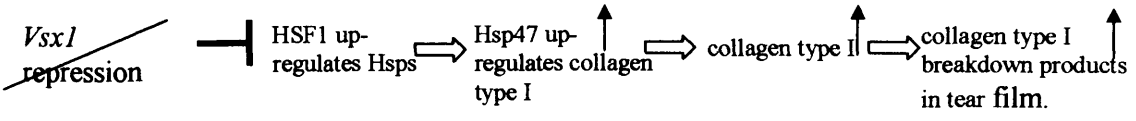


Fig 4.1. Flow diagram illustrating one hypothetical function of *Vsx1* in the cornea and the consequences of its inactivation. Arrows pointing up indicate an increase of amount/expression; the open flat arrow shows repression.

4.2.1 Hypothesis

The *paired-like* homeobox gene *Vsx1* acts as a repressor of genes in the cornea. It has been shown to bind and repress the transcription factor *HSF1* that up-regulates the production of collagen type I via the up-regulation of heat shock protein 47. In keratoconic patients there is a 2- to 5-fold increase of collagen type I breakdown products (crosslaps) in the tear film. In *Vsx1* -/- mice there is no mechanism to repress the up-regulation of collagen type I and therefore there will be an increase in crosslaps in the tear film of these mice.

4.3 Methods

4.3.1 Specimen collection

Mouse tissue of 129/sl background KO and WT was kindly provided by Dr Bob Chow at the University Of Victoria, Canada. Adult corneas were rapidly excised and wrapped in Clingfilm™. Excised corneas had their orientation maintained throughout removal and the 12 o'clock position and epithelial side were identified and marked. After wrapping they were immediately frozen at -80°C for storage and transport and stored until needed for examination. Whole murine globes of WT and KO mice were extracted and immediately placed in RNAlater® (Ambion, Inc.) for transport. Mice were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

4.3.2 SAXS data collection

Data was collected as described previously in the general Methods section. To ensure that any results that were gained were real and not due to a change in hydration the wet weight of the cornea was measured before and after scanning.

4.3.3 SAXS analysis

Data was analysed as described previously in the Methods. Statistical significance of the means was compared using a standard student's *t*-test.

4.3.4 WAXS data collection

Data was collected as described previously in the Methods. Exposure time for each pattern was 45 seconds and a step size of 0.2mm was used. Rasta scans were used to scan the entire surface of the cornea. No other changes were made from that of the general methods.

4.3.5 WAXS analysis

Data was analysed as described previously in the Methods.

4.3.6 Statistical analysis of WAXS patterns

In order to determine the variation between WT and KO mouse patterns that takes into account the inherent variability in murine corneal X-ray patterns, additional quantification was needed. Mice, as with other species, exhibit a certain distribution and pattern of collagen that makes up their corneas and although this is a distinct pattern it is found to be more intrinsically variable than corneas for higher order species such as humans and chickens. It became clear that an established control of a wild type cornea was needed in order to identify the common elements that make up this distinct pattern in mice. These control patterns would include a map of the preferred fibrillar orientation that is particular to mice; a topography map of the correlation between corneas at each point of the map and a topography map of the average distribution of aligned collagen. Unfortunately no methodology existed to

quantify the preferential alignment in order to establish statistical differences between two data sets so this was developed in order to quantify the data gathered here.

The procedure developed was as follows. Taking the linear intensity profiles for any two individual X-ray scatter patterns allows them to be correlated against each other as seen in figures 4.2-4.3. As we are mainly interested in the preferred fibrillar direction, the important part of these intensity profiles that we are looking at is the position of the peaks rather than their size, as size is indicative of collagen mass as seen in figures 4.4. By correlating the two plots we get a correlation coefficient that tells us how similar the preferred direction is between them, with a correlation >0 giving us positive correlation and <0 negative correlation. By using this method for all plots we can construct correlation coefficients for all polar plots in each sample provided that they are in the same geometric location within the cornea.

In order to establish a control corneal pattern, the exact centres of each individually mapped cornea were identified overlaid with their orientation maps. A central grid of 11 x 11 patterns was taken from each cornea in order to make sure that the positions of each WAXS pattern are comparable to each other (see fig 4.5). The aligned data for each pattern was then taken and a correlation coefficient was calculated between paired samples. This pair-wise correlation was then repeated for each cornea in a genotype resulting in a grid of coefficients between all the samples (see fig 4.6). An average correlation coefficient was then calculated for each point in the central square grid generating a grid of averaged correlation coefficients for the central 2.2mm of a hypothetical control cornea. This was displayed graphically as a topography map. In addition, the points from each KO sample were then compared with the average to see if there were any statistical differences at that point. In addition to the topographic map showing correlation a second topography map was constructed to show the differences in the distribution of the average aligned collagen mass. This was constructed by simply averaging the data for each point from each of the individual samples across the whole surface of the cornea.

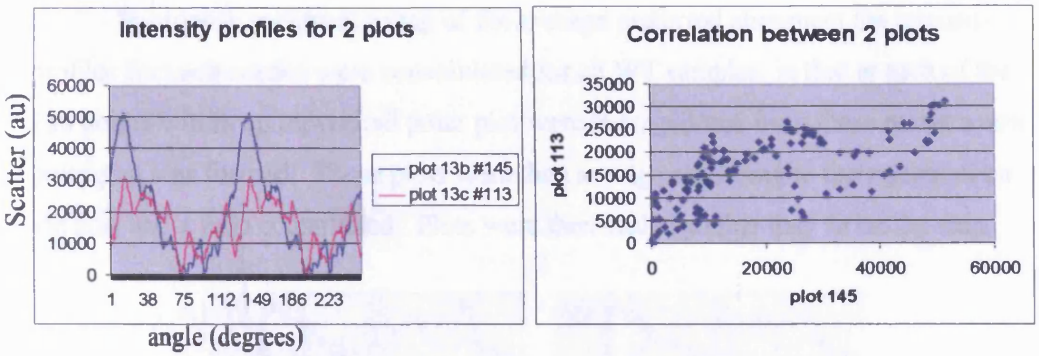


Fig 4.2. Linear intensity profiles and correlation between 2 polar plots.

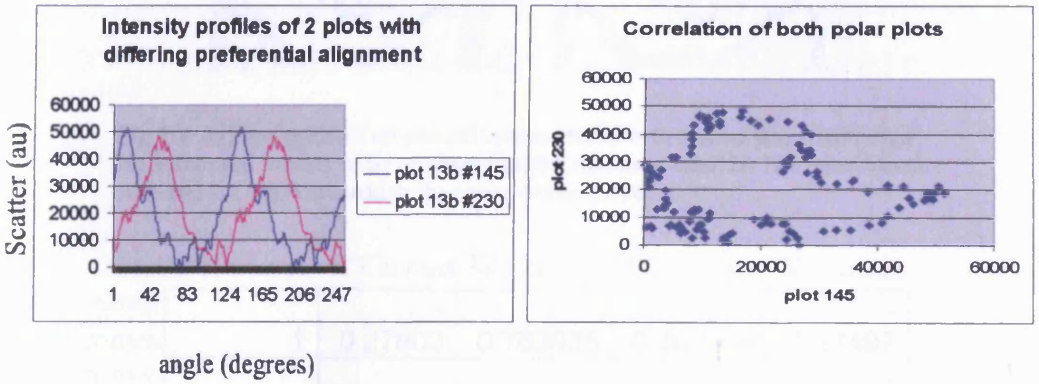


Fig 4.3. Linear intensity profiles and correlation between 2 polar plots that have different fibrillar alignments.

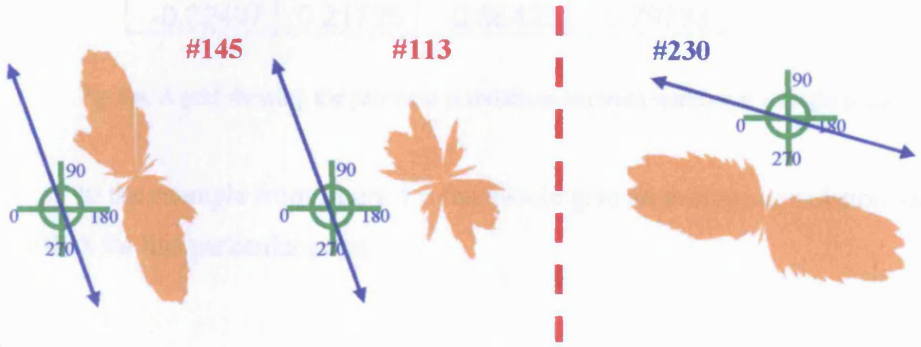


Fig 4.4. Polar plots showing their size and their preferred fibrillar alignment. The arrows show the direction of preferential alignment.

In order to construct a map of the average preferred alignment the intensity profiles for each cornea were consolidated for all WT samples, in that at each of the 256 points within an individual polar plot were averaged and from these points a new polar plot was formed. These plots were then arrange according to their position on the grid and a map constructed. Plots were then scaled so that they fit on the map.

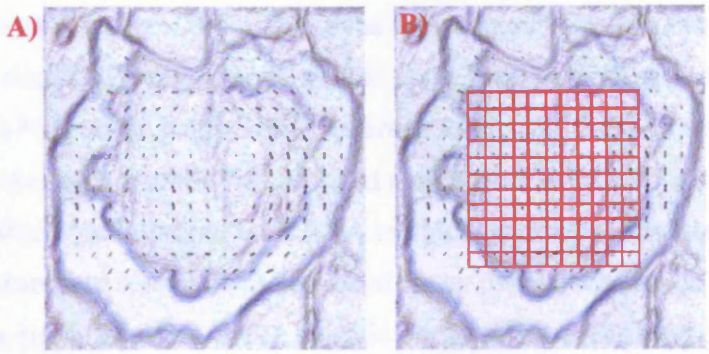


Fig 4.5. A) Photograph of an excised mouse cornea with corneal and scleral edge enhanced, and overlay of its resultant fibrillar alignment map. B) The same cornea with an 11x11 grid demarking the centre of the cornea.

	WT cornea	WT cornea	WT cornea	WT cornea	WT cornea
WT cornea	1	-0.27803	0.783935	0.267609	-0.32497
WT cornea	-0.27803	1	-0.34669	-0.21867	-0.21725
WT cornea	0.783935	-0.34669	1	0.771891	-0.66428
WT cornea	0.267609	-0.21867	0.771891	1	-0.79733
WT cornea	-0.32497	-0.21725	-0.66428	-0.79733	1

Fig 4.6. A grid showing the pair wise correlations between samples at a single point

In the example from figure 4.6 this would give an average correlation value of -0.10238 for this particular point.

4.3.7 RNA isolation and amplification

Total RNA was isolated using the RNEasy® Mini kit (Qiagen) according to the manufacturer's protocol and extracted RNA was quantified using spectrophotometry. RNA was separated into aliquots to avoid the need of repeated freeze/thaw cycles and RNA was transcribed to cDNA using the Quantitect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.
cDNA was amplified using Sigma SYBR® Green Jumpstart™ Taq ReadyMix™ for Quantitative PCR on the Roto-Gene™ (Corbett Research). Samples were performed in triplicate and each reaction was repeated three times. In order to ensure similar starting product concentrations each RNA sample was measured and then aliquoted so that each subsequent reaction was optimised for an RNA concentration of 0.5µg
Genes were amplified using cDNA specific primers for the following genes:

Name	**Primer sequence 5' - 3'**	**temp °C**
Vsx1 F	5'-ACTGCCCAGTTACCCACAAT-3'	55
Vsx1 R	5'-AAGTGGCGTAAGCGAGAGAA-3'	55
Aqp5 F	5'- ATCTACTTCACCGGCTGTTCC-3'	60
Aqp5 R	5'- GTCAGCTCGATGGTCTTCTTC-3'	60
Hsf1 F	5'- TGATGAAGGGGAAACAGGAG-3'	60
Hsf1 R	5'- CTGCACCAGTGAGATCAGGA-3'	60
Hsp47 F	5'- CCTGAGGTCACCAAGGATGT-3'	60
Hsp47 R	5'- CCATCTGCAGCTTCTCCTTC-3'	60
Col8a2 F	5'-GGTAAAGTATGTGCAGCCCA-3'	60
Col8a2 R	5'-AGTAATACCTGAGGGACCAG-3'	60
RpL19 F	5'-GGGAAGAGGAAGGGTACTGC-3'	55
RpL19 R	5'-GGACGCTTCATTTCTTGGTC-3'	55

Table 5. Primer sequences used for amplification, F- forward, R – reverse, tmp – temperature. All primer sequences are 5' to 3'.

Rpl19 was used as a housekeeping gene for normalization and all primer sequences were designed to be intron spanning. Primer sequences were designed against the following accession sequences:

Vsx1 - #NM_054068
Aqp5 - # NM_009701
HSF1 - # NM_008296
Hsp47 - # NM_001111043
Col8a2 - # NM_199473
RPL19 - # NM_009078

4.4 Results

Heterozygous samples were tested along with the WT and KO samples but only 2 were available so they were included here for completeness.

4.4.1 Differences in interfibrillar (IFS) and intermolecular (ImS) spacings and average fibril diameters (FD) between genotypes.

Genotype	Cornea #	L/R	Avg FD (nm)	IFS (nm)	IMS (Å)
WT	1	R	31.0	43.0	16.4
WT	2	L	30.7	45.5	17.2
WT	11	R	31.3	42.3	17.2
WT	12	L	32.8	47.7	17.2
WT	13	R	30.7	43.7	14.5
WT	14	L	29.3	43.6	17.2
Mean	----------	-----	**30.9 ±1.13**	**44.3 ±1.98**	**16.7 ±1.1**
KO	5	R	30.2	42.3	16.4
KO	6	L	35.1	40.4	17.2
KO	7	R	30.6	45.5	17.2
KO	8	L	30.1	45.7	16.5
KO	9	R	31.1	45.1	17.2
KO	10	L	32.4	45.7	17.2
KO	15	R	30.6	41.0	17.2
KO	16	L	31.6	45.1	16.5
Mean	----------	------	**31.5 ±1.65**	**43.9 ±2.24**	**16.9 ±0.39**
Het	3	R	31.6	43.8	17.2
Het	4	L	30.4	43.1	17.2
Mean	----------	------	**31.0 ±0.89**	**43.5 ±0.87**	**17.2 ±0**

Table 6. Measurements taken for all corneal samples measured. FD – Fibril diameter, IFS – Interfibrillar spacing, IMS – intermolecular spacing. Measurements are Bragg spacings. Measurements are ±SD.

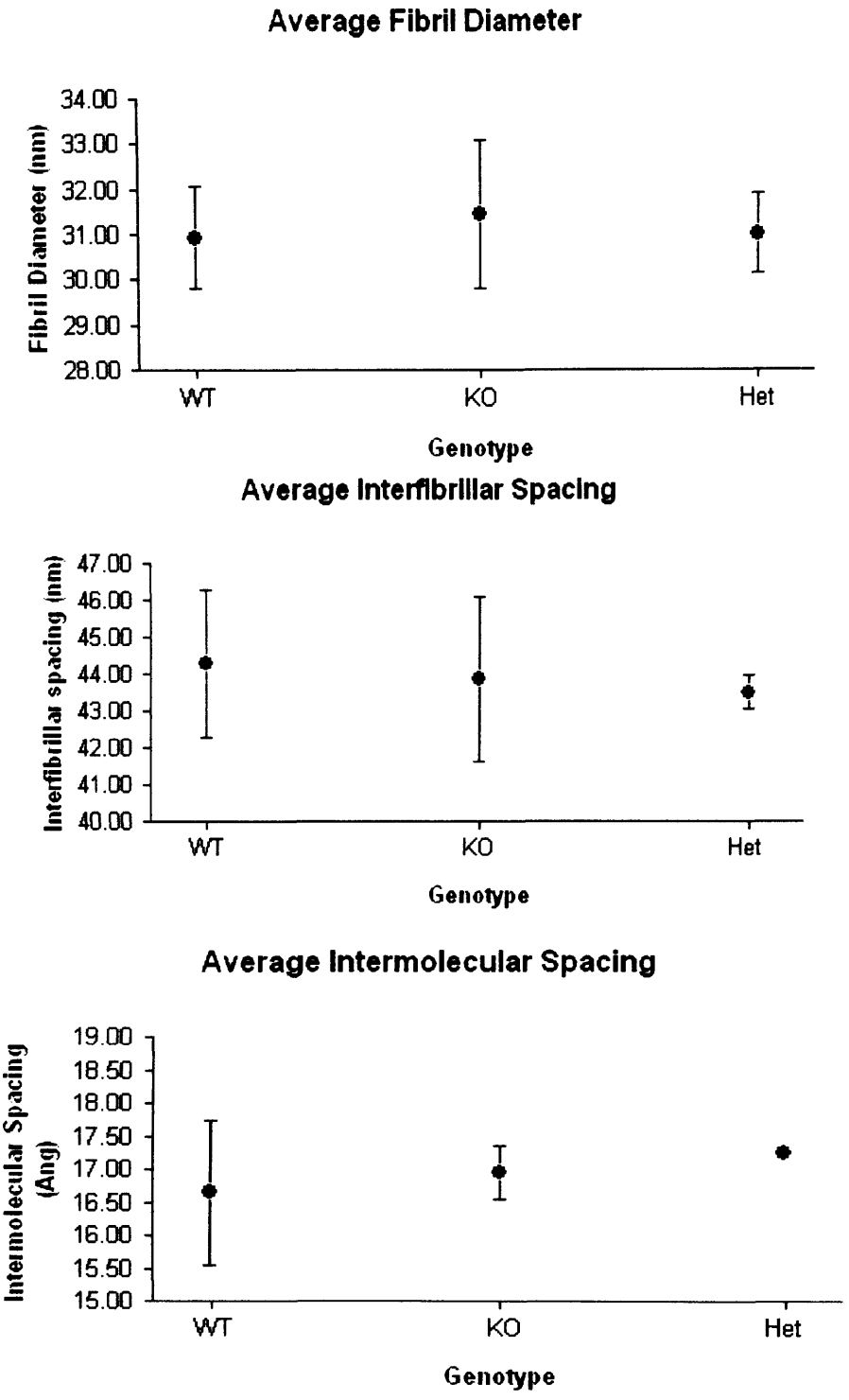
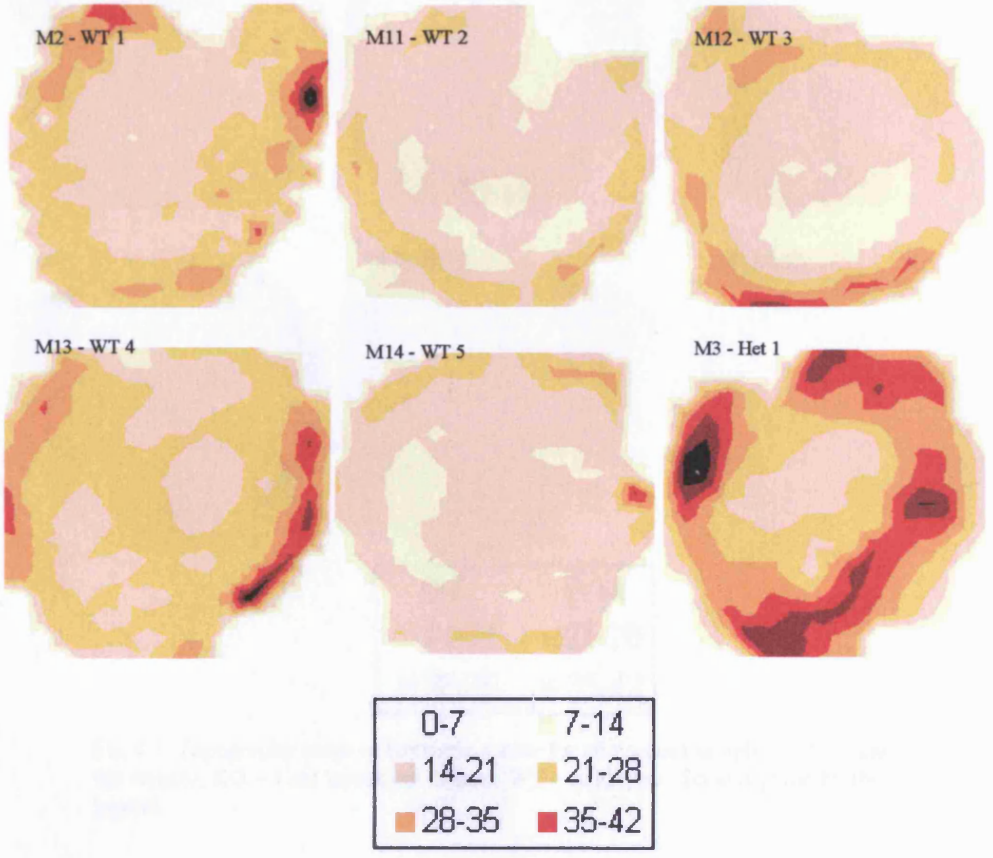


Fig 4.7. Measurements of the cornea comparing the effect of *Vsx1* deletion. Measurements of the interfibrillar spacing and the average fibril diameter are in nanometres while the intermolecular spacing is measured in angstroms. Measurements shown are means ± SD. WT – wild type +/+, KO – knock out -/-, Het – heterozygous +/-.

Figure 4.7 shows that there are no significant differences in the interfibrillar spacings and average fibril diameter between any of the genotypes (students *t*-test average fibril diameter P = 0.244, interfibrillar spacing P = 0.365, intermolecular spacing P = 0.246). It should be noted that heterozygous animals were included in the study to aid in identifying any erroneous results if any differences found were on the border line of significance.

4.4.2 Distribution of total collagen between genotypes



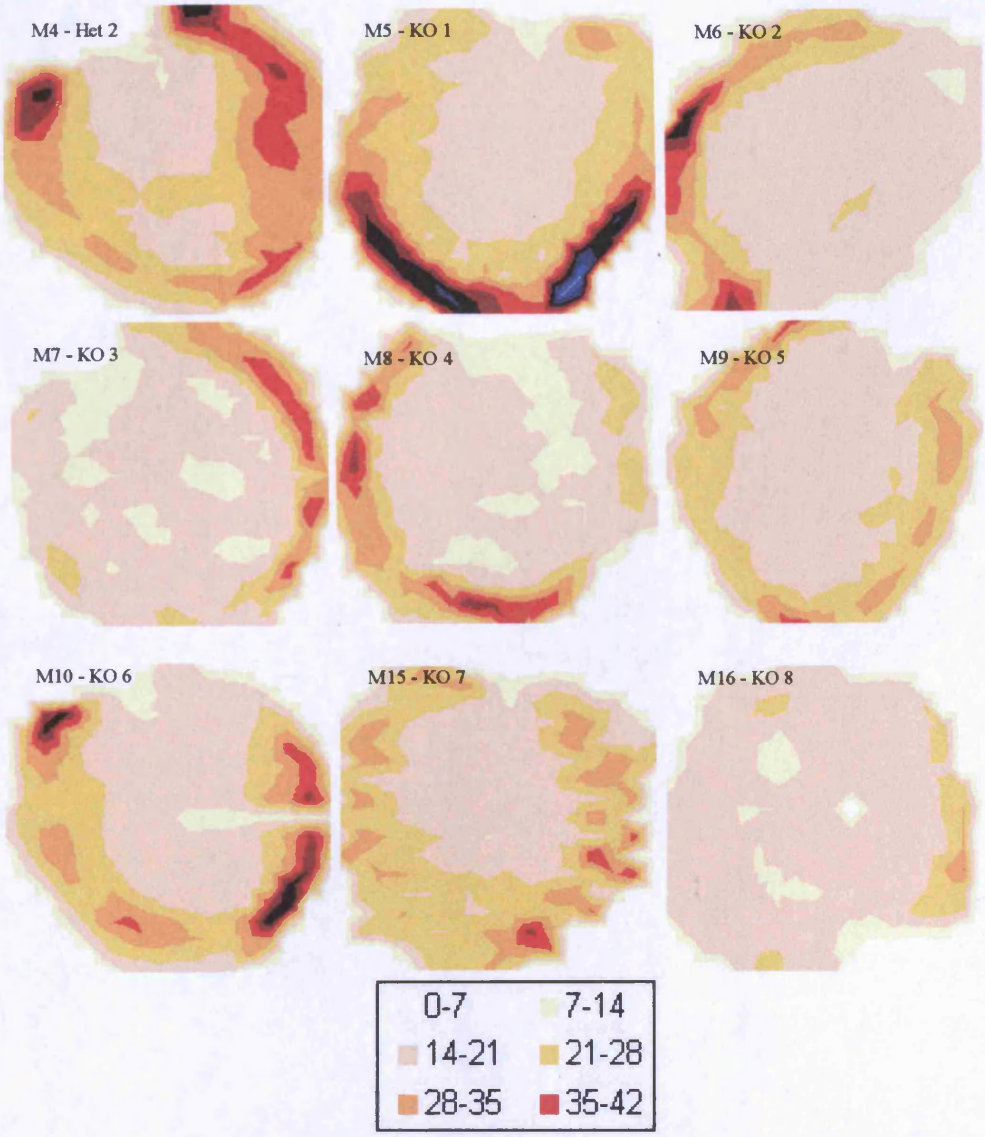
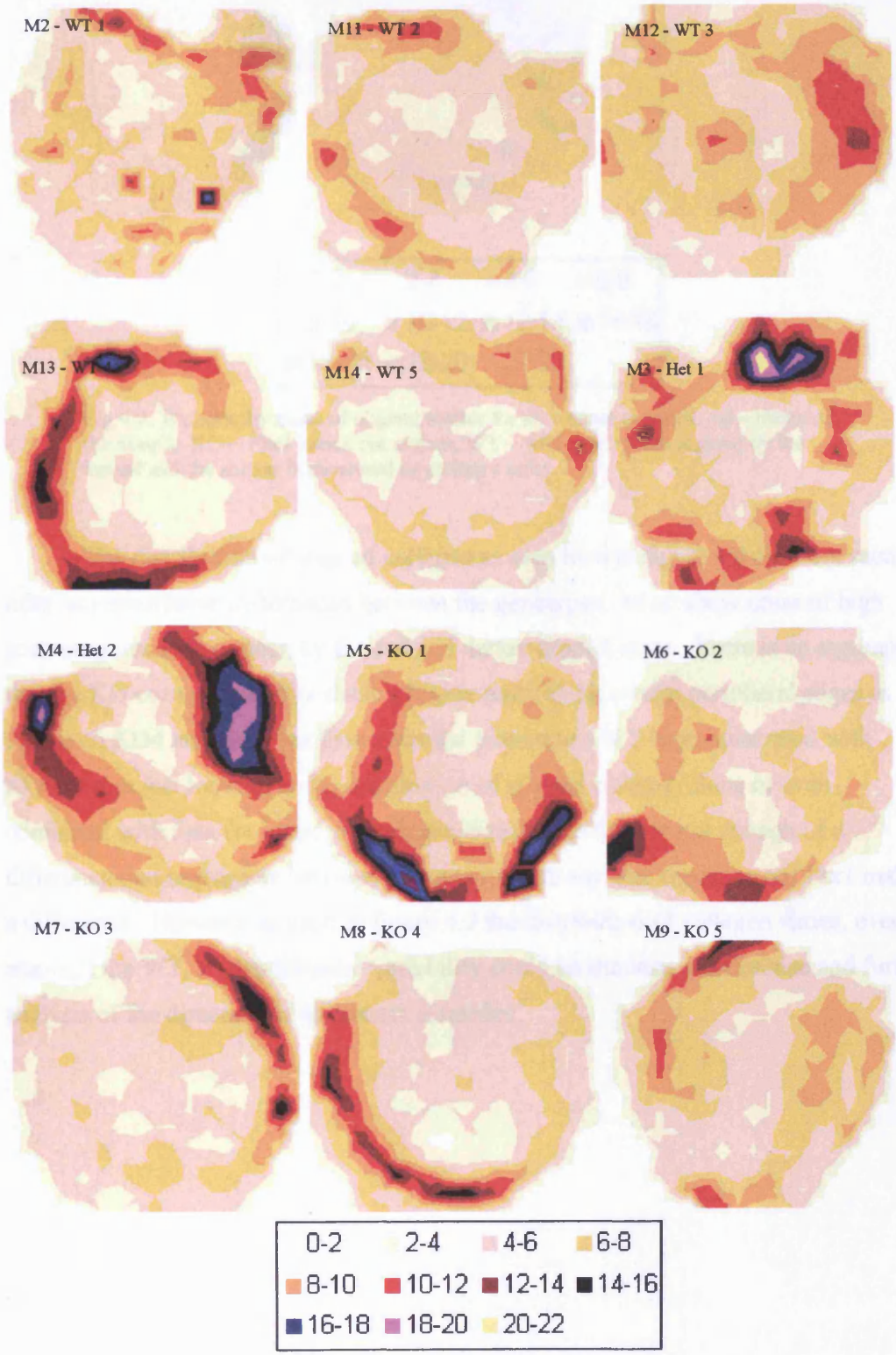


Fig 4.8. Topography maps of isotropic scatter for all corneas sampled. M – name of the sample, KO – *Vsx1* knock out cornea, WT – wild type. Scale is given by the legend.

Shown here in figure 4.8 are the topography maps for all genotypes. All corneas show a higher scattering in the limbal areas. There is a degree of difference in the intensity of scatter around the edges of these corneas that by itself does not offer any qualitative difference between the genotypes. The increases in intensity are themselves explained by an increase in actual collagen mass in the peripheral regions of the cornea.

4.4.3 Distribution of collagen that shows a preferred alignment



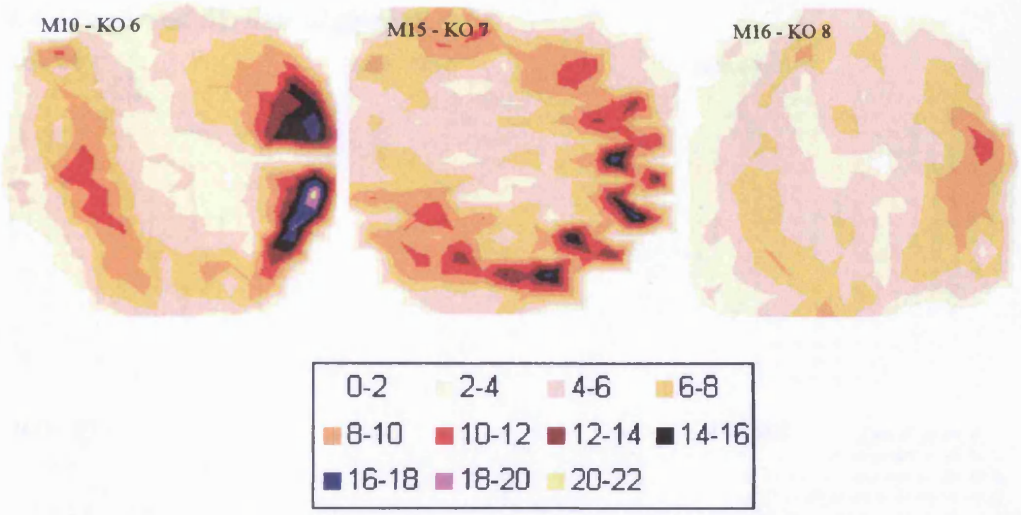
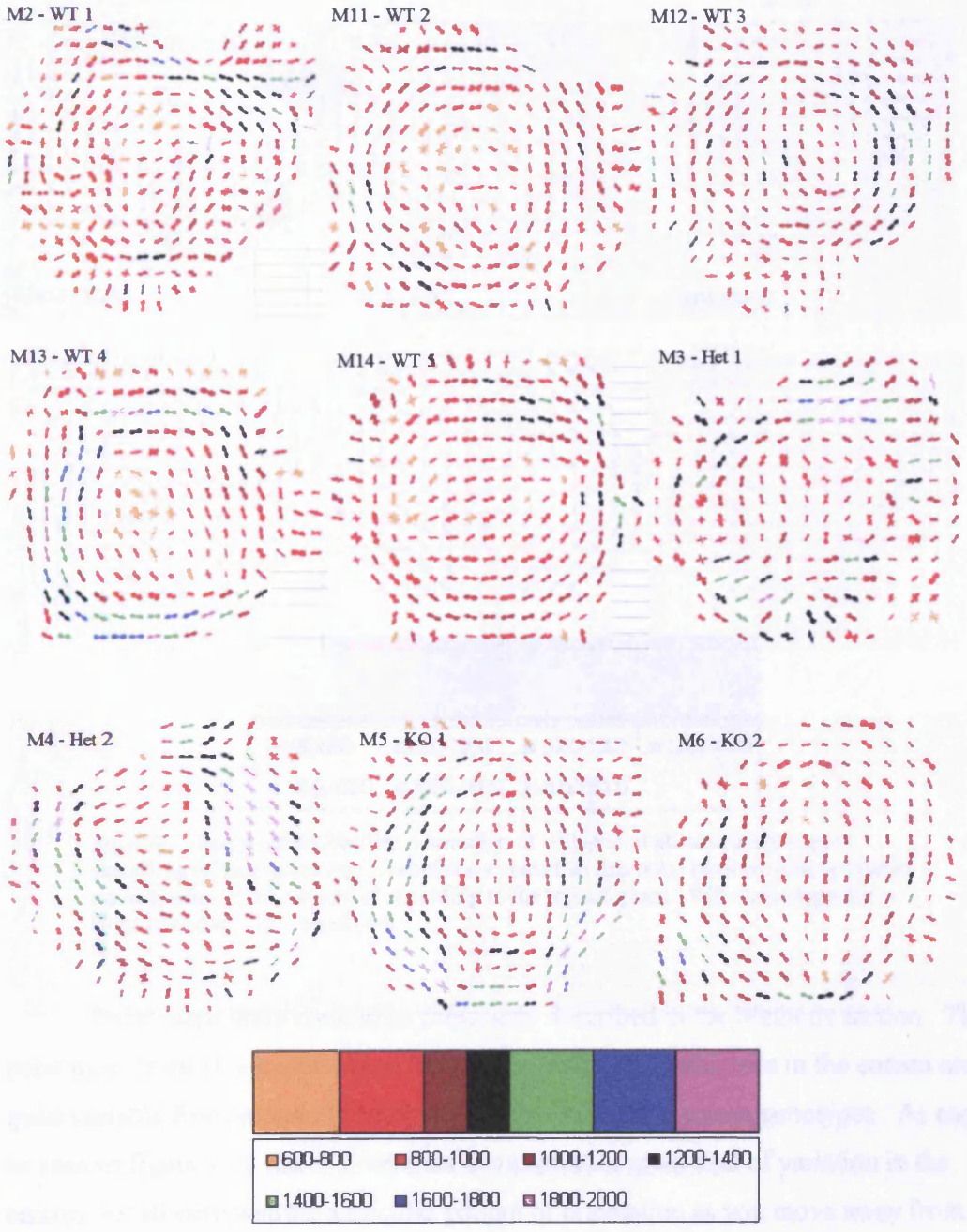


Fig 4.9. Topography maps of aligned scatter for all corneas sampled. M – Name of the sample, KO – *Vsx1* knock out cornea, WT – wild type. Scale is given by the legend and the scatter is measured in arbitrary units.

The distribution of aligned collagen as seen here in figure 4.9 does not seem to offer any qualitative differences between the genotypes. Most show areas of high scattering intensity as seen by the red and darker shaded areas. There is an argument that the KO corneas possibly show a higher mass along certain peripheral edges as seen with KO4 and KO3 but then a similar pattern to a WT is demonstrated with KO6. From just looking at the distribution of aligned collagen alone or even combined with data for isotropic collagen distribution there is not enough of a difference that segregates between the genotypes to say that knocking out *Vsx1* makes a difference. However as seen in figure 4.9 the distribution of collagen varies, even amongst the WT, so this inherent variability could be masking a difference and further analysis of the direction of alignment is needed.

4.4.4 Preferred fibrillar alignment



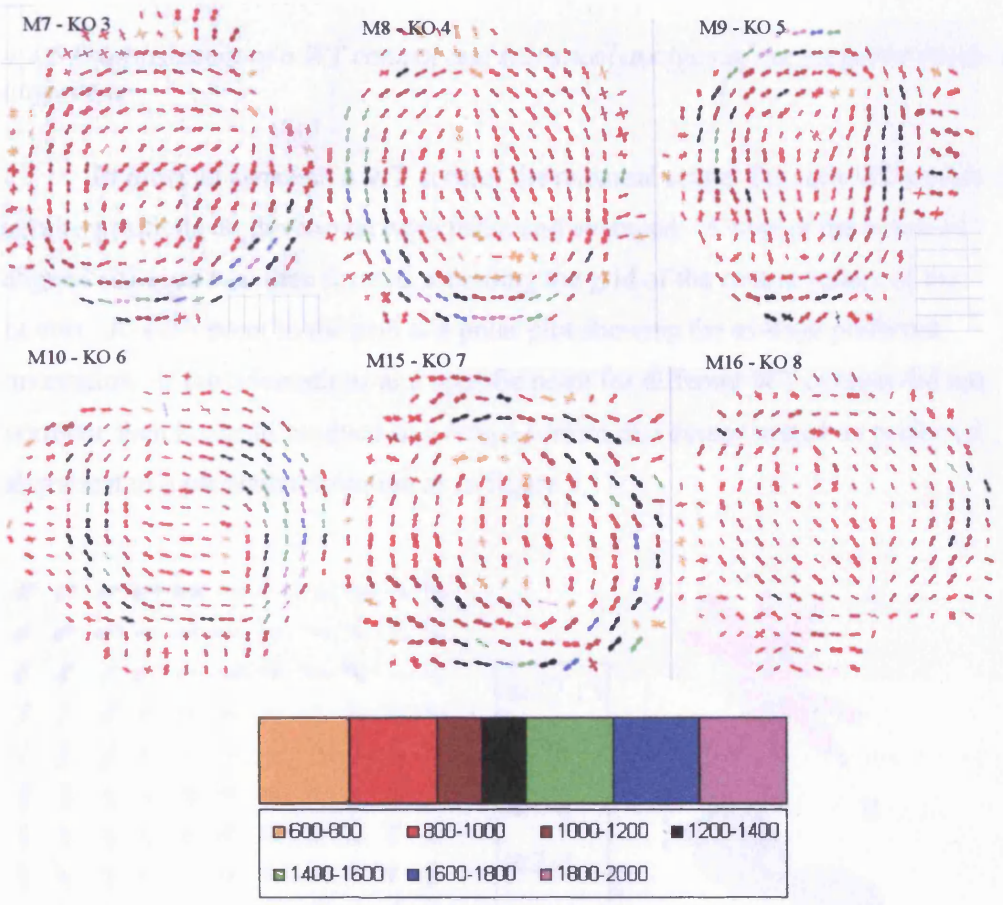


Fig 4.10. The preferred fibrillar orientation of collagen of all samples grouped according to their genotype. Samples are scaled so that polar plots are clearly visible on each map. Plots are scaled according to the legend given. WT – wild type, het – Heterozygous, KO – knock out.

Polar maps were created as previously described in the Methods section. The polar plots from all samples show that mouse collagen orientations in the cornea are quite variable from sample to sample and this can be seen across genotypes. As can be seen in figure 4.10 the WT samples demonstrate a great deal of variation in the centres but all demonstrate a circular pattern of orientation as you move away from the centre out towards the limbus. The same can be said from the other genotypes, there is a strong circular orientation of fibrils around the limbal and peripheral regions of the corneas as opposed to the centres. In all samples the circular patterns are themselves surrounded by plots that show lateral alignment leading away from the corneas, which is similar to what is found in other species including humans (Hayes et al., 2007a). The orientation maps make it very difficult to determine if there is a difference between genotypes as the WT samples are themselves quite variable.

4.4.5 Establishment of a WT control and statistical analysis of the preferred fibrillar alignment.

In order to establish a WT control the maximal scatter for each WT cornea at similar positions on the cornea were taken and averaged. A map of the averaged aligned collagen was then formed according the grid of the central square of the cornea. At each point in the grid is a polar plot showing the average preferred orientation. If the orientations at a specific point for different WT corneas did not correlate then it would produce an average pattern that demonstrated no preferred alignment in a particular direction as in figure 4.11.

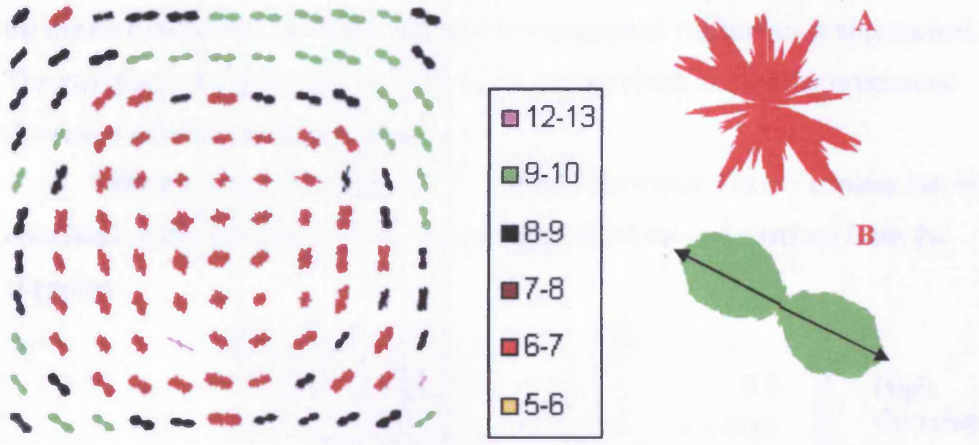


Fig 4.11. Map of aligned collagen showing directions of preferential alignment. Individual plots are scaled according to the amount of collagen present at each point. Alignment mass is measured in arbitrary units. A). Polar plot showing no preferred fibrillar alignment. B). Polar plot showing a high degree of fibrillar alignment.

The pattern produced for the average WT mouse cornea is remarkably circular around the limbus and the peripheral areas of the cornea with very little alignment in the centre. This is as expected very similar to the pattern of mouse corneas previously published (Quantock et al., 2003). This gives a very good control for the fibrillar alignment in the cornea.

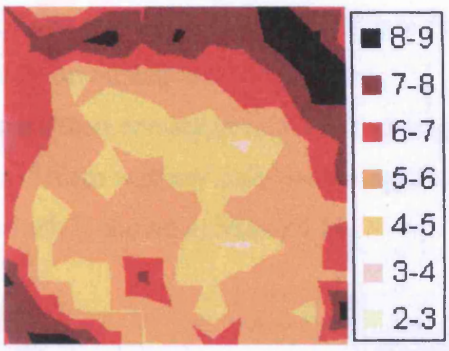


Fig 4.12. Distribution map of averaged aligned collagen for WT 129/s1 control mice. Amounts of collagen are measured in arbitrary units and are shown in the key.

Figure 4.12 shows that most of the preferentially aligned collagen mass lies on the outside towards the limbus of the cornea. There is a fair degree of aligned mass in the centre (yellow and orange regions) that increases as the limbus is approached. The topography of distributed collagen is consistent with its fibrillar orientation showing a circular pattern of intensity.

With the establishment of a WT control, individual *Vsx1*-/- corneas can be correlated to the average to see if there are any discernable deviations from the alignment.

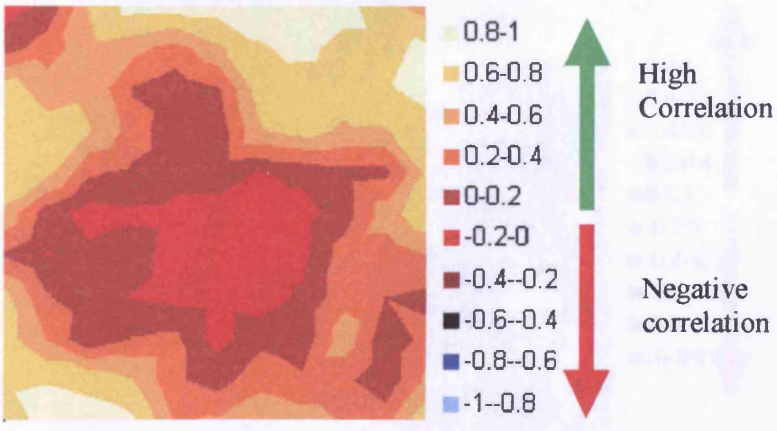
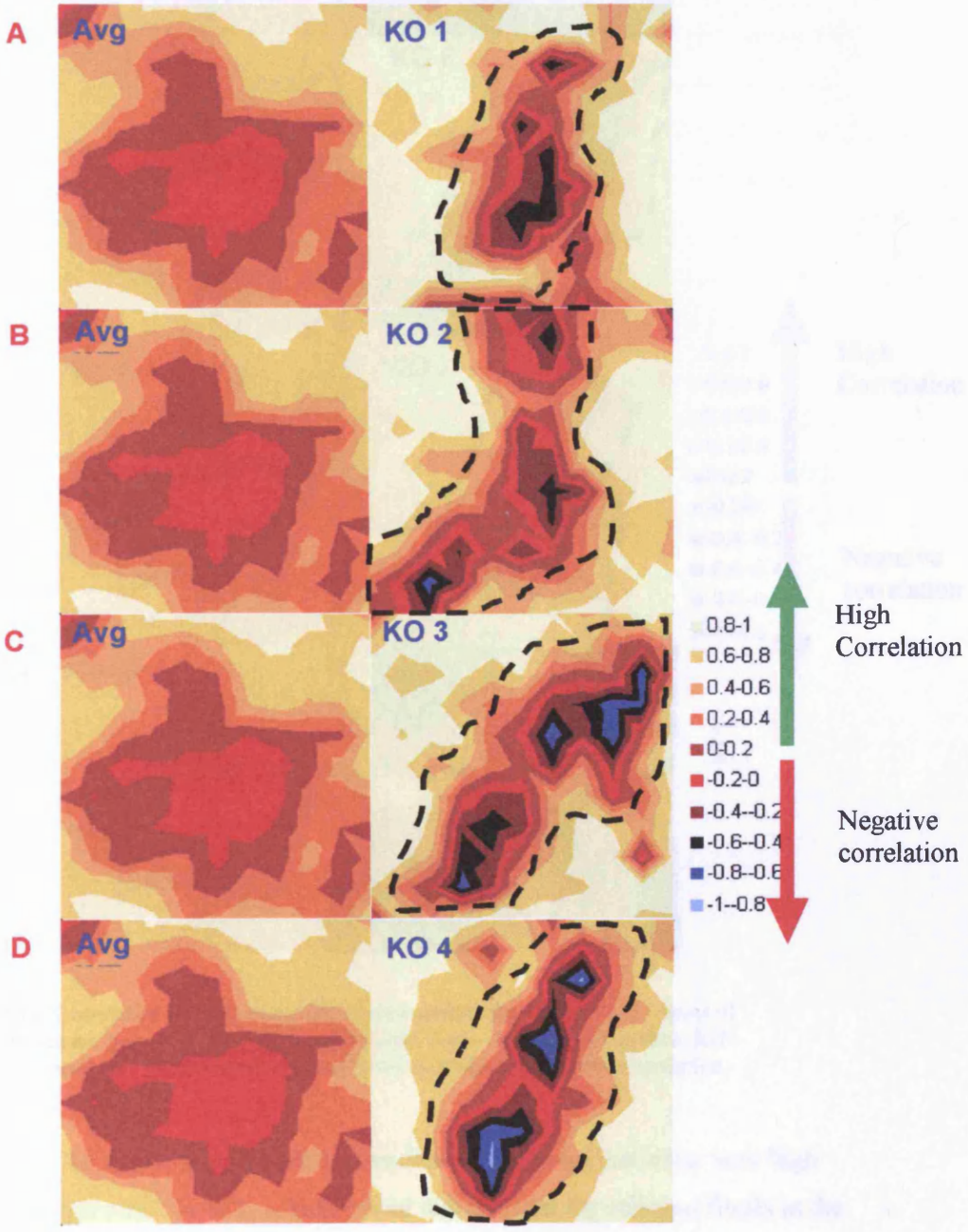


Fig 4.13. Topography map of the average correlation between individual points from different corneas. The amount of correlation is given in the accompanying legend.

Figure 4.13 shows that the average correlation for preferential alignment increases away from the centre and is very high round the area of the limbus. What figure 4.13 also shows is that the correlation outside the centre does not drop below 0 indicating that the fibrils from each of the wild type samples do not show any negative correlation, i.e. that the fibrils do not show an altered preferential alignment. The

centre of the averaged corneas appears to have very little or no correlation indicating that the fibrils are randomly orientated within the centre.

Vsx1-/- corneas were then correlated to the WT average to see if there was a similar pattern and to see if there was any statistically significant divergence in the preferential alignment, which is shown in figure 4.14.



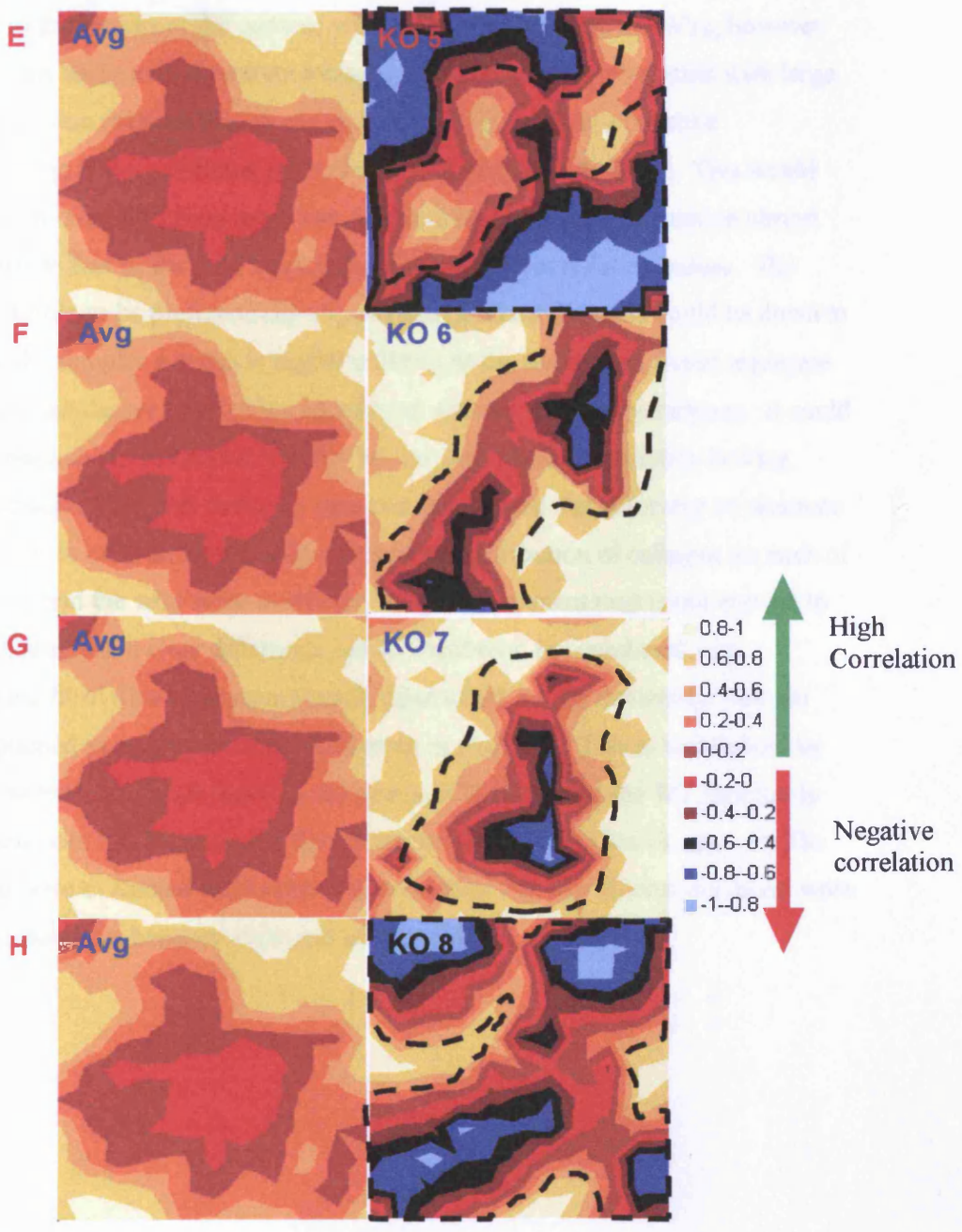


Fig 4.14. Correlation of each single KO cornea against the average WT. Areas of correlation are given by the scale bar at the side. Avg – Average WT cornea, KO – *Vsx1-/-* knockout. Black dotted lines mark the area of no or negative correlation.

Figure 4.14 shows that in each cornea there are regions that show very high areas of correlation with the WT, which would indicate that the collagen fibrils in the knock-outs show a very similar preferred orientation. This can mainly be seen in the limbal regions (light yellow-orange colours). There also appears to be very little

correlation in the centres of the corneas which is also seen within the WTs, however almost all maps seem to demonstrate a diagonal schism in their orientation with large regions (red – blue colours) that do not correlate and even show a negative correlation. This is demonstrated most clearly in figures 4.14 E and H. This would indicate that the fibrils in these areas can demonstrate a preferred orientation almost perpendicular to that of the wild types depending on their correlation values. The reasons for fibrils to be preferentially aligned in a different direction could be down to handling of the samples but this is highly unlikely as the differences found segregate and show distinct degree of negative correlation with the different genotypes. It could be that the mechanism of support that the WT corneas require is possibly lacking within the knockouts or that there is a strain on the corneas that is forcing an alternate alignment. On its own the topography maps of the distribution of collagen for each of the knockouts and the wild types combined with the alignment map is not enough to be able to distinguish a clear difference just qualitatively; but combined with correlating the fibril directions against each other offers a clear distinction that can only be explained as arising from the difference in genotype. This is highlighted by figure 4.15 as the correlation between the Average WT map and the WT samples is very high and does not demonstrate the differences found in the knockouts. All The corneas that were evaluated were right eyes indicating that any differences found were not due to differences between right and left corneas.

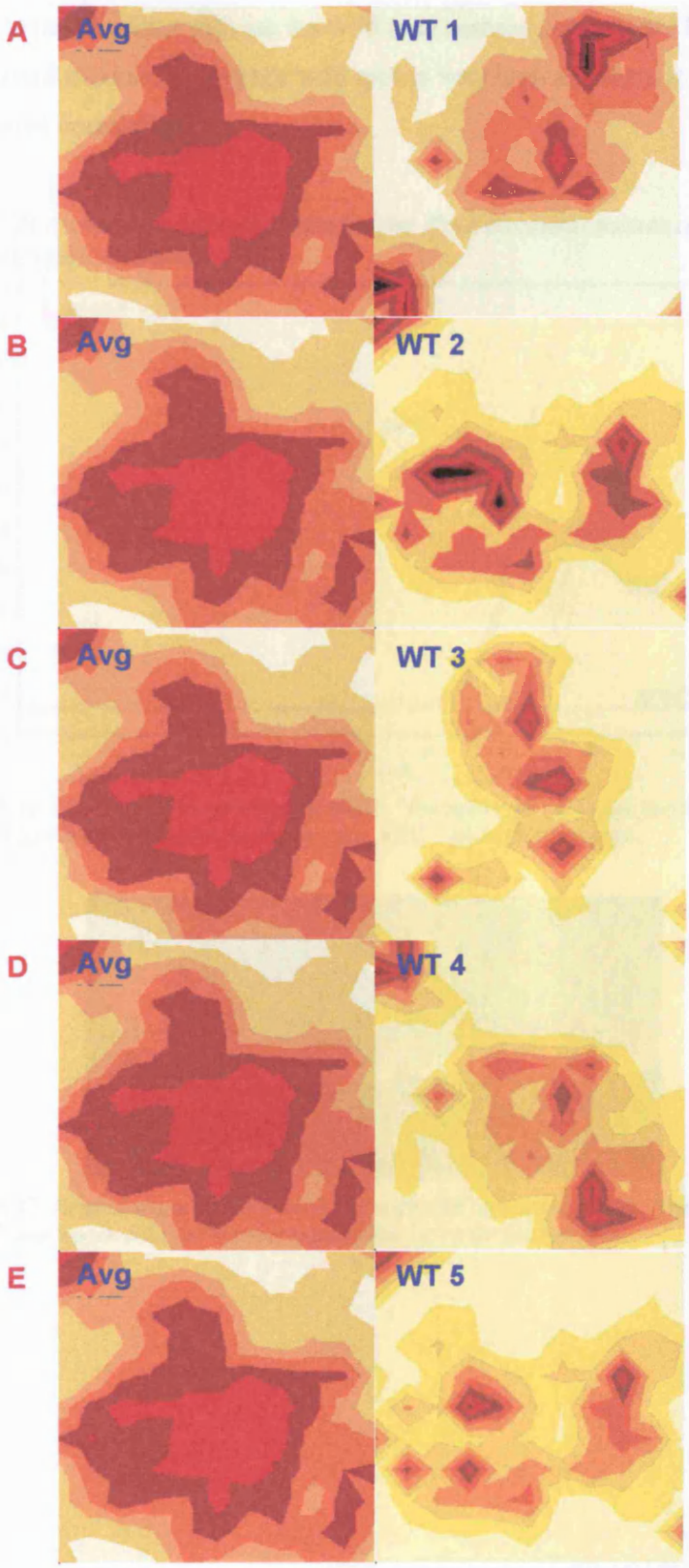


Fig 4.15. Correlation of each single WT cornea against the average WT. Areas of correlation are given by the scale bar at the side. Avg – Average WT cornea

In contrast to the knockout corneas the wild type corneas demonstrate large areas where the correlation to the average wild type is very high and there is a distinct lack of any negative correlation.

4.4.6 RT-PCR expression changes between the Vsx1 knockout mouse and background matched littermate controls.

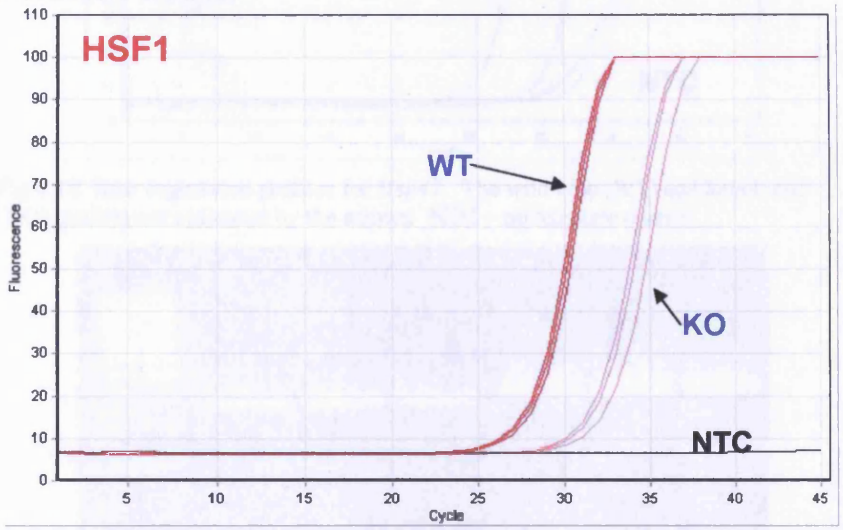


Fig 4.16. Raw expression profiles for *HSF1*. The wild type (WT) and knock out (KO) profiles are indicated by the arrows. NTC – no template control.

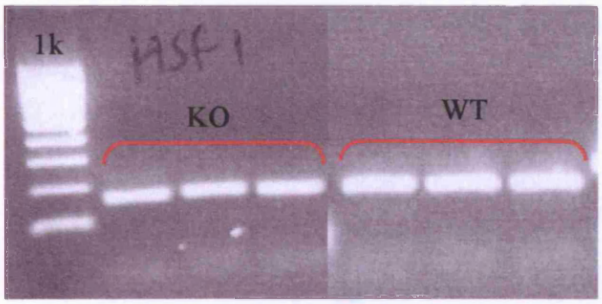


Fig 4.17. Amplification products of *HSF1* separated on an agarose gel. The wild type (WT) and knock out (KO) products are indicated by the red brackets

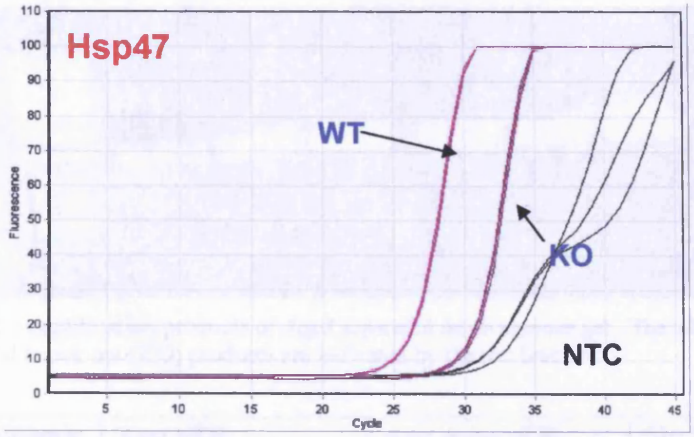


Fig 4.18. Raw expression profiles for *Hsp47*. The wild type (WT) and knock out (KO) profiles are indicated by the arrows. NTC – no template control.

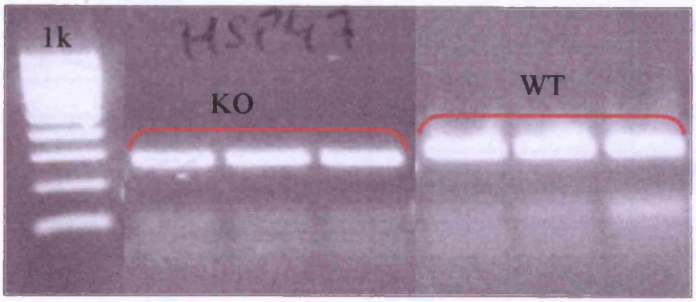


Fig 4.19. Amplification products of *Hsp47* separated on an agarose gel. The wild type (WT) and knock out (KO) products are indicated by the red brackets

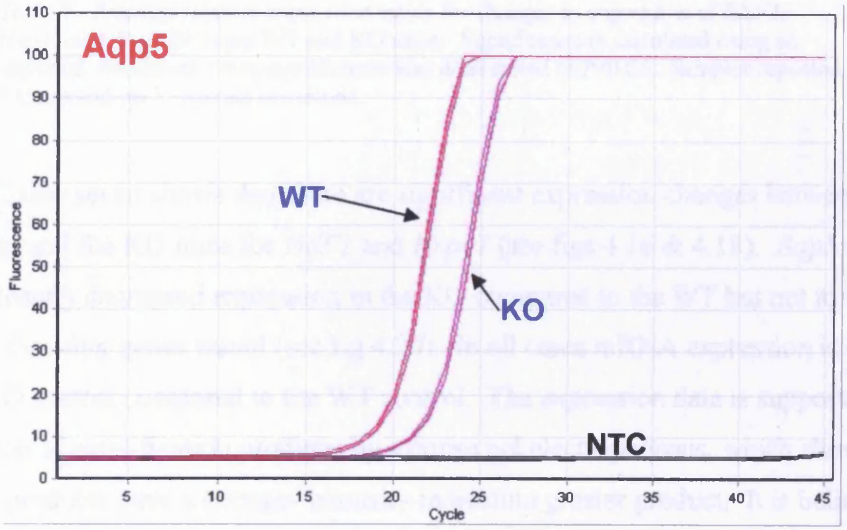


Fig 4.20. Raw expression profiles for *Aqp5*. The wild type (WT) and knock out (KO) profiles are indicated by the arrows. NTC – no template control.

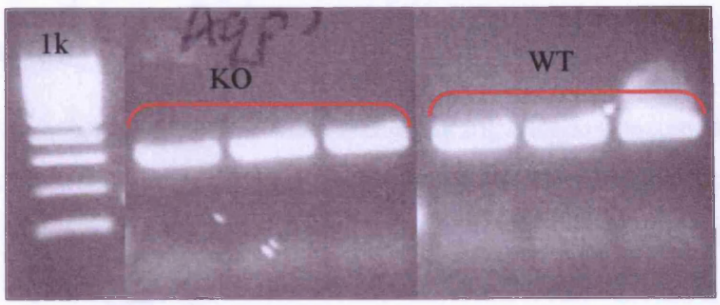


Fig 4.21. Amplification products of *Aqp5* separated on an agarose gel. The wild type (WT) and knock out (KO) products are indicated by the red brackets

Gene/ Sample	Avg rER	Avg gene rER	Sig P<0.05
HSF1			
Sample 1	0.106		
Sample 2	0.087	8.16e-2	P = 0.00001
Sample 3	0.0592		
Hsp47			
Sample 1	0.0528		
Sample 2	0.0528	5.5e-2	P = 0.00001
Sample 3	0.0461		
Aqp5			
Sample 1	0.178		
Sample 2	0.526	0.43	P = 0.011
Sample 3	0.586		

Table 7. Average relative expression ratios for changes in expression of *HSF1*, *Hsp47* and *Aqp5* between WT and KO mice. Significance is calculated using an unpaired two-tailed *t test*, significance was determined at P<0.05. Samples repeated 3 times and on 3 separate occasions.

Table seven shows that there are significant expression changes between the WT mice and the KO mice for *HSF1* and *Hsp47* (see figs 4.16 & 4.18). *Aqp5* also has a significantly decreased expression in the KO compared to the WT but not to such a level as the other genes tested (see fig 4.20). In all cases mRNA expression is lower in the KO system compared to the WT control. The expression data is supported by separation of amplification products by agarose gel electrophoresis, which shows that the WT products have a stronger intensity indicating greater product. It is believed these results are real as repetitions and both positive and negative controls were used. All expression changes were normalised against the housekeeper and calibrated to the WT. The WT was given an expression of 1 and the KO was measured as a fold change against the WT. Expression of *Col8a2* was not detected in the WT or the KO (see appendix B)

4.5 Discussion

There is a debate as to whether the changes in collagen structure are what trigger keratoconus or whether it is keratoconus that causes the collagen changes. Keratoconus is a complex disorder; however it would be far more accurate to describe it as a pure ectasia as it has many associations with other diseases so it is more likely to be a general umbrella term for the clinical appearance rather than a specific condition. Current information about the changes that are being associated with keratoconus is pointing towards a picture of how the ectasia develops. The theory in the mainstream literature is that oxidative stress is the causative factor that leads to the degradation and the thinning, and while this has strong supporting evidence, it does not explain why it occurs in the first place. There must be an initial change that starts the process that divulges from normal development and maintenance to ectasia or at least a breakdown in the system that causes these changes to occur. The actions of oxidative stress would appear to act as an accelerator of the condition rather than the cause. The *Vsx1* model offers and explanation as to what this initial change could be in a particular subset of keratoconus.

At first inspection the *Vsx1* knockout does not appear to be a good candidate as a keratoconus model as initial examination of the cornea using light microscopy and EM did not show anything unusual (Chow et al. 2004). However the findings cannot be dismissed entirely as the mice that were examined were of a relatively young age. This is an important point as keratoconus as previously mentioned does not present from birth and progresses over a course of many years. It could be that the mice examined were too young to show any gross changes that would be picked up at the histological level or the progression was below the threshold for classification. EM pictures (Chow et al. 2004) taken of the cornea were taken from the central regions of the cornea covering ~20% of the total surface area of the cornea (Bob Chow private communication). This potentially misses the initial area of change in the cornea as results presented in this chapter show that the greatest area of change happens in the periphery. The EM results also show altered cell and mitochondria ratios but this has not been analysed further (Bob Chow private communication). The findings and observations of the cornea in these mice have lowered the interest in pursuing a possible connection with keratoconus and at present appear to be the only

information that does not favour the idea that it is a good model. In fact this information has been cited in the literature on many occasions by other researchers to bolster an argument that the *Vsx1* gene should be dismissed from its involvement with keratoconus altogether (Aldave et al. 2007). However it is still a worthy model for studying *Vsx1* in conjunction with keratoconus as the results presented here demonstrate.

The only other mouse model proposed for keratoconus is the MHC-linked mouse model (Tachibana et al. 2002), which has not been evaluated through X-ray diffraction so it is unknown what the collagen structures in this model look like. These mice were examined at 4 weeks of age, which is a fairly young age when compared to when a human keratoconic would start to present and the animals demonstrated quite large and visible changes in the appearance of their corneas. However it is possible that the degree of change in such a short period of time is a point against its suitability as a model or at least is a case for the small percentage of keratoconics that have rapid progression. It would be interesting to see what would be found using X ray diffraction in the corneas of these mice and is a possible future piece of work that lies outside the scope of this study.

4.5.1 Collagen fibril patterning is statistically different from WT littermate controls

X-ray analysis combined with a novel method of quantifying preferential alignment presented in this study shows that there is a significant difference in the direction of collagen fibrils between the knockout and its wild type counterparts. It is possible that the fibril changes that are being seen in the knockout mice are preceding signs that these mice would develop a keratoconic phenotype in later stages of life. This is similar to studies on human clinical recurrent keratoconus where they first show signs of fibril orientation changes before any visible thinning has occurred (Hayes et al. private communication). The patients were diagnosed as recurrent keratoconus through videokeratography and so obvious thinning of the corneas were not apparent. The X-ray study was carried out on the excised corneal button 8mm in diameter, which covers the entire cornea and part of the sclera.

The knock out mouse patterns presented (see figures 4.16 - 4.20) all have areas where the alignment of collagen fibrils is statistically different when compared to an average wild type mouse pattern. These differences seem to be demonstrating a

diagonal area of disruption across the cornea from the superior-nasal quadrant to the inferior-temporal quadrant. This could indicate stretching that the cornea is undergoing. Instead of the fibrils appearing in a circumferential pattern they appear to be distorted and skewed. Some individual fibril directions even appear to be tangential to the wild type fibril. This is most apparent in the peripheral areas of the cornea where the correlation of fibrils within wild type mice is highest. This possibly indicates a mechanical weakness that other forces, such as eye pressure, are acting on (see fig 4.22).

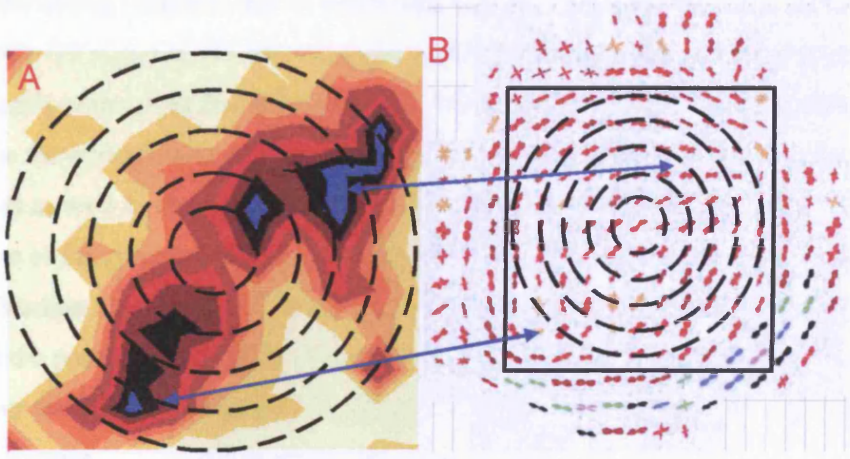


Fig 4.22. Statistically different preferential alignment of collagen fibrils as they relate to an individual cornea. A) A contour map showing the areas of statistically different fibril orientation of knock out cornea 3 (KO3) with an schematic overlay of the circular alignment of the WT cornea. B) Actual orientation of fibrils as measured in a cornea. The area being compared in all corneas is the area inside the square, so the central part of the cornea. The overlay of the WT cornea is also displayed within the square. Arrows highlight the areas that are different with some fibril orientations tangential to the WT schematic. Sample shown is knockout 3.

As can be seen in figure 4.22 the preferential alignment of fibrils is sometimes tangential to the normal circular orientation, which could indicate stretching of the cornea in that direction. It is not believed that this stretching effect is due to the stretching of the tissue during sample preparation as all samples were prepared in the same way and this effect is only seen with the knockout corneas. The changes in fibril direction would precede any visible signs of thinning in the cornea; the fibrils distort as the intraocular pressure starts to act on the weakened collagen structure. It would be interesting to examine very old mice to see whether this effect is more apparent as well as any signs of visible thinning, either through histology or X-ray diffraction

4.5.2 Limitations of performing X-ray diffraction experiments on mouse corneas

The greatest limitations imposed on these experiments are due to the inherent properties of mouse corneas. Mouse corneas are much more difficult to handle then human corneas due to their small size and thickness so special care had to be taken in order to preserve the shape of the cornea when excised from the globe. Compared to other species' corneas that have been used for X-ray diffraction experiments, mice corneas are the smallest with an average diameter of 2-2.5mm. In order to overcome these limitations excisions and to make sure that the most accurate maps were obtained a 0.5 mm rim of sclera was also excised with the cornea in order to provide additionally strength to the excised tissue. These tissues were wrapped in clingfilm to preserve the hydration of the tissue and the 12 o' clock position marked on the clingfilm as well as the epithelial side of the tissue. This helped to maintain the gross structure of the cornea and also aided the handling of the sample later during the X-ray diffraction experiments. Ensuring precise handling of the tissue at this early stage limited the possibility of artefacts that could have arisen during image processing. However this did not completely eliminate all the problems associated with isolating mouse corneas.

The method of preservation of mouse corneas is an important question when preparing for X-ray diffraction experiments. In this study we wanted to collect data about the interfibrillar, intermolecular spacings and the average fibril diameter using small angle X-ray diffraction (SAXS) and match them with their data exploring the collagen fibrils using wide angle X-ray diffraction (WAXS). In order to get both sets of data on the same corneas the samples had to be frozen after the corneas had been excised. This would not have been possible if the corneas had been chemically fixed as this alters the interfibrillar and intermolecular spacings (Fullwood et al., 1992). It should be noted however that chemical fixation preserves mouse tissues for WAXS better than freezing as the shape and curvature of the cornea are preserved, but this would not allow you to gather both WAXS and SAXS data sets of the same cornea. In order to take full advantage of using chemical fixation new cells for holding the sample in place during the experiments would have to be constructed; but the advantages you get in terms of the data are minor if you take full precautions in preparing frozen samples and cost of the design and construction of new sample cells.

The problem associated with the size of the sample is somewhat mitigated after this stage as the cornea is wrapped in clingfilm for the entirety of the experiment. This is primarily to maintain the hydration of the. The aid of a clingfilm wrap eases handling issues and maintains the shape of the cornea for the duration of the experiment. The corneas are wrapped is such a way that only a single layer is either side of the specimen. This is then further stabilised by two Mylar sheets that hold the clingfilm parcel in place in front of the X-ray beam. Although Mylar and clingfilm both produce diffraction patterns they are outside of the pattern produced by collagen fibrils and so are not detrimental to the experiment. The clingfilm also offers a degree of protection from the beam itself, where prolonged exposure can burn through the specimen; however the possibility is remote as the exposure time for each diffraction pattern was kept low.

4.5.3 Developing a novel method for quantifying the degree of preferential alignment in order to compare it to an average control

In order to create an average wild type map to use as a control several steps were needed in order to ensure that each individual point in a map could be accurately compared with others at the same position. Photographs were used to precisely determine the centres of each cornea so that exact positions in each cornea tested could be compared to each other. Samples were then compared using the central point as a reference and a grid of 11 diffraction patterns by 11 diffraction patterns were selected on each cornea. This was the optimal way in ensuring that similar points in each cornea were being compared. It was necessary to calculate the central point using photographic overlays rather than ensuring the same positions were measured during diffraction as although the central areas of the corneas were maintained during tissue preparation the overall shape of the tissue being extracted was not. It is not possible to distinguish scleral collagen from corneal collagen fibres until after data processing.

The process of comparing each fibril patterning used the raw data from each diffraction pattern, which is a process that has been developed by our lab and is well established. Comparing the data points was automated using an algorithm which was written specifically for this purpose and was rigorously tested prior to application. This eliminated human error from the calculation and sped up the process by several

months. The largest problem to overcome in calculating whether the points in one set of data were statistically significant to the other was the largest limitation that needed to be overcome. This was especially important as the data, which in its present forms is essentially qualitatative, cannot be statistically compared without greater additional calculations. The techniques that were available look at pattern differentiation and K-nearest neighbour pattern analysis, which is unsuitable for this type of data. However the data permitted the use of correlation to compare single like-for-like points that could be averaged over an entire data set, avoiding compound errors. The correlation coefficient can be a highly variable statistic. Very large sample sizes can reduce its variability; however, such samples are not always available in empirical settings. One means of addressing the problem of variability is repeatedly to compute correlation coefficients and take the average as a more accurate estimate of the population correlation, which was achieved in this case.

It should be noted though that a set of data is actually a single point in the same location from each of the corneas in a genotype, not a number of data points for the same cornea. This is important as a single correlation value cannot be assigned to an individual cornea, which would be the best solution, but what this does give is the ability to tell if regions within a knock out cornea are statistically different and by extension the cornea itself as a whole. When wild type control corneas are compared to their computed average they do not highlight any areas that are statistically different, although it is conceivable that artefacts within the cornea may produce areas that appear to be statistically different when using this method of analysis. However these types of aberrations and artefacts are usually quite obvious and can be accounted for. This method of analysis works because the standard deviations of the preferred orientations in the wild type are quite low and so this is thought to be the largest limiting factor. This further enhances the need for careful preparation of samples when dealing with mouse models. Conceivably this method of analysis can be converted to use with any other species and would produce better results as the tissues from other species are not as delicate as mouse. It should be kept in mind that if the intention is to use this method of analysis then this should be planned for as preparation of the files can be lengthy depending the number of diffraction patterns taken for each cornea.

4.5.4 Collagen mass distribution shows indications of changes between wild type and knockout mice

There are indications that the distribution of both total and aligned collagen masses are different between the two genotypes however nothing can be shown to be significant in the data's current form. This shows that there is a large amount of collagen being laid down around the periphery of the cornea forming an annulus around it, which is similar to human corneas. The total collagen maps appear similar in both the wild type and knockout corneas with the majority of mass around the annulus of the cornea. The central regions of the cornea in both genotypes seem to show some degree of variability as the collagen mass is not uniform in most cases. The greatest indication that there is a significant difference in the distribution of collagen mass comes from the aligned collagen. The wild type corneas all display an even distribution of aligned collagen around the periphery of the cornea. When this is compared to the aligned collagen from the knockout mice, there is evidence that the distribution of aligned collagen shows a preference for one side of cornea (see fig 4.23 & 4.24).

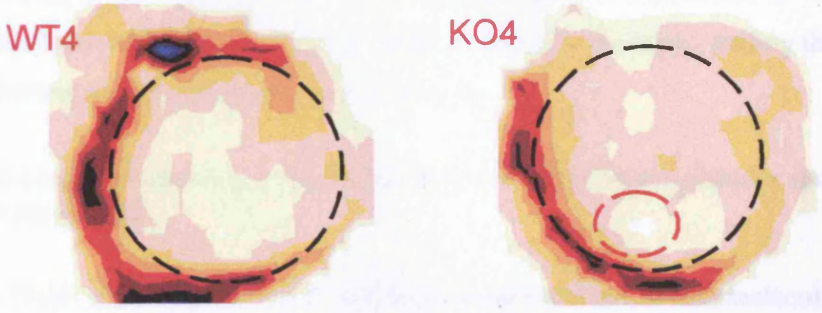


Fig 4.23. Possible effect on the distribution of aligned collagen by the removal of *Vsx1*. WT- wild type cornea #4, KO- knock out cornea #4. Black dashed circles show the area of the cornea. Red circle shows the possible location of a developing cone.

Figure 4.23 demonstrates a possible effect on the distribution of aligned collagen due to the removal of *Vsx1*. Most of the knockout corneas examined demonstrate a preference of aligned collagen to be distributed along one side of the cornea. This effect is similar although not so pronounced to human keratoconic corneas (see fig 4.24).

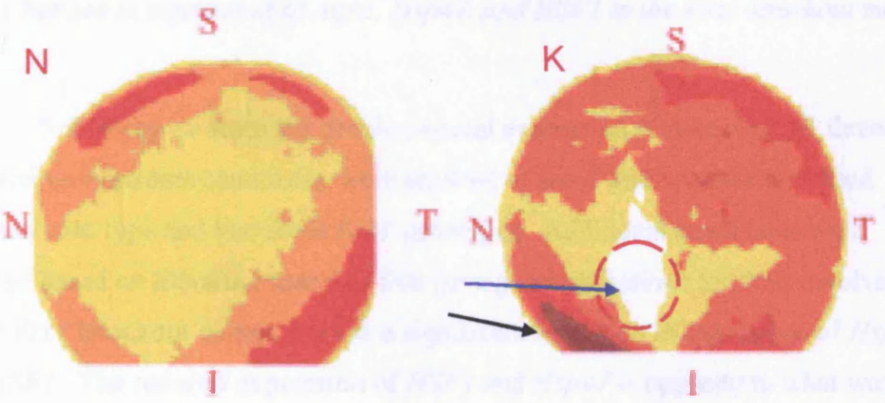


Fig 4.24. Aligned collagen distribution compared in normal and keratoconic corneal buttons. N – normal human cornea. K – Keratoconic cornea. Red circle indicates the position of the developed cone, Blue arrow shows the reduced mass of collagen tissue. Black arrow shows the area of increased aligned collagen mass. S,N,I,T indicate quadrants of the cornea, Superior, Nasal, Inferior and temporal. Figure adapted from Meek et al. 2005.

However it is difficult to determine whether this effect is real or just due to the inherent variability of mouse corneas as the data is not very conclusive due to inherent mouse variability. This could be determined through a larger sample size; however for this study it was not possible. It is suggested that this effect is due to redistribution of the aligned collagen fibrils as the cone develops, pulling the fibrils down leading to an aggregation below the cone.

4.5.5 Interfibrillar spacing, average fibril diameters and intermolecular spacings remain unchanged

There are no significant differences found between the intermolecular and interfibrillar spacings or the average fibril diameter between genotypes. This mirrors the findings between human keratoconics and clinically normal patients (Meek et al., 2005) further strengthening the argument that this is indeed a good model for the disease. In this study we have calculated the average interfibrillar spacing for a normal WT mouse as 44.29nm which is lower than previously stated (Beecher et al. 2006). This has since been confirmed independently (Meek et al. private communication) and is suggested to be a truer value for the average interfibrillar spacing. The average fibril diameter and intermolecular spacings were similar to previously reported.

4.5.6 Changes in expression of Aqp5, Hsp47, and HSF1 in the Vsx1 knockout mouse model

Following on from the developmental expression studies on *Vsx1* three potential downstream candidates were selected to see if their expression varied between wild type and knockout *Vsx1* genotypes. Additional candidates were selected based on literature searches that gave good indications for their involvement In the *Vsx1* knockout cornea there is a significant reduction in expression of *Hsp47* and *HSF1*. The reduced expression of *HSF1* and *Hsp47* is opposite to what was expected in the initial hypothesis that stated that with the removal of *Vsx1* expression should increase the expression of *Hsp47* and *HSF1*. The initial rationale for this hypothesis was based on findings that showed that *Vsx1* binds acts as a repressor of genes. This was demonstrated using *GAL4-HSF1* binding assays to assess the transcriptional activity of both *Vsx1* and *Chx10* (Vsx2) (Dorval et al., 2005). However it appears here that *Vsx1* or a *Vsx1* related mechanism is acting to enhance expression of *HSF1* and *Hsp47* as their expression is reduced in the knockout. It is not unknown for genes to have both enhancing and repressive functions based on their binding capabilities and binding positions. *Hsp47* is a direct target of *HSF1* and its decrease in expression is explained by the proportional decrease in expression of its activator.

Heat shock factors (HSFs) are transcriptional regulators of heat shock proteins (Hsps), which function as molecular chaperones in protecting cells against proteotoxic damage. Mammals have three different HSFs that have been considered functionally distinct: *HSF1* is essential for the heat shock response and is also required for developmental processes, whereas *HSF2* and *HSF4* are important for differentiation and development (Akerfelt et al., 2007). Currently it is not known whether *HSF1* is a direct target of *Vsx1* or the reduction in expression is due to other changes affected by *Vsx1*'s removal. There are certain indicators that it could be a direct target or at least present in a signalling cascade downstream from *Vsx1*. The amount of reduction in expression of *HSF1* is significant compared to the reduced expression of *Aqp5* indicating that the gene itself could be affected. However the action of *HSF1* changes depending on its situation and it is the change in function that could vary across the cornea. *HSF1* is expressed in keratocytes. Under stressed conditions it acts to activate the heat shock response due to various stresses. Under non stressed

conditions however it acts in the collagen production pathway by helping to chaperone pro collagens for assembly into fibrils. In keratoconus there is a non-stressed inflammatory response so the action of *HSF1* could be working in both ways. On the one hand it is helping to produce collagen but on the other it is reacting to stresses such as increased mechanical and oxidative stress. This could contribute to the lengthy progression of the disease as competing functions of the same protein mean that neither is being undertaken at their full capacity. The actions of *HSF1* in the cornea under both stressed and non stressed conditions in the *Vsx1* model would be a valuable next step in determining if this is the case.

There is also a reduced expression of *Aqp5*, although the reduction is not as significant as *HSF1* or *Hsp47*. It is difficult to know the role of *Aqp5* in keratoconus and its relationship with *Vsx1*. The aquaporin family of proteins (AQPs) constitute a collection of water channel proteins that play a critical role in transcellular water movement in many tissues (Liu et al., 2005; Liu and Li, 2005). It appears that AQPs are increasingly playing a role in corneal diseases such as Fuchs dystrophy and Bullous keratopathy (Kenney et al., 2004). *Aqp5* has been characterised as a water channel protein that only transports water and excludes small solutes (Oen et al., 2006). At least 5 AQPs are found in the eye, of which only *Aqp1* and *Aqp5* are found in the cornea. In Fuchs dystrophy and Bullous Keratopathy *Aqp4* was found to have lower expression (Kenney et al. 2004). *Aqp5* transcripts were later found to be absent during gene expression profiling for keratoconus and this has led to speculation that the disruption in the cornea in keratoconus could, in part be due to reduced water movement. Despite this study very little research has been conducted to investigate the possible role of *Aqp5* in keratoconus. The study presented here shows that there is reduced expression of *Aqp5* in *Vsx1* knockout corneas. This reduction is puzzling as there have been no previous indications of any interactions between the two genes.

I do not believe that the change in *Aqp5* is due to any direct relationship between *Vsx1* and *Aqp5* nor for that matter *HSF1* or *Hsp47*. It is more likely that the change in *Aqp5* expression is due to the structural changes that are occurring within the cornea as a result of the removal of *Vsx1*. The lack of transcripts for *Aqp5* was proposed to be used as a marker for keratoconus (Rabinowitz et al., 2005). Direct sequencing of the gene revealed no changes in the *Aqp5* so it was suggested that the gene was being suppressed in keratoconus corneas. However two separate studies have not identified any difference in expression levels between *Apq5* in human

keratoconus and normal controls (Garfias et al., 2008; Kenney et al., 2004). The reduction shown within the results presented here seems to add to the controversy of *Aqp5* involvement. However the reduced or lack of expression could be explained in the situations that regulate *Aqp5* expression. Both shear stress and nitric oxide have been shown to regulate *Aqp5* expression (Nagai et al., 2007; Sidhaye et al., 2008). Both act to reduce cell surface *Aqp5*. It appears that shear stress has a great impact on epithelial function, which could be as a result of keratoconus cone formation. If this is true then it is another indication that the removal of *Vsx1* plays an important role in the development of a keratoconus like phenotype.

The lack of expression of *Col8a2* in both the normal and the knockout would appear to suggest that the expression of *Col8a2* is temporally regulated. Expression of *Col8a2* has been found in the cornea during development (see chapter 3). There is no evidence in the literature to state that *Col8a2* is expressed in adult tissue only that the expression of *Col8a2* is found within the eye, more specifically as a large constituent of Descemet's membrane (Hopfer et al., 2005). *Col8a2* has been previously associated with PPCD (Biswas et al., 2001) and to a lesser extent keratoconus by virtue of the link between *Vsx1*, PPCD and keratoconus. Sequencing of the *Col8a2* gene in patients with keratoconus has not revealed any pathological mutation (Aldave et al., 2007a; Yellore et al., 2005). The lack of expression in the *Vsx1* knockout mouse is intriguing as it offers support to the theory that the two genes are linked. The expression of *Vsx1* and *Col8a2* in development (see chapter 3) offers suggestions that *Col8a2* is a direct downstream target of *Vsx1* and this has also been speculated upon elsewhere (Hopfer et al. 2005). The possibility of this interaction is strengthened by studies performed on *Col8a2* knockout mice that demonstrate that these mice have a keratoglobus-like protrusion of the anterior chamber with a thin corneal stroma (Hopfer et al. 2005). Although mutations in this *Col8a2* do not seem to cause disease, the complete ablation leads to sever anterior segment dysgenesis.

4.6 Conclusion

It can be concluded from the X-ray diffraction data conducted on the *Vsx1* knockout that the removal of *Vsx1* does cause a structural change to occur within the cornea, although further investigation is needed to see exactly what components are affected. In the *Vsx1* knockout mice the average interfibrillar, intermolecular spacings and average fibril diameters do not change, which is also the case with keratoconic and normal human corneas. It has been suggested that fibril orientation is linked with the visual acuity, but this disruption of the fibrils is apparent in both human and mice, that have very different fibril alignments. Therefore, even though both human and mouse are different the effect of this possible underlying defect is the same. Fibril orientations are statistically different in the knockout, which gives possible indications of the onset of a keratoconus phenotype mirroring data gained from recurrent keratoconus phenotypes. There are also indications that the distribution of collagen mass is different between wild type and knockout mice, which are one of the main features of keratoconus. *Vsx1* knockout mice have reduced expression of *HSF1*, *Hsp47* and *Aqp5*. Previous studies have linked reduced *Aqp5* expression with keratoconus and this is demonstrated within the knockout mice. This is the first study to show a decreased expression of *HSF1* and *Hsp47*, genes that are involved in the collagen production pathway. When combined with the X-ray, data these findings act to strengthen the view that the *Vsx1* knockout mouse is a potential model for keratoconus. If confirmed by the wider scientific community that this is indeed a good model then the data presented in this thesis offers potential avenues of future experimentation.

Chapter 5. Mutational study of human *VSX1*

5.1 Aims

- To screen a cohort of patients with keratoconus and compare them to matched background controls to see whether there are any changes present in the *VSX1* gene.

5.2 Introduction

There is an increasing amount of evidence that keratoconus is inherited in some patients and families and that the cause in all patients could have a genetic contribution. A number of candidate loci have been documented for keratoconus including 2p24, 3p14-q13, 15q22, 16q23, 20p11 and 21q (Brancati et al., 2004; Hughes et al., 2003; Rabinowitz et al., 1992; Rabinowitz et al., 2000; Tyynismaa et al., 2002). Current research suggests a number of possible genetic aetiologies for keratoconus; on the one hand keratoconus may be a multifactorial disease, caused by the interplay of environmental factors and many genes, but on the other hand it may be primarily monogenic in some rare families and individuals. To date two candidate genes, *VSX1* and *SOD1*, have been associated with keratoconus and mutations in these gene have been determined to be disease causing (Bisceglia et al., 2005; Heon et al., 2002; Mintz-Hittner et al., 2004) for *VSX1* and (Udar et al., 2006) for SOD1. A total of nine different mutations have been documented between the two genes with eight of them appearing in *VSX1*. Mutations in *VSX1* have also shown to lead to craniofacial anomalies in addition to keratoconus in one family (Mintz-Hittner et al. 2004).

There has been much debate as to whether the associated mutations found with *VSX1* are actually disease causing, as subsequent screening of *VSX1* in much larger sample groups has found the reported disease-causing changes in normal controls (Aldave et al., 2007b; Liskova et al., 2007a; Tang et al., 2008). The principle hypothesis that we are investigating is that keratoconus is a disease in which mutations in single genes may cause disease. Our rationale for this is that, in the gene *VSX1*, eight mutations have been found segregating in families and have been shown

to cause keratoconus. The mutations were also shown to be inherited within these families and although we believe that there may be other genes like *VSX1* that play a role in this disease this is our candidate of choice by virtue of its reported links and previous mutational studies. By gathering our own cohort of keratoconus patients we planned to search for novel mutations as well as confirm existing mutations in a population within the UK.

5.2.1 Hypothesis

- Sequence changes within the *VSX1* gene cause keratoconus.

5.3 Methods

5.3.1 Patient recruitment and ethical approval

Initially ethical approval was granted to recruit patients locally by an internal ethical committee (see appendix D). Later ethical approval was gained from the Cardiff and Vale NHS Trust and the National Multicentre Research and ethics committee (MREC) granting multi-centre ethical approval for recruitment (see appendix E). Patients with keratoconus were either recruited locally from the University Eye Clinic (Cardiff University) or were referred to the study from the University Hospital (Cardiff and Vale NHS Trust) by Dr Vinod Kumar with additional patients recruited from Bristol Eye Hospital by Dr Mike Johnson. Once informed consent was given, patients were enrolled into the study. In total 19 patients with clinical keratoconus at various stages of the disease were recruited. 53 normal background-matched controls were also recruited, representing 106 chromosomal controls. There were no exclusion criteria with regards to age, sex, race or family history. If there was a family history of keratoconus present then family members were asked to participate as well if they were known sufferers. Patients were only included if they had clinically diagnosed keratoconus either from the University Eye Clinic or recommended by collaborating consultants. All stages of keratoconus were included in the study, and the stages for each patient was known.

5.3.2 Proteinase K buffer preparation

10mM tris-HCl pH 8.0 was added to 700ml of dd H_20 on a vortex stirrer. To this 1mM EDTA and 0.5% SDS was also added then the solution was brought to a pH of 7.8 and made up to a total volume of 1 litre.

5.3.3 Proteinase K extraction for buccal cells

Buccal cells were obtained either via a mouthwash of 0.9% saline or via a cheek swab that was then placed into 15ml of 0.9% saline. Samples were then left to settle at 4°C for 30 minutes then centrifuged at 3500rpm for 5-7 minutes. The supernatant was then poured off and 380µl of Proteinase K lysis buffer was added and mixed by pipetting to ensure the pellet dissolved. Samples were then frozen and stored at -20°C. After thawing at 37°C, the samples were vortexed and spun, 20µl of Proteinase K 10mg/ml was added to each, and they were incubated for 2 hours at 37°C in a water bath with continuous shake (~100rpm). Samples were then centrifuged at 14000rpm to pellet insoluble material and the supernatant transferred to a silicon grease eppendorf tube. Finally, the samples were cleaned via a phenol/chloroform extraction, resuspended in 51µl of TE (10mM Tris, 1mM EDTA) and incubated for 15minutes at 37°C to ensure the DNA dissolved. Quantification was then performed to obtain DNA concentration.

5.3.4 Primer Design and PCR conditions

The primers used were taken from Bisceglia et al. 2005. They were optimised across a gradient of 20°C. Optimisation of human primers was carried out on normal human genomic DNA supplied by Dr Votruba, School of Optometry at Cardiff University. A list of human primers is given in table 8.

Exon	Sequence 5'-3'	Product size (bp)	Annealing tmp (°C)
Exon 1 F	CAGCTGATTGGAGCCCTTC	----------	58
Exon 1 R	CTCAGAGCCTAGGGGACAGG	599	58
Exon 2 F	GCACTAAAAATGCTGGCTCA	----------	59
Exon 2 R	GCCTCCTAGGAACTGCAGAA	393	59
Exon 3 F	CATTCAGAGGTGGGGTGTT	----------	62
Exon 3 R	TCTTGTGGTGCCTTCAGCTA	419	62
Exon 4 F	GATCATGCTCGGGAGAGAAG	----------	59
Exon 4 R	CGTTGCTTTGCTTTGGAAAT	394	59
Exon 5 F	CCCCAGAGATAGGCACTGAC	----------	59
Exon 5 R	TGGACAATTTTTGTCTTTTGG	495	59

Table 8. *VSX1* primers and primer conditions. Primers taken from Bisceglia et al. 2005.

PCR products were then separated on an agarose gel as previously stated.

5.3.5 Sample preparation for sequencing

PCR products were prepared for sequencing using the SureClean PCR product cleaning kit (Bioline Plc). Three times the volume of the SureClean reagent was added to the sample and a colour change of purple to yellow was observed. The PCR product sample was then spun down and a pellet was produced according to the manufacturers' instructions. The pellet was then resuspended in 12µl of sdH_2O and frozen ready to be sequenced.

5.3.6 Mutation detection and direct sequencing

Mutation detection and sequencing were carried out at the Wales Gene Park Facility (Cardiff UK). Prepared PCR samples were cleaned and sent to the facility to undergo SNP detection and verification followed by sequencing and characterisation of variants found. PCR setup is carried out in an amplicon free environment on a Beckman Biomek FX platform (Beckman Coulter Inc). Following amplification on MJ Tetrad thermocyclers (GMI Inc), the products are analysed on 3 HT Transgenomic Wave platforms using automated software. This includes automated pattern recognition software for the identification of heteroduplexes. SNP verification is carried out through sequencing of PCR product on an ABI 3100 DNA Sequencer.

5.4 Results

5.4.1 All VSX1 sequence changes found in this study

Gene	Fragment	Base change with 20 bases upstream	Seq change	aa change
VSX1	X1	ACCATGACCGGCCGGGACTC **K**	c.18G>T	S6S
VSX1	X3	CCCAGGGGACATGTGCCCAC **Y**	c.3 -24 C>T	Intronic
VSX1	X3	CTGGAAGAGTTGGAGAAGGC **R**	c.546A>G	A182A
VSX1	X3*	GTCTGGGGTCCCTTTTCTCC **R**	c.650G>A	R217H
VSX1	X3*	GTGAGCCAATCAGCAGTCCC **W**	c.711T>A	P237P
VSX1	X5#	TCCTTTTTATTTTTTTTTTT **T**	c. 809 -6 ins T	Intronic

Table 9. Sequence changes found by screening the *VSX1* gene. Fragment denotes what exon the sequence change was found in, X1 shows exon 1, X3 – exon 3, X5 – exon 5. The bold letter shows the base that is altered. K – G or T, Y – C or T, R – A or G – W – A or T. * indicates that this sequence change was found only in the short isoform, # indicates that this sequence change was only found in the long isoform.

In total 6 polymorphisms were detected in the patients screened (see Table 9). This included two intronic sequence changes in both the third and fifth exon. Three of the sequence changes found, 18G>T in exon one, 546A>G in exon three and 711T>A also in exon three, code for silent changes resulting in no change in amino acid. The other change found in exon three codes for an amino acid change of an Arginine to a Histidine (650G>A) which is likely to be a polymorphism as it occurs in both normal and affected populations (for chromatograms of mutations found see appendix F).

5.4.2 Existing sequence changes of VSX1

Reference SNP ID reported by	Position in the gene		Nucleotide change		Protein change	
	Variant 1	Variant 2	Variant 1	Variant 2	Isoform A	Isoform B
rs8123716	Exon 1	Exon 1	c.18G>T	c.18G>T	p.(S6S)	p.(S6S)
Aldave et al. [34]	Exon 1	Exon 1	c.174G>T	c.174G>T	p.(P58P)	p.(P58P)
rs6115023	Exon 1	Exon 1	c.315C>A	c.315C>A	p.D105E	p.D105E
rs6037016	Exon 1	Exon 1	c.339C>T	c.339C>T	p.(G113G)	p.(G113G)
rs6050307	Exon 1	Exon 1	c.391C>A	c.391C>A	p.R131S	p.R131S
Heon et al. [17]	Exon 2	Exon 2	c.432C>G	c.432C>G	p.D144E	p.D144E
Novel 1	Intron 2	Intron 2	c.504-10G>A	c.504-10G>A	-	-
Novel 2	Intron 2	Intron 2	c.504-24C>T	c.504-24C>T	-	-
rs12480307	Exon 3	Exon 3	c.546A>G	c.546A>G	p.(A182A)	p.(A182A)
rs6138482	Intron 3	Exon 3	c.627+23G>A	c.650 G>A	-	p.R217H
Bisceglia et al. [31]	Intron 3	Exon 3	c.627+84T>A	c.711T>A	-	p.(P237P)

Table 10. Existing sequence changes found by screening the *VSX1* gene in either familial studies or cohorts of unrelated individuals. Table adapted from Liskova et al. 2007.

As can be seen from table 10, most of the reported changes found in this study have all been previously reported. There are two new intronic sequences that are novel for *VSX1*, these are the 809 -6 ins T for exon 5 and the 3 -24 C>T. It is interesting that the majority of changes that occur in exon three are in the short isoform.

5.4.3 Demographic of sequence changes found in this study

A

Gene	Fragment	Seq change	aa change	genotype ab	genotype bb	# normal controls	# keratoconics
VSX1	X1	c.18G>T	S6S	10	1	11/53	0/19
VSX1	X3	X3 -24 C>T	Intronic	1	0	1/53	0/19
VSX1	X3	c.546A>G	A182A	20	1	21/53	2/19
VSX1	X3	c.650G>A	R217H	23	3	26/53	3/19
VSX1	X3	c.711T>A	P237P	20	1	21/53	2/19
VSX1	X5	c. 809 -6 ins T	Intronic	11	1	12/53	0/19

B

Gene	Fragment	Seq change	aa change	% incidence in controls	% incidence in keratoconics
VSX1	X1	c.18G>T	S6S	20.8 %	0 %
VSX1	X3	X3 -24 C>T	Intronic	1.9 %	0 %
VSX1	X3	c.546A>G	A182A	39.6%	10.5%
VSX1	X3	c.650G>A	R217H	49.1%	15.7%
VSX1	X3	c.711T>A	P237P	39.6%	10.5%
VSX1	X5	c. 809 -6 ins T	Intronic	22.6%	0 %

Tables 11A. Patient numbers who have *VSX1* sequence changes. Genotype refers to the sequence change as follows: allele 'a' is the nucleotide of the reference sequence so 'aa' is an unaffected individual, 'ab' is a heterozygous sequence change, 'bb' is a homozygous sequence change. B. Table showing % incidence of polymorphism in normal and affected populations

In total only 3 polymorphisms were found in keratoconic patients in the *VSX1* gene. The polymorphisms found in exon 3 have a high incidence in unaffected controls, each occurring in over 35% of normal controls screened.

5.4.4 Demographic of existing sequence changes

Reference SNP ID reported by	Nucleotide change		Protein change		Observed genotypes			Ethnicity of probands with alleles ab or bb
	Variant 1	Variant 2	Isoform A	Isoform B	aa	ab	bb	
rs8123716	c.18G>T	c.18G>T	p.(S6S)	p.(S6S)	76	9	0	Asian (3), White (5), Unclassified (1)
Aldave et al. [34]	c.174G>T	c.174G>T	p.(P58P)	p.(P58P)	83	2	0	White (2)
rs6115023	c.315C>A	c.315C>A	p.D105E	p.D105E	83	2	0	Black (2)
rs6037016	c.339C>T	c.339C>T	p.(G113G)	p.(G113G)	83	2	0	Black (2)
rs6050307	c.391C>A	c.391C>A	p.R131S	p.R131S	80	4	1	Black (5)
Heon et al. [17]	c.432C>G	c.432C>G	p.D144E	p.D144E	84	1	0	White (1)
Novel 1	c.504-10G>A	c.504-10G>A	-	-	84	1	0	Unclassified (1)
Novel 2	c.504-24C>T	c.504-24C>T	-	-	82	2	0	Asian (2)
rs12480307	c.546A>G	c.546A>G	p.(A182A)	p.(A182A)	32	44	9	Asian (15), White (24), Black (11), Unclassified (3)
rs6138482	c.627+23G>A	c.650 G>A	-	p.R217H	67	17	1	Asian (4), White (13), Black (1)
Biasceglia et al. [31]	c.627+84T>A	c.711T>A	-	p.(P237P)	32	44	9	Asian (15), White (24), Black (11), Unclassified (3)

Table 12. Numbers of patients affected by each sequence change according to inherited allele and ethnicity. Genotypes are as follows, allele 'a' represent the nucleotide of the reference sequence so 'ab' represents an individual who has a heterozygous sequence change and 'bb' an individual who has a homozygous sequence change. Table adapted from Liskova et al. 2007.

From tables 9 and 10 it can be seen that the changes found within this study are not thought to be disease-causing as they appear in control individuals and do not meet the criteria for pathological mutation.

5.5 Discussion

5.5.1 Changes found in the VSX1 gene in a population of patients from South Wales.

In this study a total of 19 patients representing 38 tested chromosomes with clinically diagnosed keratoconus were screened for changes in the *VSX1* gene. This was compared with 53 background-matched control patients representing a control panel of 106 chromosomes. There were no sequence changes found in this study in the *VSX1* gene that can be said to be disease-causing as they occur in both the normal and control populations. Two of the polymorphisms that have been found in both keratoconic and normal patients are silent polymorphisms in that the change in base does not cause a change in amino acid. This means that the *Vsx1* protein would be unchanged. The final polymorphism, R217H causes an Arginine residue to be exchanged for a Histidine residue. Arginine residues carry the strongest positive charge and this is being replaced with a residue that has a weak charge. This could

theoretically affect binding and the polymorphism occurs in the homeobox domain. However the residue is flanked by another Arginine residue and an Aspartate residue indicating a change from a positive to a negative charge. The loss of an Arginine residue at this point would not be that severe, which is possibly why this polymorphism does not have a great impact on function as some of the other reported sequence changes. Several previously reported polymorphisms were also found (see table 19). The reported polymorphisms appear to be very abundant in the control population and all but one of the polymorphisms has been documented in previous studies. It is interesting that there appears to be a high incidence of synonymous polymorphisms in this gene in the normal population and in keratoconus. The high incidences of polymorphisms demonstrate that the *VSX1* gene could be quite robust in its sequence and is not greatly hindered by small changes to its sequence. *VSX1* may not be a crucial gene in development as removing it does not cause lethality (Cow et al., 2004) but may be far more crucial as a temporally expressed gene needed for maintenance and repair. This could offer an explanation as to why mutations are not so detrimental and these changes have been able to propagate among the general population.

5.5.2 Previous mutations in VSX1

The ongoing debate surrounding the *VSX1* gene and its association with keratoconus lies in the previously reported sequence changes within the gene that are said to be disease-causing. The controversy surrounding *VSX1* is whether the sequence alterations are disease causing or whether they are silent polymorphisms.

Six changes have been reported in *VSX1*, leading to protein changes that are suggested to be pathogenic or disease-causing. These are:

p.L17P, Leucine - Proline
p.D144E, Aspartate - Glutamate
p.L159M, Leucine - Methionine
p.R166W, Arginine - Tryptophan
p.H244R, Histidine - Arginine
p.P247R, Proline - Arginine

There are several factors that suggest that sequence alterations are disease-causing. The main reason for a change to be highlighted in a study is that the change only appears in an affected individual, and not within the control group. However,

this on its own is not enough to justify its status as disease-causing. The change must also be shown to segregate within a family of affected individuals and happen in an area of the gene that is thought to affect the final structure of the protein; for example a base change in the gene that codes for a residue in an important domain such as a binding domain and that the changed residue will impair the final function of the protein. Another example of this would be a change in the gene that codes for a stop codon resulting in a truncated protein that is non functional. Of the changes that have been suggested as disease-causing (above), D144E, L159M, R166W, H244R and P247R have now also been reported in unaffected control individuals in larger studies that have been carried out in addition to the studies that originally proposed them as disease-causing (Aldave et al., 2005; Gwilliam et al., 2005; Tang et al., 2008) Although these changes have been found in control individuals their presence indicates that the *VSX1* gene is undergoing rapid changes and future polymorphisms if found, could produce stronger associations with keratoconus.

The L17P change (Bisceglia et al. 2005) results in a change from a Leucine residue to a Proline residue and occurs in the first exon, which means that it lies outside of the crucial homeodomain and CVC domain. However the mutation results in a change in amino acid that is highly conserved in all species where *VSX1* has been identified (Bisceglia et al. 2005). As yet no studies have attempted to explain what may be the actions of these mutations within the gene, such as do the amino acid changes seen affect the folding of the protein? A possible theory explaining the effect of this particular mutation is that it affects the binding of other proteins that help to chaperone the nuclear export of the protein. The mutation occurs outside all the domains identified thus far in the gene. The closest domain to the mutation is the highly conserved nuclear export signal (NES) that is coded for in the first exon (Knauer et al., 2005). The presence of a NES is often sufficient to cause nuclear export of a protein. However the activity of the NES of some proteins e.g., the protein kinase inhibitor (PKI) and the adenovirus E4 34-kDa protein, only becomes exposed after the binding of another protein (Dobbelstein et al., 1997; Wen et al., 1994; Wen et al., 1995). This exposure is normally affected by an adjacent effector domain. Such a domain has not yet been characterised in the *VSX1* gene but it is possible that the action of this mutation is to affect this domain and subsequently, the nuclear export of *VSX1*. In order to determine if this were the case then a nuclear export assay that compares normal *VSX1* to the L17P mutated variant would need to

be carried out. The amino acid change could also give a clue as to the gene's dysfunction. Leucine is an amino acid that has no charge and is an isomer of Isoleucine and both are hydrophobic. In contrast Proline is not hydrophobic and promotes turns in the transcribed protein. This could potentially influence the secondary and tertiary structure of the protein causing impaired function.

The D144E change results in a missense mutation that changes the amino acid from an Aspartate to a Glutamate residue. This mutation has been shown to lead to keratoconus and a wide expression of the disease, from very severe cases to mild aberrations (Bisceglia et al., 2005; Eran et al., 2008; Heon et al., 2002). It is difficult to say whether this mutation itself leads to keratoconus due to the varying forms of keratoconus that this mutation has been associated with. This mutation was also identified in a patient with glaucoma who had a normal cornea (Heon et al. 2002). The position of this amino acid is highly conserved throughout all species (Heon et al. 2002) and lies in the second exon upstream of the homeodomain. Functionally Aspartate and Glutamate share many traits, they are both negatively charged hydrophilic and acidic residues with similar molecular weights. The difference lies in Glutamate that has an extra CH_2 chain in the centre of the residue. This could alter the binding properties of the protein or affect exon splicing, as the residue is close to the intron/exon boundary.

The L159M variant demonstrated segregation in four families with keratoconus. The residue at this position is not highly conserved between species however the change in amino acid at this point is the most drastic change seen compared to the other mutations reported. The aliphatic Leucine residue is replaced by a sulphur-containing Methionine residue. There is no difference in the pH or charge of the residue at this position but Methionine is an initiator on translation. Methionine is one of two amino acids that are coded for by a single codon and is normally found in all proteins at the N-terminus. The change from Leucine to Methionine in this case could be responsible for initiating translation of *VSX1* in the incorrect place in the gene, resulting in a truncated and incomplete protein. This would help to explain why it was found among many families. However this mutation has been found in normal controls (Tang et al. 2008). The action of this mutation is difficult to interpret as the change must not be that severe as to cause KC on its own. It may help that Methionine is not produced by the body so other external factors work to counteract the deficiencies this mutation creates.

Arginine to Tryptophan occurs at position 166 in the *VSX1* protein and crucially occurs in the homeodomain (Heon et al. 2002). In titration experiments it was shown that proteins bind to the *VSX1* WT gene with greater affinity than to the mutated R166W variant (Heon et al. 2002). This mutation has also been examined in the possible functions of *VSX1*; functional assays have revealed that the R166W variant represses expression at a much lower level compared to the wild type protein (Dorval et al. 2005). The Arginine residue has the strongest positive charge and like other charged residues is hydrophilic, which greatly aids binding. Tryptophan on the other hand is the largest amino acid and is uncharged. The presence of a Tryptophan residue in the homeodomain, which is the main binding domain of *VSX1*, would reduce the binding of *VSX1* significantly. Given this it is surprising that later experiments have demonstrated this mutation in normal controls (Tang et al. 2008). A possible explanation for this is that Tryptophan promotes hydrogen bonding, which may overcome the conventional binding properties given by an Arginine residue.

The remaining two mutations that have been found occur in normal control populations. The H244R mutation occurs in the highly conserved and critical CVC domain, which is thought to play a part in binding and repression (Dorval et al. 2005). The Histidine at this position is 100% conserved between all species so it is interesting that while this mutation was detected in keratoconus patients it was also detected in normal controls. If it is suggested that the CVC domain is a binding domain then the presence of and Arginine residue at this position would strengthen the binding. Both Histidine and Arginine are charge amino acids, however Arginine has a much stronger charge due to the differing side chains. It is conceivable that the action of this change would be to prevent or reduce dissociation of *VSX1* from its target protein once it has bound and repressed its target. The P247R mutation was initially found co segregating with the G160D mutation. The mutation had been inherited by a keratoconus proband from her father who was unaffected. The mutation was later found to segregate with keratoconus patients in another study. This mutation also occurs in the CVC domain results in a change from a Proline residue to an Arginine residue. Proline residues as mentioned previously promote turns in the protein sequence and are not charged or hydrophobic. Replacing this with an Arginine would create a residue that has strong binding capabilities. This could conceivably affect the secondary or tertiary structure of the protein increasing its binding affinity for its target, which could disrupt dissociation. If the action of the

VSX1 CVC domain is to repress then this increased affinity could lead to longer inactivation of the target. Other external factors appear to play a part as by itself the mutation does not unconditionally cause keratoconus but it may affect the susceptibility keratoconus to occur.

5.5.3 Importance of Single Nucleotide Polymorphism (SNP) changes

SNPs have conventionally been used as markers within the genome and due to their size as single nucleotides very fine mapping can be carried out. However the importance of SNPs in genes already under investigation changes as now it is the properties that these changes bring to a gene that is of interest. The amount of SNPs found within a gene is a reflection of how fast the gene is evolving. With age the amount of SNPs in a gene can increase and this can lead to changes in its behaviour. In the past it has been thought that synonymous single nucleotide polymorphisms (sSNP) were silent, in that their change did not have an effect on the overall structure of a protein. These polymorphisms are often found whilst searching for disease causing mutations within a gene. These changes are often disregarded in the disease-affected individual because they are found in the control population. This begs the question what is the point of listing polymorphic variants that are not disease causing? It is gradually emerging that these silent changes are becoming more important than was once thought. Cumulatively SNP changes that, individually are silent, could add to the susceptibility to a particular disease. Recently it was found that synonymous SNP changes within the ABCB1 gene can affect protein conformation and function (Sauna et al., 2007). In the case of keratoconus, which is a complex disorder, the silent polymorphisms that are occurring could be acting to increase the susceptibility of patients with sSNPs to keratoconus. If this is the case, it would help to explain why mutations that were previously thought to be disease-causing in *VSX1* have been found in a small number of unaffected controls leading them to be questioned in the disease's involvement.

5.5.4 Limitations of screening unrelated patient samples in mutation screening.

There is a difficulty in recruiting patients for genetic studies of a disease such as keratoconus despite the prevalence of keratoconus being high. Patient reliability in terms of completing the mouthwash themselves and the rate of participation often is poor, which means that the effectiveness of obtaining samples is relatively low. In this study, DNA was gained from buccal cell samples obtained from a mouthwash. The sample was either gained from the patient on site or via the post. Samples from either method yielded suitable quantities of DNA as well as viable amplification products. Specificity of product was ensured through human-specific primers. It was felt that these measures were sufficient in order to obtain accurate sequences for each sample. DNA remained stable for lengthy periods of time frozen at -20°C however samples were good enough not to warrant multiple uses. It should be noted however that if samples are to be stored for longer periods of time then storage at -80°C is recommended. Recruiting unrelated patients has disadvantages compared to finding large families that appear to have an inherited form of keratoconus. Familial keratoconus offers the opportunity to explore any polymorphisms to a greater extent such as seeing whether any mutation found segregates with the disease. Familial studies also let us determine if any inheritance pattern of a gene can be found. Despite the disadvantages of screening unrelated patients the information this type of screening study can provide is useful in that it can help to determine if existing mutations that have been identified are real. It has mainly been through unrelated patient screens that have questioned some of the polymorphisms found so far.

5.5.5 Should screening of VSX1 continue?

Despite the studies that are suggesting the contrary, the screening of *VSX1* should continue alongside the investigation of other candidate genes. Subsequent studies question some of the mutations uncovered thus far, however there are still mutations that are thought to be disease-causing within the *VSX1* gene. Future screening will also discover more polymorphisms, which in light of the increasing roles they are playing makes the screening doubly important. A recent study has also revealed two new variants of the *VSX1* gene with two new exons (Hosseini et al.

2008). These have yet to be characterised and could potentially reveal different roles the gene is thought to play. It is unlikely that keratoconus is a single gene disorder and that a single polymorphism within that gene would result in the disease. It is more likely that changes within the *VSX1* gene alter the susceptibility of that patient to developing keratoconus through a *VSX1*-linked disruption. From the mutation studies alone *VSX1* should definitely remain a candidate gene for this disease, maybe not necessarily as direct causative gene but perhaps as a susceptibility gene for the disease. Combined with functional studies, further molecular characterisation will in time elucidate the roles *VSX1* has to play. However to dismiss the gene entirely from the keratoconus story at this stage would be short-sighted.

Chapter 6. General Discussion

6.1 Summary of results

- *Vsx1* expression was examined during mouse postnatal development at P5, 12 and 17. Significant differences in expression were found between postnatal day 5 and postnatal day 12.
- *Col8a2* expression increased significantly from postnatal days 12 to 17.
- Mouse corneal fibrils develop at postnatal day 10, with the circumcorneal annulus forming at this time point as well.
- Mouse corneal structure continues to develop until fully formed at postnatal day 28.
- Mouse corneas demonstrate a circumferential fibrillar orientation pattern.
- *Vsx1* KO corneas have a statistically significant difference in the fibrillar alignment compared with normal WT controls.
- There is evidence that corneal collagen mass distribution is altered between KO and WT mice. Regions of thinning in the posterior region of the cornea are apparent in the *Vsx1* -/- mice
- There are no significant differences detected in the intermolecular and interfibrillar spacings and no difference in the average fibril diameters between the *Vsx1* KO mouse and WT controls. This mirrors findings with human keratoconic corneas and normal controls using X-ray diffraction.
- There are significant reductions in expression of *Aqp5*, *HSF1* and *HSP47* between the KO and the WT corneas.
- *Col8a2* in not detected in either WT or KO corneas in adult mice.
- Mutational screening of keratoconus patients in South Wales increases the number of SNP detected in the *VSX1* gene but failed to find any novel mutations.

6.2 The *Vsx1* KO mouse as a keratoconus model

In terms of keratoconus, the *Vsx1* KO mouse model offers a good stepping stone for investigation into keratoconus. Although the visual phenotype needs characterising at an older age, the similarities between human keratoconic corneas and mouse *Vsx1* KO corneas are quite striking (see chapter 4). When compared with the other mouse models, such as the MHC keratoconus model, there are greater similarities between KC and the *Vsx1* KO model. Human keratoconus has yet to be linked in any way to the MHC, whereas there is an association with human *VSX1*. The X-ray studies provide strong evidence to support the *Vsx1* knockout mouse model and show statistically different alterations to the structure. Of course, any animal model can only be a stepping stone as a tool to investigate the ectasia and does not offer a full basis for comparison, but it is the best currently available.

The combination of gene expression changes in the model offers possible insights into previously published changes found in keratoconus. *Aqp5* transcripts were found to be absent in keratoconus (Rabinowitz et al. 2005), however, mutations in *Aqp5* in keratoconic corneas were absent (Garfias et al. 2008). From the expression work carried out in this study (see chapter 3 & 4) *Aqp5* itself is not a causative factor in keratoconus; however removal of *Vsx1* in mouse corneas causes the reduction of its expression. It has been reported that shear forces exerted by keratoconic corneal progression and increases in oxidative stress act to reduce the expression of *Aqp5* (Sidhaye et al. 2008, Nagai et al. 2007) and it is logical to assume that this is happening in this case. It should be noted that while *Vsx1* expression has been documented during wound healing in the cornea (Barbaro et al. 2006) *Aqp5* expression during wound healing in the cornea remains unchanged (Song et al. 2004). This strengthens the viewpoint that *Aqp5* expression is not mediated by *VSX1* directly, but by a 'keratoconic environment', which is an environment that leads to formation of a cone. It would be advisable to include *Aqp5* expression tests in any future models of keratoconus to see whether this is indeed caused by the changes in environment rather then by direct action of *Vsx1*. The reductions in expressions of *HSF1* and *Hsp47* have not been previously reported. At present it cannot be stated if these are features of keratoconus and this would need to be clarified with additional experimentation.

6.3 *Vsx1* expression in the cornea is most likely temporally expressed by activated keratocytes

The issue of whether *Vsx1* is expressed in the cornea has added to the controversy surrounding its involvement in disease, specifically *PPCD* and keratoconus. We have found that *Vsx1* is expressed within the cornea at P5, P12 and P17 of development and it is the temporal expression of *Vsx1* which is its most important aspect. This is the most likely explanation as to why expression in other studies has been so variable. This was the first study to show *Vsx1* expression in the mouse cornea over an important period of development (see chapter 3). Recent studies have also demonstrated *VSX1* expression in human neonatal corneas (Hosseini et al. 2008) which is not surprising given the results in chapter three demonstrating *Vsx1* mouse postnatal expression and other studies that have presented mouse neonatal expression (Ohtoshi et al. 2001). So far the location of expression of *VSX1* in the cornea has been only been demonstrated in active keratocytes during wound healing (Barbaro et al. 2006). Although it is possible that it could be expressed elsewhere in the cornea it is likely that *VSX1* temporal expression is going to be linked to periods of keratocyte activity. This would help to explain why expression occurs at these three time points in development as keratocytes are active at this time (Song et al. 2003).

6.4 *Vsx1* is not linked with *PPCD*

The initial linkage of *VSX1* to *PPCD* and keratoconus (Heon et al. 2002) offered researchers opportunities to probe the possible causes of these diseases. However these two diseases are quite distinct and present quite different symptoms, so it does raise the question as to how the same gene can be involved in two diseases that are so distinct. However, there are other examples of genes which appear to be involved in two quite specific diseases with very different phenotypes, so this situation would not be at all unusual, for example the BIGH3 gene. Over expression of a mutant form of BIGH3 in transgenic mice causes retinal degeneration (Bustamante et al., 2008), however other mutations are known to cause Avellino corneal dystrophy (El-Ashry et al., 2003; Huerva et al., 2008; Igarashi et al., 2003). Keratoconus is a multifactorial complex disorder that leads to the cornea developing a cone shape through progressive degradation of its ultrastructure. Posterior

polymorphous corneal dystrophy (*PPCD*) is an autosomal dominant endothelial corneal dystrophy characterized by vesicular lesions and epithelial-like changes of the corneal endothelium. The association between *VSX1* and *PPCD* has not been verified by sequencing of the *VSX1* gene in other studies (Aldave et al. 2005, Krafchak et al. 2005, Gwilliam et al 2005, Liskova et al. 2007, Hosseini et al. 2008). *PPCD* is a posterior corneal disorder and is most likely due in part to disruptions of the Tcf8 gene and its actions on Col4a3 (Krafchak et al 2005). It is likely that *PPCD* is caused by multiple genes such as Tcf8 and Zeb1 (Krafchak et al., 2005; Liskova et al., 2007b; Liu et al., 2008).

6.5 Possible functions of the *Vsx1* gene

6.5.1 Is the action of VSX1 to enhance or is it to repress?

VSX1 is a gene that contains at least one crucial binding domain. It contains the homeodomain and the CVC domain, which are of unknown function. It is suggested that the homeodomain is involved in binding to target genes and then mediating repression. The R166W mutation of *VSX1* has been used in conjunction with binding assays to show that this mutation reduces repression of activated reporter genes (Heon et al. 2002, Dorval et al. 2005). Currently it in unknown what downstream genes are the target of *VSX1*, however expression studies conducted here (chapter 3 & 4) demonstrate that *Col8a2* and *HSF1* may be two possible targets. During postnatal development at the time of eye opening *Col8a2* expression increases significantly at a time point after *Vsx1* expression also increases (see chapter 3). This may be due to eye opening itself. However as *Vsx1* is expressed in keratocytes, which is the point of collagen synthesis, it is possible that *Col8a2* may be a direct downstream target. If this were the case, then the action of *Vsx1* would be to act as an enhancer of transcription rather then a repressor. Similarly in *Vsx1* KO mice, expression of *HSF1* is decreased, which possibly indicates its expression is not being enhanced and further supporting the case for a role as an enhancer. Direct functional studies would be required in order to test these genes as possible downstream targets and would help to elucidate whether *VSX1* has duel functionality.

6.5.2 Is Vsx1 involved in collagen synthesis and reorganisation in the cornea?

An appealing prospect is that *VSX1* aids in the synthesis of collagen fibrils in the cornea, or at least helps to organise collagen fibrils in the corneal stroma. X-ray diffraction studies (see chapter 4) have demonstrated that the fibril organisation in the corneal stroma in *Vsx1* -/- mice is altered from that of the WT. This may occur in two ways: either the production of fibrils themselves is downgraded due to a shortfall in fibril constituents, or the proteoglycans that aid organisation of fibrils are disrupted. The reduced expression of *HSF1* and *Hsp47* (chapter 4) is an indication that the fibrils themselves are being disrupted through downgraded fibril production. Hsp47 is involved in chaperoning collagen type I during non-stress situations (Koide, 2004; Koide et al., 1999; Koide et al., 2000; Koide et al., 2006; Koide et al., 2002; Makareeva and Leikin, 2007; Tasab et al., 2000) and if the chaperoning of these constituents is down regulated then this can lead to incorrect helix formation. It has been proposed that folding of the triple helix requires stabilization by preferential binding of chaperones to its folded, native conformation, which is achieved largely by Hsp47. It has also been proposed that it takes over 20 Hsp47 molecules to stabilize a single triple helix at body temperature. The required 50–200 μM concentration of free Hsp47 is not unusual for heat-shock chaperones in the ER, but it is 100 times higher than used in reported *in vitro* experiments, which did not reveal such stabilization (Makareeva and Leikin 2007). In this case if the expression of Hsp47 is reduced, through a lack of up-regulation by *HSF1* and in turn by *VSX1*, and then there would be a shortage of correctly formed helices. The energetically preferred conformation of type I collagen at body temperature is a random coil, which would need to be degraded. This would in turn account for the increase in degradation seen in keratoconus. Functional studies on *Hsp47* in keratoconic corneas would need to be examined and compared with similar experiments in the *Vsx1* KO mouse. Although this helps to explain how incorrect synthesis of collagen helices may occur, the direct targeting of *Vsx1* to either *HSF1* or *Hsp47* must be established before the interaction of these 3 genes and their role in keratoconus can be confirmed (see fig 6.1).

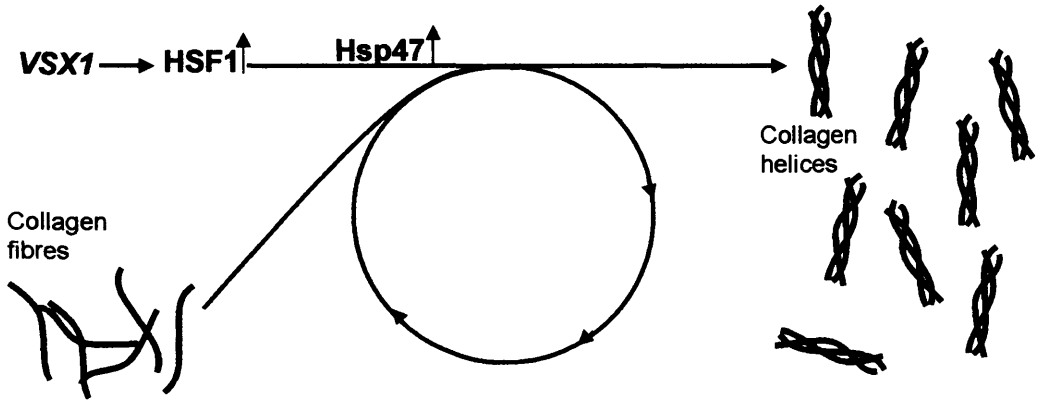


Fig 6.1. A schematic proposed for the actions of *VSX1*, *HSF1* and *Hsp47* in the correct folding of collagen helices.

The second possibility is that the proteoglycans are altered as a result of *VSX1* disruption. The *Vsx1* KO model would provide an excellent tool to examine the proteoglycans to see if this is the case. Proteoglycans help organise and maintain collagen fibrils in the cornea. In keratoconus at least three proteoglycans, decorin, biglycan (Funderburgh et al., 1998) and keratocan (Wentz-Hunter et al., 2001) are known to be increased in keratoconic corneas. The disruption of collagen fibril orientations in the *VSX1* KO may possibly indicate a functional pathway. Given the proposed repressive functions of *VSX1* increased proteoglycan content in *Vsx1* -/- mice would offer a good starting point for further study.

6.6 The possible mechanisms and causes of keratoconus

Keratoconus is a complex disorder that is progressive up until a point where it normally arrests. The time period that keratoconus progresses over can vary depending on the severity and is unique to each patient. The findings presented in this thesis do offer possible explanations as to how certain mechanisms can lead to the reported changes associated with keratoconus, and combined with previous research, can help to provide a theory as to its possible cause.

6.6.1 VSX1 is a susceptibility gene for keratoconus

Previous mutation studies indicate a role for *VSX1* in keratoconus. The mutations that have been discovered all lead to missense mutations within the gene that can lead to alterations in binding properties or to protein conformations (see chapter 5). When *Vsx1* expression is removed in the mouse there is a clear and statistically altered corneal defect that results (see chapter 4) in a keratoconus phenotype. Collagen fibril alignment becomes disrupted and there is a possibility that the mass distribution is altered as well. Combined with these alterations, key genes also have a reduced expression; *Aqp5* expression is reduced as well as *HSF1* and *Hsp47*. These findings indicate that *VSX1* missense mutations alone may not be enough to cause keratoconus, but are enough to trigger a series of events in the cornea that will lead to keratoconus depending on other susceptibility factors.

When *Vsx1* expression is ablated it leads to a keratoconic-like phenotype. The mice that were analysed were all young adults so this could explain the degree of disruption that was seen in our X-ray experiments. Combined with the inherent properties of mouse corneas (the thickness and the size), this would offer an explanation as to why no gross changes have been witnessed under histological examination (Chow et al. 2004). The electron microscopy was performed in the centre of the cornea at a young age (Dr R. Chow personnel communication) and this may have missed other changes that were occurring in the periphery of the cornea, which is usually the first place for any signs of keratoconus to occur. Mutations in the *VSX1* gene are being associated with keratoconus; however individual mutations are not enough to fully cause keratoconus by themselves as most of these mutations have

been found in normal controls. The severity and likelihood of a mutation being associated with keratoconus depends on the location of the mutation and the nature of the mutation. Changes in residue in the crucial binding domains appear more frequent than mutations that lie outside these domains (see chapter 5). If the mutation is likely to strengthen or weaken binding, such as an introduction of an Arginine residue, then the likelihood of keratoconus is increased. It is therefore logical to assume that only complete disruption of *VSX1* will trigger the start of the disorder but missense mutations that do not completely eliminate function require other factors to allow the initial disruption to progress (see fig 6.2).

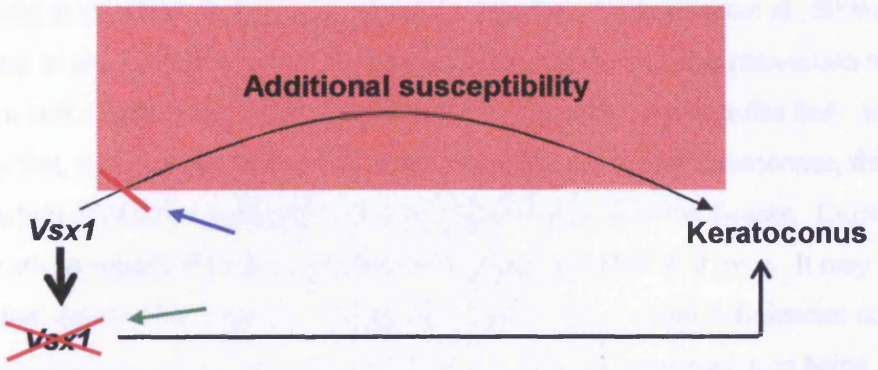


Fig 6.2. Proposed initiation of keratoconus by *VSX1*. The blue arrow indicated missense mutations of *VSX1* that does not completely destroy the function. The green arrow indicates no expression of *VSX1* at all.

If the premise that *VSX1* is involved in the synthesis/organisation of collagen in the cornea is correct then it is most likely that the initial injury caused by *VSX1* inactivation would occur during development, as so far this is the only time in the cornea when *Vsx1* expression has been recorded (see chapter 3). This would also fit in with the slow progressive characteristics of keratoconus. Human keratoconus presents in the teen years and this presentation may be due to an acceleration of the growth the entire body undergoes during this time acting on the initial impairment. Cases of keratoconus from birth are rare if not non-existent and the presentation of keratoconus makes it difficult to screen potential keratoconus candidates for changes in *VSX1* before clinical presentation.

6.6.2 Superoxide Dismutase 1 (SOD1) is a susceptibility gene for keratoconus

In similar fashion to *VSX1*, SOD1 has also been proposed as a candidate gene for keratoconus (Udar et al. 2006). A unique genomic deletion within intron 2 close to the 5' splice junction of the SOD1 gene was identified in three patients with KC. Moreover, mRNA from one affected individual also had two transcript splice variants (LE2 and LE2E3) that others have shown to code for proteins lacking the active site of the SOD1 enzyme (Udar et al. 2006). Superoxide dismutase isoenzymes are altered in keratoconus (Behndig et al., 2001; Behndig et al., 1998) and there is a long established connection between oxidative stress and keratoconus (Atilano et al., 2005; Behndig et al., 2001; Brown et al., 2004; Buddi et al., 2002; Chwa et al., 2006a; Kenney et al., 2000; Kenney et al., 2005). Since establishing this connection there has been a lack of follow up within the scientific community to pursue this link. It is likely that, whereas *Vsx1* disruption would trigger the onset of keratoconus, the disruption of SOD1 would act to prevent the acceleration of the disease. Currently there are no reports that have examined the cornea in SOD1 KO mice. It may be the case that detailed investigation of a SOD1-/- cornea may reveal deficiencies consistent with keratoconus but at the moment it is best to class this association as being susceptibility rather than causative. SOD1 null mice do have associations with cataract formation (Olofsson et al., 2007a; Olofsson et al., 2007b), so it may be the case that further eye-related abnormalities are yet to be found. The association made thus far is valid and important in understanding the mechanisms of keratoconus but further investigations are needed.

6.6.3 Keratoconus is susceptibility dependent and accelerated by oxidative stress to the point of arresting

The theories presented in the wider scientific literature for keratoconus often rely on a single causative factor to explain the initial onset of the disease. However with a complex disorder surely this cannot be the case. It is more likely that keratoconus has multiple susceptibility factors, which combine together and allow an initial trigger to start the degradation. This degradation is then accelerated by an incremental build up of oxidative stress that eventually leads to the degradation arresting through oxidative stress mediated crosslinks (see fig 6.3).

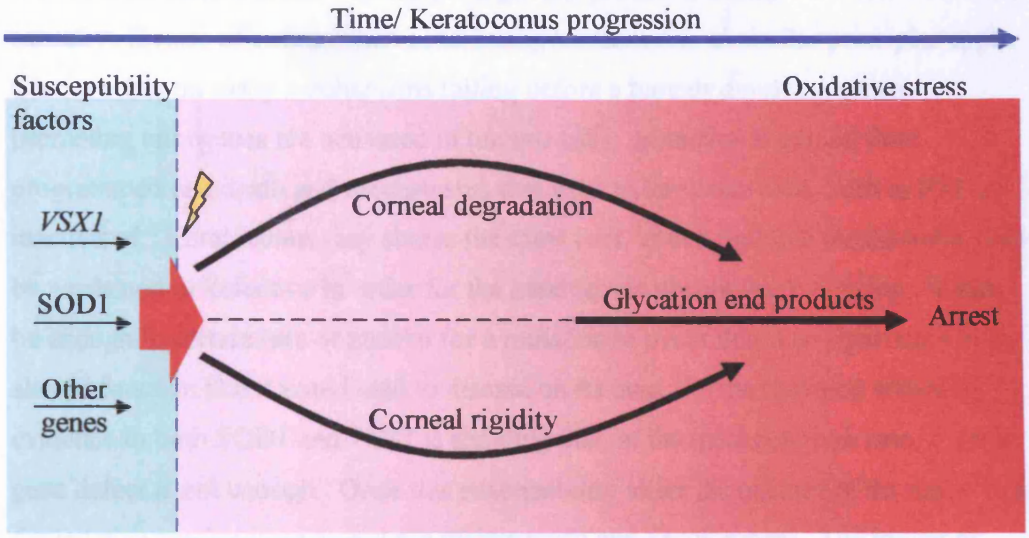


Fig 6.3. A flow diagram for keratoconus over time. Green area indicates a time for susceptibility factors to become unbalanced. The red arrow indicates the transition into keratoconus. The yellow flash denotes the trigger that starts the degradation. The red area shows the amount of time where the condition is accelerated by oxidative stress. The blue arrow indicates the passage of time. The dashed line indicates the normal meridian for corneal rigidity and degradation.

Figure 6.3 is a proposed theory for keratoconus that tries to explain the different mechanisms that occur during the progression of the disease. In order for keratoconus to develop one or more susceptibility factors must be present in order to create an environment that allows the normal cellular mechanisms to become unbalanced. When this occurs keratoconus is triggered, which leads to increasing levels of oxidative stress over time. Over time as the amount of oxidative stress increases the cornea degrades and loses rigidity due to stress related mechanisms, either mechanical or cellular accelerated by the increased oxidative stress environment. This continues to increase until the formation of glycation end products starts to reduce the degradation by increasing rigidity through artificial crosslinks. This eventually leads to arrest but not quick enough to prevent long term structural changes to the cornea as a whole. The severity and degree of keratoconus progression would be dependent on the number of susceptibility factors and nature of mutations within them.

The susceptibility factors that have been associated with keratoconus have traditionally been associated with mechanical stressors, such as the tendency to eye rub and eye lid pressures on a weakened cornea (Donnenfeld et al., 1991;

McMonnies, 2008; Nash et al., 1982; Ozcan and Ersoz, 2007; Weed et al., 2008). However all factors eventually require a genetic defect. It is helpful to think about the causative factors of cancer when considering keratoconus, as similar principles apply. Cancer relies on many mechanisms failing before a tumour develops; cancer promoting oncogenes are activated in tumour cells; protection is gained from programmed cell death and mechanisms that exist to terminate cells, such as P53 are inactivated. Keratoconus may shares the same trait, in that multiple mechanisms must be weakened or defective in order for the keratoconic phenotype to develop. It may be enough in certain rare occasions for a mutation to occur that is so significant in its altered function that it could lead to disease on its own, but the mutation screening evidence in both SOD1 and *VSX1* is showing that, in the most common case, a single gene defect is not enough. Once this susceptibility alters the balance in the tissue then degradative mechanisms overcome maintenance and development. The trigger of keratoconus is a subject of great debate, being a complex disease it is unlikely that there is a single causative trigger. In accordance with figure 6.3 the trigger would be created when there is an imbalance in the processes necessary for normal corneal growth and repair, which is brought about by an accumulation of susceptibility factors.

The increased degradative processes generate free radicals and reactive oxygen/nitrogen species (Chwa et al., 2006b) that act to further increase their production. This also propagates (Brown et al., 2004) the additional characteristics seen in keratoconus such as increased mitochondrial damage (Atilano et al., 2005), increased proteinase activity (Collier, 2001; Collier et al., 2000; Sawaguchi et al., 1994; Sawaguchi et al., 1989; Smith et al., 1998), decreased proteinase inhibitors (Opbroek et al., 1993; Sawaguchi et al., 1990) and loss of transcripts of alpha-enolase and beta actin (Srivastava et al., 2006). The progression of keratoconus normally arrests on its own and this is also likely due to the actions of increased oxidative stress. This would happen by a similar mechanism to the new treatments that are trying to form artificial crosslinks by promoting increased reactive oxygen to cause advanced glycation end products to accumulate in the corneas of patients with keratoconus (Dawczynski et al., 2002). These have the potential to form crosslinks that lock the cornea in place preventing further degradation arresting the condition.

6.7 Future Experiments

Based on the work carried out in this thesis a number of avenues of research should be investigated to see whether the theories presented here can be justified with supporting biological evidence. The majority of keratoconus research is directed at discovering new genes using mutational studies in order to establish a single causative gene, which is probably not the case. However, additional research is needed to clarify the existing gene connections that have been established in order to determine whether there are any polymorphisms present that may affect their function. Existing linkage studies have identified candidate genes (see chapter 1, table 1) which may prove to be additional susceptibility factors for keratoconus; each represent opportunities to further elucidate functional pathways that can lead to keratoconus if unbalanced.

To further clarify the role of *VSX1* in keratoconus, the proposed *Vsx1* KO model should be used to investigate the levels of degradative enzymes and inhibitors of degradation to see if similar results to human keratoconus corneas can be found. Additional EM should also be undertaken in order to clarify the changes that are happening in the periphery of the cornea where initial changes start to occur. Additional EM should also be undertaken on older mice in the centre of the cornea in order to see whether there is any collagen degradation or evidence of fibril slippage. The location of *Vsx1* in mouse development should be investigated in order to see whether the expression of *Vsx1* does indeed occur in stromal keratocytes or if it is present elsewhere. If *Vsx1* is expressed in activated keratocytes then this would demonstrate that similar mechanisms are at work in development and wound healing. This would also offer support to the role of *Vsx1* in collagen synthesis. Additionally if *Vsx1* is involved with collagen organisation, which may be apparent due to the altered fibril patterning (see chapter 4), then there is a good chance that proteoglycans such as decorin, are in some way altered. These glycoproteins should be characterised in the *Vsx1* KO. The model can also be used to examine the development of the cornea without *Vsx1* expression, similar to the developmental X-ray studies conducted here in wild type mice (see chapter 3). This would provide the opportunity to see whether there is a potential trigger effect that allows an initial defect to manifest and then progress to a keratoconic level of alteration. This may determine whether there

are developmental cues that act as an additional accelerator to keratoconus which would provide insights as to why most keratoconus cases present in their teens.

The development of the circum-corneal annulus in the mouse was seen at postnatal day 10, a time point which also saw significant changes in the expression of *Vsx1* and *Col8a2*. However day 10 precedes the crucial period of eye opening. To investigate whether the changes presented here are due to eye opening it is necessary to repeat the same expression studies and X-ray experiments on mice that do not open their eyes. An approach would be to artificially prevent the eyelids from opening and carry out the same developmental studies that were performed here. Cell culture studies using corneal keratocytes from postnatal mice would provide opportunities to see whether *Vsx1*is expressed while corneal keratocytes are active. If this is the case then it would strengthen the argument for *Vsx1*'s role both in development and wound healing and that the pathways involved in both processes are similar.

Chapter 7. References

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Appendix A

"Comparative study of fibrillar collagen arrangement in the corneas of primates and other mammals."

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Comparative Study of Fibrillar Collagen Arrangement in the Corneas of Primates and Other Mammals

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ABSTRACT

This study is a comparative study of the relationship between corneal structure, morphology, and function in a range of mammalian species. X-ray scattering patterns were gathered at regular spatial intervals over the excised cornea (and in most cases also the scleral rim) of humans, marmosets, horses, cows, pigs, rabbits, and mice. All patterns were analyzed to produce quantitative information regarding the predominant orientation of fibrillar collagen throughout the tissue. The predominant direction of corneal collagen varies between mammals. This variation is not related to the size, shape, or thickness of the cornea or the frequency with which the animal blinks. A relationship does, however, appear to exist between corneal collagen arrangement and visual acuity. An excess of collagen directed toward one or both sets of opposing rectus muscles is a feature of animals that have an intermediate to high level of visual acuity. There is a significant variation in the arrangement of corneal collagen between different mammalian species. This finding may be related to differences in the frequency of action and the forces generated by the various extraocular muscles during eye movement and image fixation. Anat Rec, 290:1542–1550, 2007.

Key words: cornea; species; collagen; x-ray scattering

The cornea, often referred to as "the transparent window at the front of the eye," provides a tough external barrier to protect the contents of the eye from injury while also maintaining a precise curvature to enable light to be focussed onto the retina. The mammalian cornea is composed mainly of water and type I collagen fibrils, and it is the unique arrangement of this collagen that governs the strength, shape, and transparency of the tissue. The long, cylindrical collagen fibrils have a uniform diameter, a regular separation distance, and lie parallel to each other within layers (lamellae), which are themselves stacked parallel to the surface of the cornea. The diameter and separation distance of the fibrils differs between species but the proportion of the corneal stroma occupied by hydrated collagen fibrils remains constant (Meek and Leonard, 1993).

As initially proposed by Kokott (1938) and more recently confirmed using x-ray scattering (Meek et al., 1987; Aghamohammadzadeh et al., 2004; Boote et al., 2006), most lamellae in the central region of the human

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TABLE 1. Sample details

Species	Sample details	Source	Storage
Human *Homo sapiens sapiens*	Right cornea with 3-mm scleral rim. Tagged at superior. (n = 2)	Great Wall Hospital, Bejing, China	Fixed 10% formalin
Human *Homo sapiens sapiens*	Paired left and right cornea with 3mm scleral rim. (n = 2)	Manchester Eye Hospital, UK	Frozen −80°C
Human *Homo sapiens sapiens*	Left corneas with 3-mm scleral rim. Tagged at superior. (n = 2)	Bristol Eyebank, UK	Frozen −80°C
Common marmoset *Callithrix jaccus*	Paired left and right cornea with 0.5-mm scleral rim. Tagged at superior. (n = 20)	Oxford University, UK	Frozen −80°C
Horse *Equus caballus*	Cornea with a 2-mm scleral rim. Vertical meridian marked. (n = 3)	Abattoir, Bristol, UK	Frozen −80°C
Cow *Bos indicus*	Cornea with a 2-mm scleral rim. Vertical meridian marked. (n = 5)	Abattoir, Bristol, UK	Frozen −80°C
Pig *Sus scrofa domesticus*	Cornea with a 2-mm scleral rim. Tagged at superior. (n = 3)	Abattoir, Bristol, UK	Frozen −80°C
New Zealand White Rabbit *Oractolagus cuniculus*	Cornea with a 2-mm scleral rim. Tagged at superior. (n = 3)	Heath Hospital, Cardiff, UK	Frozen −80°C
Laboratory mouse *Mus musculus*	Cornea with a 0.25-mm scleral rim. Tagged at superior. (n = 13)	Cardiff University, UK	Frozen −80°C

cornea, particularly in the deeper layers of the stroma, lie in the superior–inferior and nasal–temporal directions. As the tensile strength of connective tissue is determined in part by the orientation of collagen fibrils in relation to the direction of stress (Jeronimidis and Vincent, 1984; Hukins and Aspden, 1985), this has prompted suggestions that the preferred orthogonal orientation of fibrils in the central human cornea may be necessary to resist the mechanical forces of the four rectus muscles that insert behind the limbus at opposing positions along each meridian (Kokott, 1938; Daxer and Fratzl, 1997; Newton and Meek, 1998). A limbal annulus of collagen surrounds the human cornea and is believed to provide additional reinforcement at the point where it meets the lesser curved sclera (Maurice, 1984; Newton and Meek, 1998; Aghamohammadzadeh et al., 2004).

Based on the birefringence properties of the bovine cornea, it was suggested that a nonrandom distribution of lamellae was also present in the bovine cornea (Kaplan and Bettleheim, 1972). Small-angle light scattering of bovine and rabbit cornea confirmed this to be the case in both species (McCally and Farrell, 1982), but the authors were unable to determine the precise orientation of the stromal lamellae. Of interest, analysis of single x-ray scatter patterns obtained from the central cornea of over 50 vertebrate species (which included primates), revealed the human cornea to be unique in possessing an arrangement of stromal collagen that is preferentially orientated along both the superior–inferior and nasal–temporal meridians (Meek et al., 1987).

X-ray scattering is a powerful technique for studying the gross orientation of collagen fibrils in the cornea. As corneal collagen molecules lie near axially within the fibrils, the high-angle equatorial x-ray scattering pattern arising from the lateral packing of the molecules in the stroma can be used to determine the preferred direction of the fibrils at that position in the cornea. In recent years, this method has been used successfully to map the predominant orientation of collagen at fine spatial intervals throughout the entire human and marmoset cornea and limbus (Aghamohammadzadeh et al., 2004; Boote et al., 2004, 2006).

Given the frequent use of nonhuman species in studies of corneal biomechanics and that the size and orientation of collagen fibrils largely contributes to biomechanical calculations (Jeronimidis and Vincent, 1984; Hukins and Aspden, 1985), it is clear that a greater knowledge of the precise arrangement of corneal collagen in species other than human is needed. Here, we have addressed this issue by using x-ray scattering to map the predominant orientation of corneal and limbal collagen in the human, marmoset, horse, cow, pig, rabbit, and mouse.

MATERIALS AND METHODS

Tissue Samples

All tissue samples used in this study (Table 1) were obtained in accordance with the tenets of the Declaration of Helsinki for the use of human tissue and the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic research.

In the majority of cases, the in vivo orientation was marked on the scleral rim at the 12 o'clock position by means of a nylon suture or a surgical skin marker pen. In the case of the horse, cow, and pig cornea, where the in vivo orientation was not known, the vertical meridian was identified by the elliptical shape of the cornea. To minimize tissue dehydration, frozen samples were wrapped in Clingfilm™ (Superdrug Stores Plc., Croydon, UK) before freezing in liquid nitrogen-cooled isopentane. Each sample was defrosted at room temperature immediately before data collection.

X-ray Scattering Data Collection

High-angle x-ray scatter patterns were collected from most samples on Station 14.1 at Daresbury Synchrotron Radiation Source (Warrington, UK), using a 200 × 200

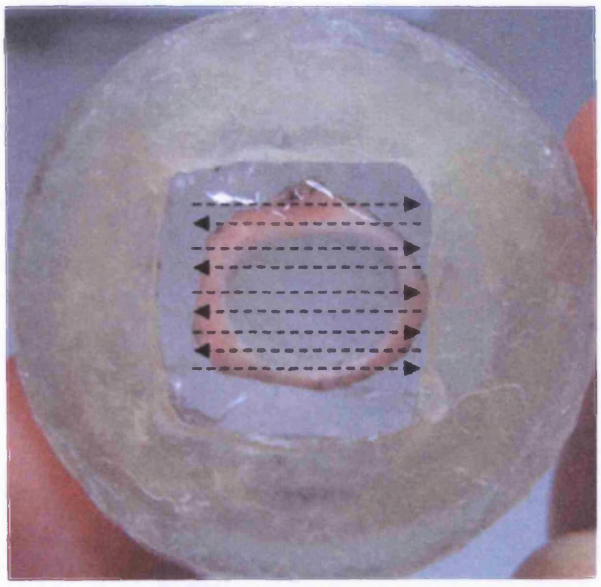

Fig. 1. A pig cornea (with scleral rim) enclosed within a sealed sample holder. X-ray scattering patterns were recorded at regular intervals across the cornea in the directions indicated by the arrows.

micron beam with a wavelength of 0.1488 nm. The images were recorded on a Quantum 4R CCD detector (ADSC, Poway, CA) placed 150 mm behind the sample. The rare opportunity to use the micro-focus x-ray beam (measuring 5 microns in diameter and with a wavelength of 0.984 nm) on Station ID-13 at the European Synchrotron Radiation Facility (Grenoble, France) was utilized to obtain comparable x-ray scattering data for an additional pig cornea.

Each cornea was placed in its correct orientation into a sealed sample holder enclosed between two sheets of Mylar (Dupont-Teijin, UK; Fig. 1). The sample holder was then carefully positioned onto a computer-operated translation stage (Newport Spectra-Physics Ltd., Newbury, UK) so that the most anterior side of the cornea faced the x-ray beam. X-ray scatter patterns resulting from an exposure time of 75 sec (human), 150 sec (marmoset), 30 sec (pig), and 45 sec (rabbit and mouse) were collected over the entire cornea and scleral rim of each specimen at regular intervals of 0.8 mm (human), 0.5 mm (marmoset), 1 mm (pig and rabbit), and 0.2 mm (mouse). Due to the large size of the horse and cow cornea and the limited capacity of the specimen holder, x-ray scatter patterns resulting from a 20-sec x-ray exposure were collected at 1-mm intervals over a 14-mm trephined corneal disc taken from the center of each cornea.

X-ray Diffraction Data Analysis

A circular x-ray scatter reflection, formed as a result of the interference of x-rays scattered by the collagen molecules within the fibrils, was present on each x-ray scatter pattern. In cases where the collagen was orientated equally in all directions within the plane of the tissue, a uniform ring of x-ray scatter was formed; however, when there was an excess of fibrils lying in a particular direction (Fig. 2A) or directions (Fig. 2B), lobes of increased scatter intensity were seen at right angles to the dominant orientation of the fibrils.

Using image analysis software (Optimas 6.5, Media Cybernetics, UK) and Microsoft Excel (UK), a 0–360 degree intensity distribution profile was produced for each x-ray pattern by measuring the intensity of x-ray scatter around the intermolecular reflection (Fig. 2C,D). Each intensity distribution profile was normalized against the exposure time and the average x-ray intensity during exposure (recorded by an ion chamber placed between the x-ray beam and the sample). At this point, the signal to noise ratio was improved by folding the intensity profile; this was possible without the loss of any data, due to the centrosymmetric nature of x-ray scatter patterns. The area under the intensity profile, which is proportional to the total amount of collagen through which the x-ray beam has passed, can be separated into two components—the scatter from collagen lying equally in all directions within the plane of the cornea (isotropic scatter) and the scatter from collagen fibrils that adopt a preferred orientation (aligned scatter) (Fig. 2C,D).

Removal of the isotropic scatter from the intensity profile leaves only the scatter from aligned fibrillar collagen. To ascertain the preferred orientation of these aligned fibrils, it was necessary to first shift the profile by 90 degrees to account for the fact that the high-angle equatorial reflection occurs at right angles to the fibril axis. Using statistical analysis software (Statistica 7, StatSoft Ltd., UK), the intensity profile was then converted into a vector plot (Fig. 2E,F), in which the distance from the center to the edge of the plot at any given angle is proportional to the intensity of x-ray scatter from aligned collagen molecules oriented in that direction. Individual vector plots were then compiled onto a grid relating to corneal position to show the preferred orientation of aligned collagen in each cornea.

The specific arrangement of corneal collagen in each species was examined alongside published values (where available) of corneal size, radius of curvature, and thickness as well as the frequency with which the animal blinks (shown as the interval between subsequent blinks) and its visual acuity (Table 2). Visual acuity, which is measured in cycles per degree of visual field, may be defined as the spatial resolving capacity of the visual system. It provides a measure of the eye's ability to distinguish one object from another in terms of degree angles of visual field.

As no values could be found in the literature for the blink interval of the pig, this value was measured by assessing videographic footage acquired from nine healthy Yorkshire/Landrace mix breed sows housed in single stalls for birthing. The time between subsequent blinks was recorded in several minute episodes using the video time counter, and the average blink interval was calculated.

RESULTS

Figure 3A shows the predominant direction of collagen in the human cornea. Within the central 5.6 mm, most fibrils lie in the superior–inferior and nasal–tem-

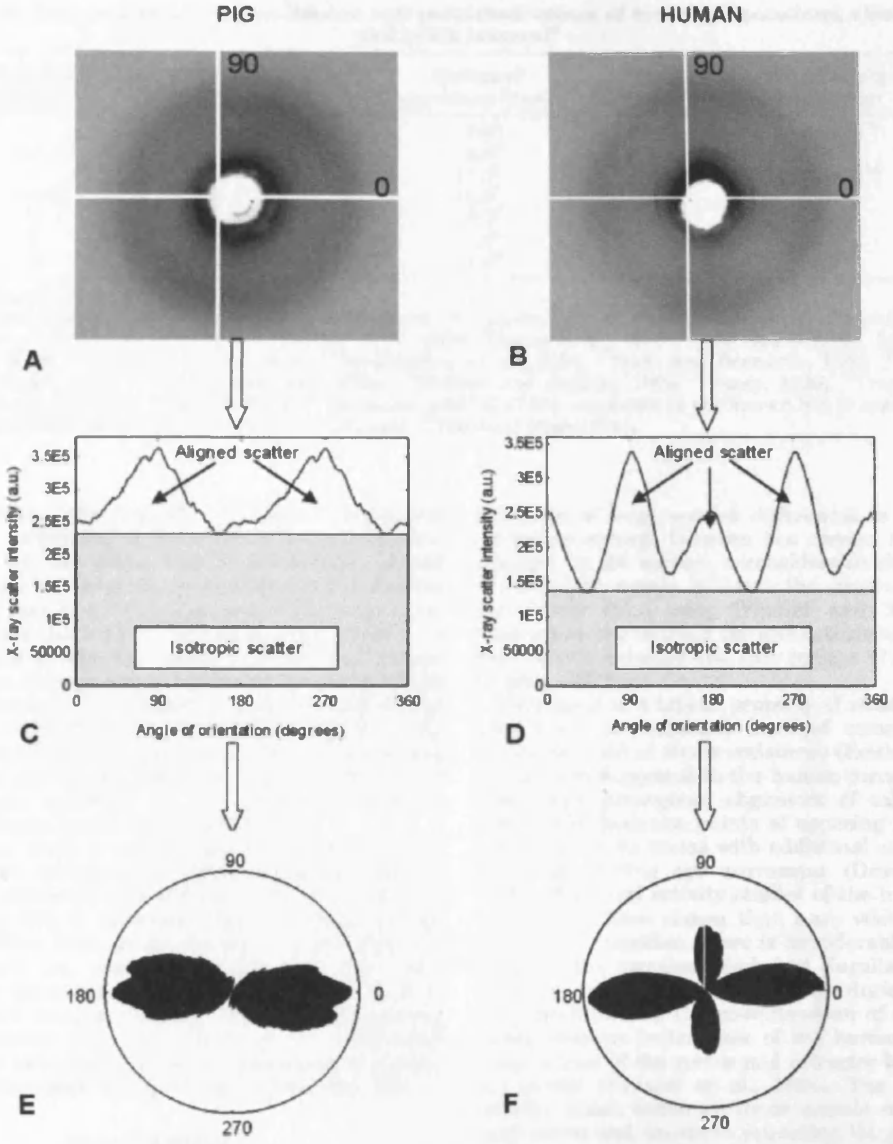

Fig. 2. **A,B:** High angle x-ray scattering patterns from the center of a pig (A) and a human cornea (B). **C,D:** The intensity of x-ray scatter around the intermolecular reflection was measured to produce a profile of total collagen scatter intensity as a function of angular position. **E,F:** The profile of x-ray scatter from aligned collagen only (isotropic scatter removed) is displayed as a vector plot, taking into account that x-rays are scattered at right angles to the fibril axis. The distance from the center of a vector plot in any given direction is representative of the amount of collagen fibrils preferentially orientated in that particular direction.

poral directions, giving rise to a characteristic cross-shaped vector plot (Newton and Meek, 2001; Aghamohammadzadeh et al., 2004; Boote et al., 2006). With increasing distance from the center of the cornea, the size of the vector plots increases, indicating that more collagen is highly aligned and the principal direction of collagen becomes increasingly tangential with respect to the edge of the cornea. A distinct annulus of aligned collagen measuring 1.6–2.0 mm spans the corneolimbal region.

A predominantly orthogonal orientation of collagen is rarely seen in the marmoset cornea (4 of 20 examined). In most cases (16 of 20 corneas examined) an excess of fibrils are oriented along the superior–inferior meridian (Fig. 3B). As with the human cornea, a well-defined limbal annulus measuring 0.5–1.2 mm is a common feature of the marmoset.

X-ray scatter patterns recorded from three equine corneas and three bovine corneas revealed that, in the central 14 mm, an excess of collagen is orientated along the

TABLE 2. Corneal collagen arrangement and published values of corneal dimensions, visual acuity and blink interval[a]

Species	Collagen direction[1]	Cornea size (horizontal × vertical) (mm)	Radius of curvature (mm)	Corneal thickness (mm)	Visual acuity (cycles/deg)	Blink interval (sec)
Human	+	11.7 × 10.6[2]	7.8[2]	0.55[9]	47.0–86.0[13]	2.8[18]
Marmoset	I (or +)	6.1 × 5.4[3]	3.5[3]	0.30[3]	30.0[3]	1.4[19,20]
Horse	I	35.0 × 28.5[4]	17.2[7]	0.81[10]	16.4–20.4[14]	2.3[19]
Cow	I (or O)	29.0 × 24.0[4]	15.8[7]	0.80[11]	12.4[15]	2.7[19]
Pig	O	15.5 × 12.5[4]	9.0[7]	0.85[9]	8.0[15]	3.8[21]
Rabbit	O	13.4 × 13.0[5]	7.3[5]	0.38[12]	4.3[16]	>30[19]
Mouse	O	3.5 horiz.[6]	1.4[8]	0.17[6]	0.5[17]	>30[19]

[a]The superscript numbers indicate the following:
[1]Corneal collagen arrangement is classified as orthogonal (+), vertical (I) or circumferential (O); [2]Tripathi and Tripathi, 1984; [3]Troilo et al., 1993; [4]Smythe, 1956; [5]Bozkir et al., 1997; [6]Zhang et al., 1996; [7]Coile and O'Keefe, 1988; [8]Schmucker and Schaeffel, 2004; [9]Wollensak et al., 2003; [10]Svaldeniene et al., 2004; [11]Scott and Bosworth, 1990; [12]Li et al., 1997; [13]Hirsch and Curcio, 1989; [14]Timney and Keil, 1992; [15]Heffner and Heffner, 1992; [16]Vaney, 1980; [17]Prusky et al., 2000; [18]Ponder and Kennedy, 1927; [19]Blount, 1927; [20]The blink interval of the marmoset is not known but is assumed to be similar to that of published values for the Sudanese monkey; [21]Personal observation.

vertical meridian (Fig. 4A,B). Collagen orientation appears to be tangential at the edge of the 14 mm trephined disks, but this effect may be an artefact caused by trephination. Of interest, examination of a further two bovine corneas that were prepared in the same manner revealed the presence of collagen lying parallel to the edge of the cornea throughout the central 14-mm region and this arrangement of collagen will be referred to as "circumferential." A predominantly circumferential arrangement of fibrillar collagen was observed in all pig (Fig. 4C), rabbit (Fig. 4D), and mouse (Fig. 4E) corneas, and as seen in the human and marmoset, the amount of aligned collagen increases at the limbus to form an annulus of collagen surrounding the cornea.

As shown in Table 2 and Figure 5, the variation in corneal collagen arrangement between species does not appear to be correlated with the size (Fig. 5A), radius of curvature (Fig. 5B), or thickness (Fig. 5C) of the cornea. The average blink interval for the pig was calculated to be 3.76 (± 2.0) sec, and when these new data were included with those taken from the literature, no relationship seemed to exist between collagen arrangement and blink interval (Fig. 5E). However, a relationship does appear to exist between the arrangement of corneal collagen and the visual acuity of the animal (Fig. 5D).

DISCUSSION

In this study, we have shown that major differences in corneal collagen arrangement exist between mammals and, as highlighted in the common marmoset and the cow, further structural differences may also exist between individuals of the same species. The observed variation in collagen arrangement does not, however, appear to be related to the size, radius of curvature or thickness of the cornea.

It has been suggested elsewhere that the presence of an annulus of aligned collagen at the limbus may play a role in maintaining the curvature of the cornea at the point where it meets the lesser curved sclera (Maurice, 1984; Aghamohammadzadeh et al., 2004; Boote et al., 2004). Of interest, a limbal annulus of circumferentially aligned collagen appears to be a common feature of all animals examined within this study group, despite the existence of large species differences in the amount of curvature change between the cornea and sclera. For example in the human, a considerable change in radius of curvature occurs between the cornea (7.8 mm) and the sclera (11.5 mm; Tripathi and Tripathi, 1984), whereas in the rabbit a far less notable change in curvature occurs between the two regions (7.5 and 9.8 mm; Hughes, 1972).

Because it is a typical property of most connective tissues that an increased level of stress produces an increased level of stress resistance (Reichel et al., 1989), it has been suggested in the human cornea that the predominantly orthogonal alignment of collagen, directed toward the insertion points of opposing rectus muscles, may provide the tissue with additional strength to resist distortion during eye movement (Daxer and Fratzl, 1997). Electrical activity studies of the human extraocular muscles have shown that, even when the eye is in the primary position, there is considerable activity in all of the rectus muscles (Bjork and Kugelberg, 1953). Furthermore, the retraction of the eye during blinking has been attributed to the co-contraction of the extraocular rectus muscles in the case of the human and the combined action of the rectus and retractor bulbi muscles in the rabbit (Evinger et al., 1984). The retractor bulbi muscle, which forms an inner muscle cone around the optic nerve and assists in retracting the globe, is present in the horse, cow, pig, rabbit, and mouse but exists only as a rudimentary structure in the monkey and is entirely absent in the human (Prangen, 1928). The presence of four extraocular rectus muscles are, however, common to all mammalian species, although the length, width, and position of insertion varies between animals (Prangen, 1928; Prince et al., 1960). For example, the rectus muscles of the monkey and human insert at locations much closer to the limbus than those of the rabbit and pig (Prangen, 1928). Despite all mammalian species possessing the same four extraocular rectus muscles, a predominantly orthogonal arrangement of corneal collagen is not a common feature of vertebrates and has only been observed in the human, a minority of marmosets (Boote et al., 2004), and the chicken (personal observation). It must be remembered at this point that x-ray scattering patterns provide an average measurement of

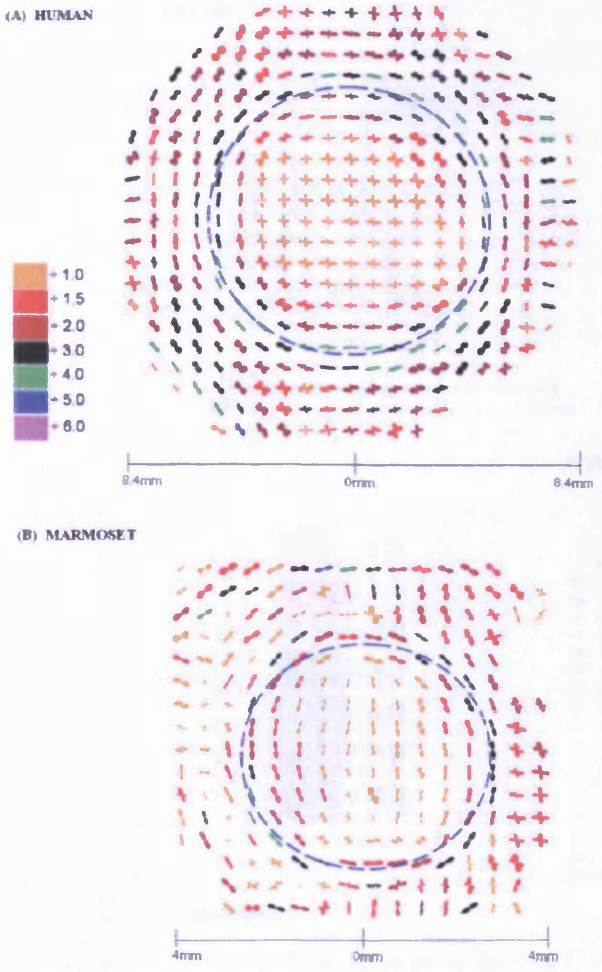

Fig. 3. **A,B:** Vector plot maps showing the predominant direction of stromal collagen in the human (A) and marmoset (B) cornea and scleral rim, sampled at 0.8-mm and 0.5-mm intervals, respectively. Vector plots are scaled down by the factors shown in the key. The position of the limbus is highlighted by a broken blue line.

collagen fibril orientation throughout the entire thickness of the cornea. It is possible therefore that other nonhuman species also possess an orthogonal arrangement of lamellae but as suggested by Gyi and colleagues (1987), they may be fewer in number or of a reduced thickness to those present in the human cornea and therefore contribute less to the overall x-ray scatter pattern. Indeed electron microscopy, a technique that can be used to provide detailed structural information at specific sites in a tissue, has provided evidence of some fibrils crossing at right angles to each other in the posterior stroma of the developing mouse cornea (Haustein, 1983), but x-ray scattering has revealed that, on average, most stromal collagen in the adult mouse cornea lie in a circumferential arrangement with respect to the edge of the cornea.

Here, we have shown that a relationship may exist between corneal collagen orientation and visual acuity (Fig. 5; Table 2). This apparent relationship may be due to animals with a higher level of visual acuity having an increased frequency of rectus muscle contraction as a result of finer eye movements (Prince et al., 1960). Primates are exclusive among mammals in their possession of a fovea—a small circular region of the retina that has a high cone photoreceptor density and affords the animal with a high level of visual acuity. The alignment of collagen in the human cornea toward the insertion points of the rectus muscles may play a role in maintaining a stable eye position during image fixation on the fovea and/or resisting corneal distortion caused by the co-contraction of antagonistic pairs of rectus muscles during blinking. It is worth noting here that, although marmosets possess a fovea, have a relatively high level of visual acuity (30 cycles/degree; Troilo et al., 1993) and likely (based on published values of the blink interval in the Sudanese monkey; Blount, 1927) blink at a similar frequency to humans, they rarely exhibit an orthogonal arrangement of corneal collagen. However, in contrast to the human retina, which shows a sharp decrease in cone density with distance from the fovea, the marmoset retina exhibits a much more gradual decline of cone density along the horizontal meridian (Troilo et al., 1993). The need for such precise horizontal eye movements during image fixation may therefore be reduced and the presence of a predominantly vertical arrangement of collagen may help to preferentially counteract the forces generated by the superior and inferior rectus muscles. It must also be considered that, if corneal collagen arrangement is indeed influenced by the stresses exerted on the cornea by the action of the extraocular muscles, then one might expect the corneas of animals born and raised in captivity to differ from their wild counterparts as a result of differences in their visual environment and, hence, the frequency and direction of eye movements. As suggested by Barmack (1976), deprivation of binocular vision in an animal that normally has excellent binocular vision may reduce the stiffness of the extraocular muscles and cause misalignments.

The need for accurate control of eye position is greatly reduced in animals that possess a retina with a visual streak (a broad strip of increased cone density), because the lateral position of their eyes affords a large field of view without the need for extensive eye movement (Prince et al., 1960; Barmack, 1976). The horse and cow possess an intermediate level of visual acuity (relative to the other species studied here), and in the majority of cases examined in this study have an excess of vertically orientated collagen within the central region of the cornea. A predominantly vertical arrangement of collagen in these species may help to counteract the forces of the superior and inferior rectus muscles, which act antagonistically to help maintain a stable retinal image on a horizontal visual streak. In lower visual acuity animals such as the pig, rabbit, and mouse, the predominantly circumferential direction of corneal collagen does not correspond to the insertion points of any of the rectus muscles. In these species, the frequency of rectus muscle contraction may be lower as a result of limited eye movement and the possession of a retractor bulbi muscle, which helps to retract the eye during blinking. Such a relationship between collagen

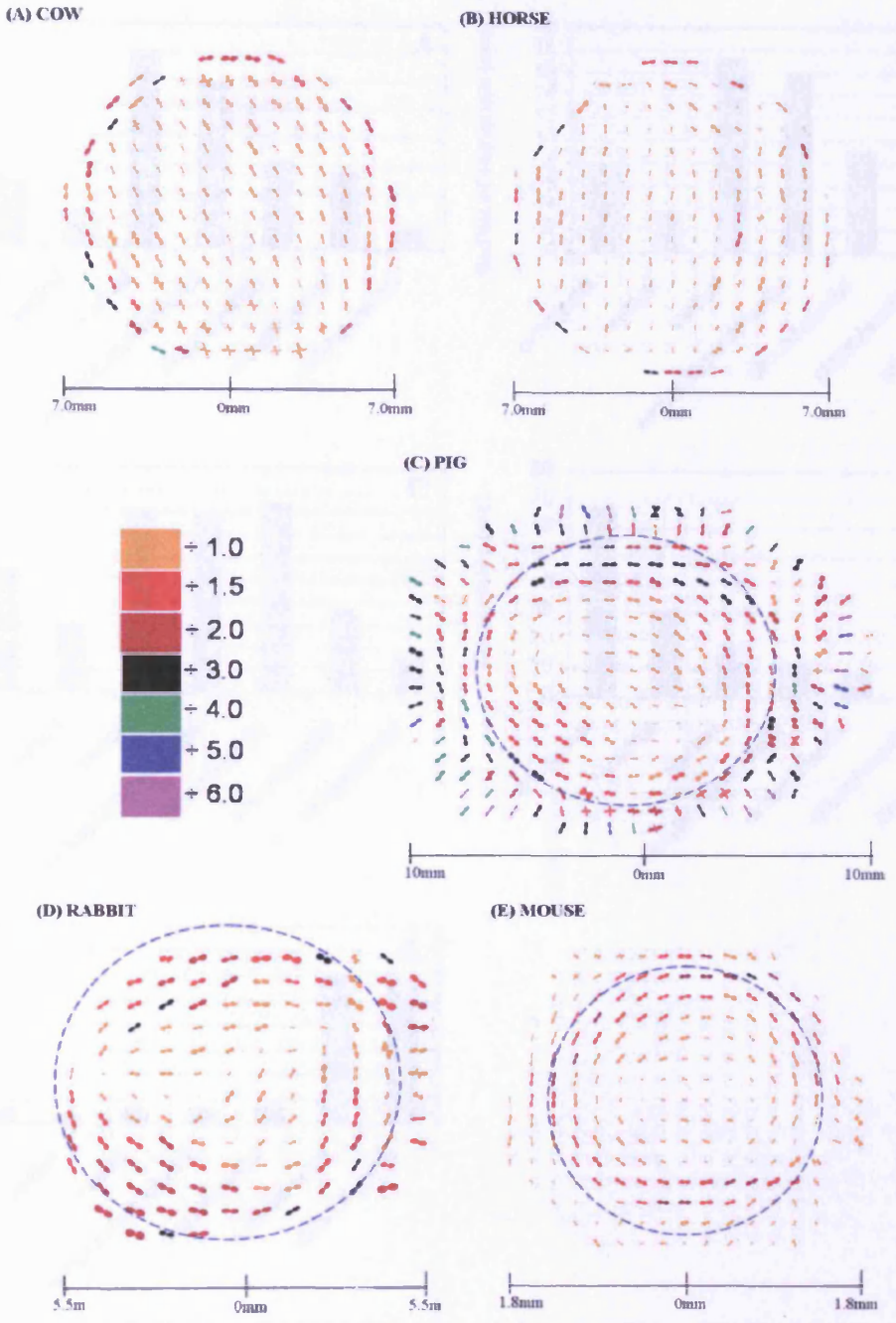

Fig. 4. **A–E:** Vector plot maps showing the predominant direction of stromal collagen in the central 14 mm of the cow (rotated anticlockwise 30 degrees off axis; A) and horse cornea (B) and in the cornea and scleral rim of a pig (C), rabbit (D), and mouse (E). Vector plots are scaled down by the factors shown in the key.

arrangement and eye movement is supported by the fact that the stiffness of both the cornea (Jue and Maurice, 1986) and the extraocular rectus muscles (Barmack, 1976) of the rabbit are far lower than that of the human.

This study has highlighted that major differences in corneal structure exist between mammalian species. These differences may be related to variations in the direction, frequency, and precision of eye movements, however, a functional study of corneal collagen arrange-

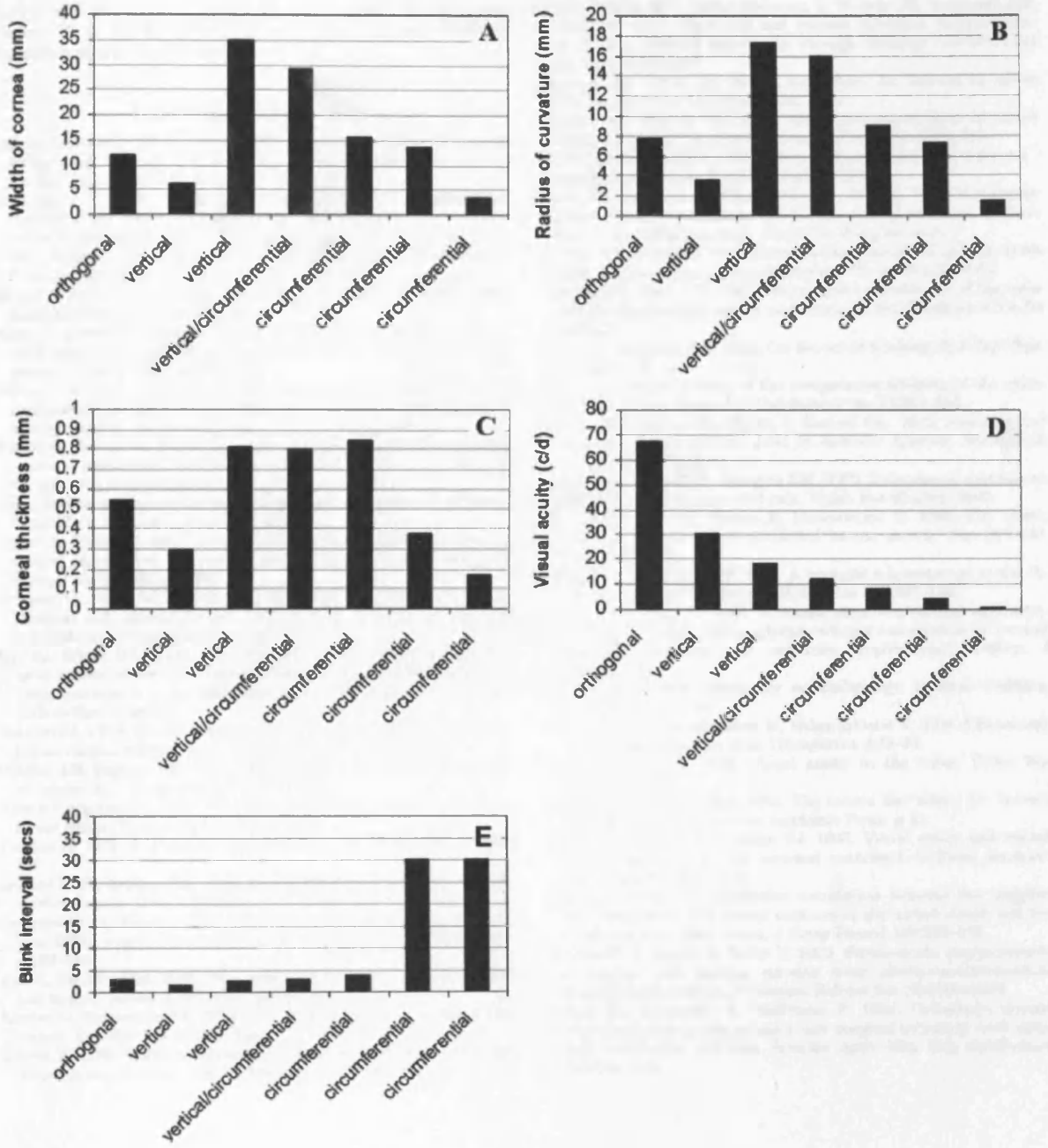

Fig. 5. **A–E:** The arrangement of corneal collagen (orthogonal, vertical, or circumferential) in the human, marmoset, horse, cow, pig, rabbit, and mouse (shown in order from left to right across each graph) is shown relative to published values of the size (A), radius of curvature (B), and thickness (C) of the cornea and the visual acuity (D) and blink interval (E) of each animal (as listed in Table 2).

ment and ocular muscle activity is needed to confirm this. As a consequence of such interspecies structural variation, one should be cautious in interpreting the clinical relevance of nonhuman corneal biomechanical studies.

ACKNOWLEDGMENTS

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Appendix B

Expression of *Col8a2* between the WT and *Vsx1* KO

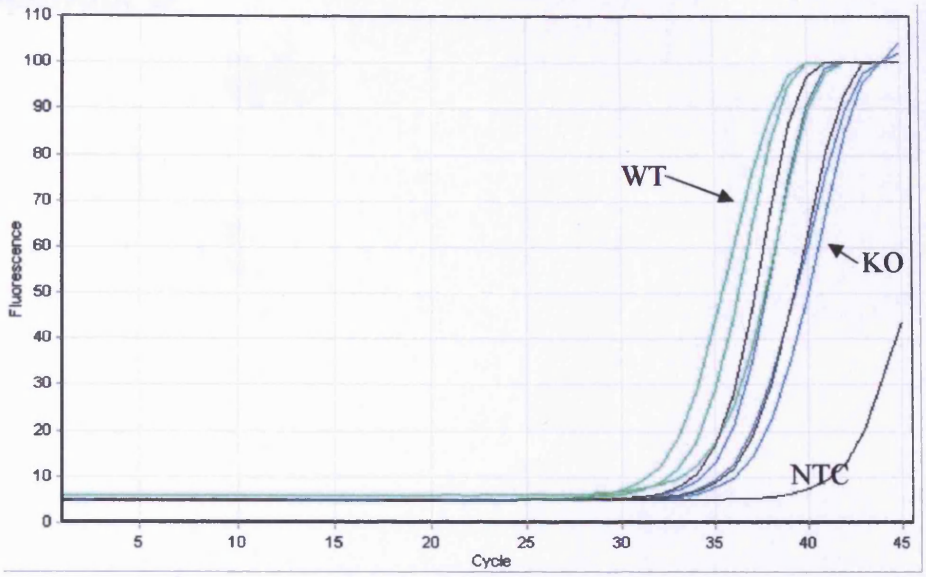

Fig X. Raw expression curves of *Col8a2*. Samples are indicated by corresponding arrows. NTC- no template control

Figure X shows that expression was gained for the NTCs along with expression of the samples of interest indicating that an incorrect product was being amplified which was not the *Col8a2* transcript. This was confirmed by melt curve analysis that heated the samples until the two strands of synthesised cDNA became disassociated. From the curves gained we could see that there was an incorrect product being formed and therefore no expression of *Col8a2* was gained (see fig Y).

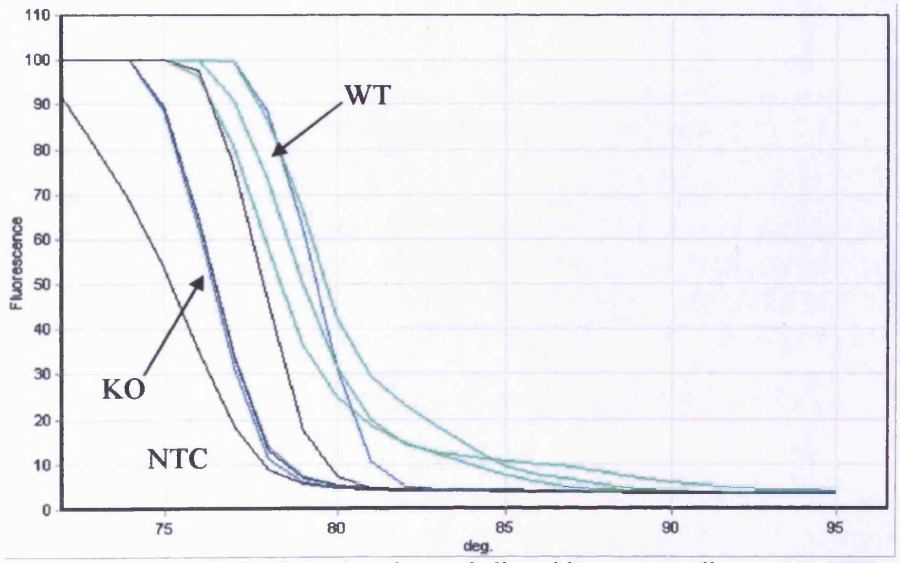

Fig Y. Melt curve for *Col8a2*. Samples are indicated by corresponding arrows. NTC- no template control

Appendix C

Internal Departmental Ethics (LREC)

KC Study Information Leaflet 11/02/05.

School of Optometry & Vision Sciences, Cardiff University
Tel: 020 2087 6163

Dear Sir/Madam

My name is Jack Sheppard and I am a research student at Cardiff University in the Ocular Genetics Research Group in the School of Optometry and Vision Sciences. Together with Dr Marcela Votruba, who is my Supervisor, we are investigating potential genes that can cause keratoconus and relating any possible mutations with structural changes using x ray diffraction.

Our School also has an Optometry Clinic that is open to the public, offering a range of services.

We are mainly investigating a gene called *VSX1* that is known to be linked to keratoconus and posterior polymorphous corneal dystrophy (*PPCD*). Other potential candidate genes that may be involved in keratoconus will also be investigated. As part of this study we are planning to screen the DNA of people with keratoconus to see if we can find any changes in the sequence or mutations that lead to keratoconus or *PPCD*. This will lead to a greater understanding of how keratoconus can begin and what factors can cause mutations within the gene. Novel mutations will lead us in new directions of research and may provide us with ways to identify patients with suspected keratoconus quicker and earlier. This research may discover new genes for keratoconus that will provide new alleys of research to be explored

Why do we need volunteers?
We are planning to conduct a study on keratoconus and are very keen to recruit as many people as we can from the community to make sure it's a success and help us to better understand the condition. This study will run concurrently with another study investigating the causes of keratoconus in subjects with Down's syndrome.

We are looking for people who have **keratoconus** to participate in this study.

What will the study involve?
When you come to the clinic, we will ask you for a sample for DNA analysis. This sample may be a mouth wash, buccal scrape (from the inside of your cheek) or a blood sample (up to 20 ml max). You can choose which you prefer. Samples will be collected by Jack Sheppard and blood will be taken by Dr Marcela Votruba in the optometry clinic. If you are unable to attend the clinic and have been approached

through a local optician, we can arrange for you to send us a mouth wash sample by post.

Where will the study be carried out?
The study will take place in the School of Optometry and Vision in Cardiff University. The clinic is situated in the Redwood Building, King Edward VIIth Avenue, which is in the centre of Cardiff.

What you should do if you'd like to participate.
If you would like to participate in this study please fill in and return the consent form enclosed. Once you have returned it we will contact you to arrange an appropriate time for you to come to our clinic, if you are not all ready visiting us. We would like to thank you in advance for your cooperation

Consent Form

Investigating the genes that cause keratoconus
Research to be carried out by Jack Sheppard, Dr Marcela Votruba.

Declaration
I have read and understood the above information and have had the opportunity to ask questions before proceeding:

I agree to participate in the project.

I am aware that:-
a) My participation is voluntary.
b) I can withdraw from this experiment at any time. My withdrawal will not affect the standard of clinical care that I receive.

Name of person participating in the study _____

Contact Details:

Full name _____

Address _____

_____

_____

_____

Telephone number _____

Signed ..... Date.....

Witness' Signature ..... Date.....

Jack Sheppard

DEPARTMENT OF OPTOMETRY AND VISION SCIENCES

HUMAN SCIENCE RESEARCH ETHICS COMMITTEE

Please complete form for consideration by assessors appointed by the Human Science Research Ethics Committee. Please submit the completed form, with any related correspondence, to the Departmental Office. **A subject information leaflet and consent form must be included.**

PROJECT TITLE:
Investigating genes that may cause keratoconus.

LEAD INVESTIGATOR(S): DEPARTMENT/ADDRESS:	PHONE:
1. Dr Macela Votruba, OPTOM	70134
2. Dr Maggie Woodhouse, OPTOM	76522
3.	
4.	
5.	

SPONSORING/COLLABORATING ORGANISATION: Cardiff University

DOES THE SPONSORING/COLLABORATING ORGANISATION PROVIDE INSURANCE:

YES/NO*

IF DRUGS ARE USED DO ANY REQUIRE A CLINICAL TRIALS CERTIFICATE OR CLINICAL TRIALS EXEMPTION CERTIFICATE?

YES/NO*

*If YES please provide a copy of the certificate.

SUMMARY OF PROJECT:

1. Starting date 1 April 2005

2. Duration 3 years

3. Location School of Optometry & Vision Sciences

4. Physical procedures mouth wash or buccal scrape or phlebotomy

5. Drugs or substances to be administered* NONE

***A substance is anything other than normal food. Chemical constituents of food stuffs, ethanol and variation of the diet are included as substances.**

SUBJECTS:

1. Number of subjects to be used
   As many as possible, above 10.

2. Age and gender of subjects
   Subjects to be above 16 years of age, male and female

3. How will subjects be recruited?
   By invitation from OPTOM Eye clinics, current Down's cohort, community

4. Will payments be made to the subjects (if so how much)? NO

5. Will any subjects be excluded and if so on what grounds?
   Lack of co-operation

6. Is the activity of the subjects to be restricted in any way either before or after the procedure (e.g. diet, driving etc.) NO

HAZARDS:

1. Please give full details of any hazards which could affect the health safety or welfare of any subject:

None other than phlebotomy, which occasionally leads to bruising

2. How do you propose to minimise these hazards?:

Routine measures for careful phlebotomy wil be taken by an experienced practitioner ie Dr M Votruba.

3. Is there any precedent for these experiments? If so, please give details with references if possible:

Yes: ...

4. Has this project been considered, or is it being considered by any other Ethics Committee? If so, please give details and decision made. NO

STATEMENT BY NAMES INVESTIGATORS, HEAD OF DEPARTMENT AND RESEARCH SUPERVISOR (if necessary).

I consider that the details given constitute a true summary of the project, and the hazards and potential risks to any subject are accurately described.

..... (Signed)..... (Date)

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..... (Signed)..... (Date)

Research protocol

Investigating candidate genes that cause keratoconus

Aims

The aim of our project is to investigate the role of genes that cause keratoconus in human subjects. The gene, *VSX1* will be the first gene to be screened for mutation in panels from subjects, but this will be followed by other candidate genes that become available during the course of the study. We plan to investigate two main groups of individuals:

1. Subjects with keratoconus
2. Subjects with Down's syndrome.

Study 1

To investigate the role of genes that are either known to cause keratoconus, or which are candidate genes for keratoconus, in a panel of subjects from the UK population affected by keratoconus, with or without a positive family history of disease.

Study 2

To investigate the genes that are either known to cause keratoconus or are candidate genes for keratoconus in a panel of subjects with Downs's syndrome and keratoconus and compare them to subjects with Downs's syndrome only.

Hypotheses

Study1

Keratoconus is a disease in which single gene defects may cause disease

Study 2

Subjects with Down's Syndrome have a higher incidence of keratoconus because they carry mutations in genes that may predispose them to develop keratoconus.

Background

Keratoconus

Keratoconus is a major ocular disease in the western world and the main candidate for corneal surgery. It is a non inflammatory progressive corneal thinning disease where the shape of the cornea is altered thus changing its optic properties and this leads to myopia and irregular astigmatism. The disease progresses until the third or fourth decade of life, when it arrests and does not continue to deteriorate or improve. However there is a possibility that it may commence again at a later stage(Rabinowitz, 1998). Keratoconus has been associated with a vast number of other systemic disorders, including Down's syndrome(Schmitt-Bernard et al., 2000). As yet there is no known cure to this disease; however numerous studies are being carried out into the structure of keratoconic corneas and its developmental mechanisms. Not much is known about the cause of the disease but there is evidence that some forms of keratoconus could be hereditary and that the cause in all patients could have a genetic contribution.

Current research suggests a number of possible aetiologies for keratoconus; on the one hand keratoconus appears to be a multifactorial ectasia, caused by the interplay of environmental factors and many genes. On the other hand mutational studies have shown that mutations in a single gene, *VSX1*, can result in keratoconus(Bisceglia et al., 2005; Heon et al., 2002; Mintz-Hittner et al., 2004). There are an increasing

number of genetic loci and mapped genes being associated with keratoconus, although this number is still currently low. There are also currently several positional and functional candidate genes, including COL4. It is very likely that with the advent of linkage and population studies this number will increase.

Visual system homeobox gene 1 (*VSX1*) is a gene that codes for a homeodomain transcription factor. *VSX1* has been shown to be essential for late differentiation and function of OFF-cone bipolar cells by regulating, either directly or indirectly 4 proteins expressed by OFF-cone bipolar cells, recoverin, NK3R, Netol and CaB5(Chow et al., 2004). Linkage analysis has been performed in several studies on patients with posterior polymorphous corneal dystrophy (*PPCD*), keratoconus or both and the results of these studies have resulted in a number of loci and candidate genes. However a positive link was made between keratoconus, *PPCD* and *VSX1* when Héon et al (2002) identified the first mutations in patients with *PPCD*/keratoconus by screening the coding sequences of *VSX1* in these patients (4 Canadian families and 2 unrelated individuals). They identified at least 4 mutations that were considered to be directly disease causing. Further mutational studies have identified more mutations. There are currently 8 mutations that have been found within this gene that give rise to keratoconus and *PPCD*(Bisceglia et al., 2005; Heon et al., 2002; Mintz-Hittner et al., 2004). Of great interest for our study is that mutations in *VSX1* were also shown to lead to craniofacial anomalies as well as keratoconus in one family(Mintz-Hittner et al., 2004).

Keratoconus and Down's Syndrome

It is well known from the literature that the prevalence of keratoconus in people with Down's syndrome (DS) is much higher than in the normal population. The incidence of Down's syndrome patients presenting with keratoconus is reported to be 15%(Stoiber et al., 2003). The onset of keratoconus is about the age of 16 years, although it is reported to be at a younger age in patients with DS. The progressive thinning of the cornea is particularly visible in individuals with DS who are known to have thinner corneas in general. The tendency to eye rub has been thought as a possible cause of keratoconus and could be significant in people with DS who have a higher tendency for eye rubbing(van Splunder et al., 2004). This could aggravate the keratoconic progression or possibly start it in the first place.

However, there is clearly a genetic component to KC in DS, just as in subjects with KC only. A striking example of an association is given by a study carried out by Lang et al (1989), who investigated a small family in Germany. The authors found that the mother had autosomal dominant posterior polymorphous corneal dystrophy (*PPCD*), the daughter had bullous keratopathy and showed signs of *PPCD* and the son had Down's syndrome with acute keratoconus(Lang et al., 1989). This suggests a link between DS and KC that is as yet unexplained. Whilst DS is due to trisomy 21, no KC genes have to date been located on chromosome 21; however a candidate locus is known to exist on the most telomeric region of chromosome 21(Rabinowitz et al., 1992).

As people with Down's syndrome have a higher prevalence for keratoconus overall, we propose to screen the candidate keratoconus genes, including, *VSX1*, in these individuals. We wish to explore if patients with KC and DS have mutations in the same genes as people with KC only.

Study Organisation
Location and Researchers
This research is being carried out in The School of Optometry and Vision Sciences by the Ocular Genetics Research Group, in collaboration with the Down's Syndrome Study Group.

Genetics:
Dr Marcela Votruba MA PhD FRCOphth
Mr Jack Sheppard BSc(Hons).

Down's Syndrome:
Dr Maggie Woodhouse
Ms Ping Ji

Samples will be collected at the Eye Clinic within the School of Optometry and Vision Sciences and in local optometric practises and analysis will be carried out in laboratories of the School.

Subjects:
Study 1: We shall study individuals with keratoconus, with or without a family history. In the presence of a family history we shall recruit the participation of other family members, both affected and unaffected. In the absence of a family history we shall recruit the participation of a spouse or friend without keratoconus.
Study 2: We shall study subjects in two groups: individuals with Down's syndrome with keratoconus and individuals with Down's syndrome without keratoconus. In the presence of a known family history of keratoconus we shall recruit the participation of other family members, both affected and unaffected.

Recruitment
Study 1: Subjects with keratoconus will be recruited from The Optometry Clinic and from the local community through local optometrists. This will be done by personal approach, letter, posters and local advertising.
Study 2: Individuals with Down's syndrome will be recruited from the existing cohort at the School of Optometry & Vision Sciences (participating in Ping Ji's study), and additional subjects with the help of local community support nurses.

Study Numbers
We hope to get at least 50 (up to 100) subjects with KC only. We have predicted that we may get 20 individuals with KC and DS and 30 individuals with DS only.

Procedures and equipment
DNA will be collected from patients via mouthwash, buccal scrape or blood depending on the choice of the patient. This will be done in the optometry clinic or mouthwash or buccal scrape samples can be sent by post. Blood samples and buccal scrape and mouth wash samples will be stored at -20C.

Samples will then be analysed using molecular genetic techniques. DNA from blood will be extracted using standard protocols and DNA from buccal cells will be extracted using the proteinase K protocol. DNA samples will be frozen until they are needed. Initially primers that have been designed for the gene *VSX1* will be used to amplify the DNA using polymerase chain reaction (PCR). Later, as other candidate genes become available, primers can be designed for them using the Primer 3 program and the DNA can be amplified in the same way. Once the DNA is amplified it can be sequenced using the BIG DYE sequencing protocol. This will allow us to search for mutations by comparing patient DNA will normal control sequences. Sequences will be analysed using the EMBOSS software suite. Mutations can be identified by comparing the frequency of the sequence change in the affected group to the control population using standard parametric statistics. A mutation would be identified if it did not appear significantly in the control group. Segregated within a family would be powerful evidence in favour of a sequence change being disease causing, as would the results of protein prediction algorithms and conserved amino acid homology searches.

Substances to be administered

NONE

Expected Outcomes
If our hypotheses are correct then the study would show that keratoconus in individuals with DS develops as a result of mutation in genes that also cause KC in individuals without DS, and not just due to those genes found on chromosome 21. The frequency of these mutations in the two populations will be of considerable interest.

We expect to present our scientific results both internally within the University, externally, and publish in peer-reviewed journals.

We shall also disseminate our results to the DS Cohort and patient support groups involved and interested in DS and / or KC.

Confidentiality and sample storage
All samples will be treated in a confidential manner and analysed in an anonymised fashion.

At the end of the Study and Ethical Approval the samples will be destroyed.

Timetable
Year 1:
First 6 months- recruitment, collection of all subject material for Study 1 and Study 2, primer design, optimisation, DNA extraction, storage
Next 6 months- analysis of first candidate gene (*VSX1*) in subjects with keratoconus and / or DS and keratoconus only
Year 2 and 3:
Completion of above analysis, including normal controls, segregation etc. Studies of additional candidate or known genes that cause keratoconus in the study populations. Family studies as made possible by material collected.

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CLINICAL TRIALS CHECK LIST

Under certain conditions it is possible to reduce the time taken to refer clinical trials to our insurers. The attached check list has been compiled using the criteria stipulated by our insurers. Please complete it and return it to Mr Ken Howells Estates.

A Definitions (from our insurers)

"Clinical Trials" an investigation or series of investigations conducted on any person for a Medicinal Purpose.

"Medicinal product" A substance or article for administration to human beings or animals for a medicinal purpose.

"Medicinal purpose"

(a) treating or preventing disease

(b) diagnosing disease or ascertaining the existence degree or extent of a physiological condition

(c) assisting with or altering in any way the process of conception or investigating or participating in methods of contraception

(d) inducing anaesthesia

(e) otherwise preventing or interfering with the normal operation of a physiological function.

B Questions **Please answer Yes or No in the space provided.**

1. Does the Clinical Trial involve volunteers outside Great Britain, Northern Ireland, The Channel Islands or the Isle of Man? _____

2. Does the Clinical Trial involve administration of a medical product without a Product Licence (as defined in the Medicine Act 1968) or outside the terms of a Product licence. _____
   (If the answer to 2 is Yes please answer the following question. If the answer to 2 is No please continue at question 4)

3. Has the manufacturer of the Medicinal Product provided a "hold harmless agreement" in favour of the involved? _____

4. Does the Clinical Trial involve the use of any procedure other than questionnaires and/or venopuncture? _____

   (If the answer to 4 is Yes please answer the following questions.)

5. Does the Clinical Trial involve any consent procedure other than the provision of written informed consent by the volunteer. _____

6. Does the Clinical Trial involve the provision of informed consent by any party other than the volunteer? _____

7. Does the Clinical Trial involve the use of volunteers who are pregnant? _____

8. Does the Clinical Trial involve the use of volunteers who are under 18 years old? _____

9. Does the Clinical Trial involve the use of volunteers who have a neoplastic condition? _____

10. Does the Clinical Trial involve the use of volunteers who have diseases of the cardovascular system? _____

11. Does the Clinical Trial involve the use of volunteers who have a neurological disorder? _____

12. Does the Clinical Trial investigate the AIDS or HIV related condition of volunteers who have such a condition? _____

13. Does the Clinical Trial involve the process of conception or contraception? _____

14. Does the Clinical Trial involve the undertaking of surgery? _____

Title of Trial

.....

Signed.....Date.....

Appendix D

Multicentre Research Ethics (MREC)

Project Registration Number:
(For R&D Office use only)

Cardiff & Vale NHS Trust
Research & Development Office

Project Registration Form (Version 2)

Completion of this form is required as part of an application for Trust scientific and risk assessment of any research proposal. The form must be completed by the Principal Investigator or their delegate for all projects, both commercial and non-commercial, that use **any** Trust resources. This includes projects led by individuals outside Cardiff and Vale NHS Trust. Trust resources include additional staff time, the use of a Trust location (including recruitment or consent in Trust clinics/wards), patient samples or patient data (including studies where medical records only are used).

Investigators must not apply to an ethics committee for ethical approval before receiving written approval from the Trust R&D Office. Research Ethics Committees require evidence of prior scientific review as part of the ethics application.

Please note that both a fully signed hard copy and an e-mail/disk version of the Project Registration together with all other relevant documentation, as specified in the list at the end of this form, must be received by the Trust R&D Office before a project can be reviewed and approved by the Trust.

You are strongly advised to refer to the R&D Project Registration Procedures and Guidelines document before sending your application to the Trust R&D Office.

Research using Cardiff and Vale NHS Trust resources must not proceed until a written letter of approval has been received from the Trust R&D Office. Failure to comply with this requirement could result in disciplinary action in accordance with the Trust Research Misconduct Policy.

This form has been protected.
Please use the tab key to take you through the form.

1.0 Details of the Principal Investigator

The person with overall responsibility for this project at this site. This person will be used as contact name in the National Research Register. This individual should ***not*** *be a student undertaking an undergraduate or postgraduate course. In these cases it must be the student's academic or clinical supervisor.*

Surname:
Votruba

Title:
Dr

First Name(s):
Marcela

Address:
School of Optometry and Vision Sciences, Redwood Building, King Edward VII Avenue, Cardiff CF10 3NB

Phone: **Bleep** **Fax:** **Email:**

2.0 Additional information required for projects which are part of an undergraduate or postgraduate course

2.1 Details of student (N.B. A student cannot be named as the principal investigator)

Surname:
Sheppard

Title:
Mr

First Name(s):
Jack

Address:
School of Optometry and Vision Sciences, Redwood Building, King Edward VIIAvenue, Cardiff CF10 3NB

Phone:	Bleep	Fax:	Email:
02920 876471	NA	NA	sheppardj@cf.ac.uk

2.2 Details of academic supervisor (indicate if same as section 1.0)

Surname:
Same as 1.0

Title:

First Name(s):

Address:

Phone:	Bleep	Fax:	Email:

2.3 Details of clinical/scientific supervisor (indicate if same as section 1.0)

Students must have a clinical supervisor when the project involves contact with patients recruited via the Trust.

Surname:
Same as 1.0

Title:

First Name(s):

Address:

Phone: Bleep Fax: Email:

2.4 Title of course/qualification that the research relates to

PhD, Cardiff University (Genetic and structural pathogenesis of keratoconus)

3.0 Permission for inclusion on the National Research Register (NRR)

Can details of this project be listed on the NRR? Yes ☒ No ☐

NB. Details of all projects using NHS R&D resources shall be entered on the NRR unless this breaks confidentiality agreements with commercial sponsors, or jeopardises intellectual property rights. It is the responsibility of the principal investigator to check that the commercial sponsor agrees to inclusion on the NRR.

4.0 Project Details: *(Information from this section will be included on the NRR)*

Title *Use the same title as on any Grant application or Ethics Committee applications.*

Investigating genes that may cause keratoconus

Shortened Name /Acronym (if appropriate)

Genes and keratoconus

Lay Summary

Keratoconus (KC) is a condition in which the cornea becomes conical in shape because of the thinning of the cornea. This impairs the vision at both near and distance. It usually starts in late childhood. It can be corrected up to a point with contact lenses, although when KC is more advanced only a surgery will restore sight. The genetic causes of KC are being investigated, but so far only a small number of genes have been directly implicated. One of these genes is called VSX1, but it may only be responsible for KC in some patients. We intend to screen genes that may cause KC to establish their contribution to the development of KC in a UK population. In the long term, this information may enable us to identify people who will develop KC in later life at an earlier stage than is now possible, and thus provide more targeted follow-up and appropriate treatment.

*This section **must** be comprehensible to a general audience without any specific knowledge of the topic. It is essential to provide a lay summary in order for the project to undergo peer and risk review (250 words maximum).*

Principal Research Question

What is the role and significance of mutations in single genes in the development of keratoconus in a UK population.

The principal question or hypothesis addressed by the piece of research. Information should be as specific as the research design permits. When a hypothesis is being tested, enter the hypothesis. If possible, this should be a single sentence

Methodology

** Use either the pick list or the free text option:*

Other

Genetic screening and mutational analysis: genetic studies involving the recruitement of subjects, obtaining DNA (by buccal swab, mouthwash and venepuncture), screening of patient DNA using PCR, Big Dye sequencing, restriction enzyme digest and other mutation detection methods.
A brief description of the methodology to be used

Sample Group(s)
Patients and their families with clinically diagnosed keratoconus (aged 16+ years).
A description of the notional population(s) from which a sample(s) is drawn for the purposes of the study

Outcome Measure
Identification of disease causing mutations in candidate genes that may cause keratoconus in both sporadic and familial cases of keratoconus.
Endpoints or factors used to evaluate health status, such as symptoms, physiological/behavioural data, survival discharge status or quality of life.

Project start date: (dd/mm/yy) 01/05/05 **Project end date**: (dd/mm/yy) 01/05/08
*It is **essential** to provide an estimate if exact dates are not known.*

5.0 NHS Ethics Committee Approval

Will ethical approval be sought? Yes ☒ No ☐ Already Obtained ☐

LREC/MREC Ref:

If you will not be seeking ethical approval please state why you consider this is not required:

6.0 Multicentre Studies

Is this a multicentre trial requiring MREC approval? Yes ☒ No ☐

For multicentre studies, state the Lead Centre.

School of Optometry and Vision Sciences, Cardiff University

Who is the Chief Investigator for the whole study?
The Chief Investigator is the authorised healthcare professional or other researcher, whether or not he/she is an investigator at any particular site, who takes primary responsibility for the conduct of the research including co-ordinating the principal investigators who take the lead at each site
Dr Marcela Votruba

If Cardiff and Vale is the Lead Centre list all other participating organisations

7.0 Funding Details
** Use pick lists: these will appear when you tab into the relevant box and click on the arrow.*

*Category	Specific name of funder	*Funding Status	Funding Requested £	Funding Received £	*Recipient

Research Council	Medical Research Council	Awarded			Other

In most cases commercial funding status will be pending.

8.0 Staff time associated with the project

** It is **essential** that the approximate number of hours devoted to the project by each investigator be specified (**including** the principal investigator).*

Post (e.g. Research Nurse, Consultant)	**Name** **Title**	**Forename**	**Surname**	**Department / Directorate**	*** Hours devoted to project** (Per week)	**Employer** (Trust, UWCM, Other – *please specify*)
Honorary Consultant	Dr	Marcela	Votruba	Ophthalmology and School of Optometry and Vision Sciences	5	Other: Cardiff University
Other	Mr	Jack	Sheppard	Ophthalmology and School of Optometry and Vision Sciences	40	Other: Cardiff University

9.0 Anticipated impact on patient care services

	How many patients/ volunteers will be affected by this research?	What will be the effect of your research on each patient/volunteer?		Study location e.g. clinic, ward (state if Trust or UWCM)
Out-patients	100	No of additional visits **per patient/volunteer**	none to Trust/ UWCM (in some cases up to 1 visit to Cardiff Univ.)	Neither: Cardiff School of Optometry and Vision Sciences
		Length of each additional visit	30 mins	
		No of extended routine visits	0	
		Extra time **per routine visit (min)**	0	
In-patients	0	No of additional day stays **per patient**	0	0
Day cases	0	No of extra procedures performed **per patient**	0	0
Operating theatre time	0	No of additional hours **per patient**	0	

If there is an anticipated reduction in outpatient visits/inpatient day stays/procedures give details:

NA

Specify if outpatients' visits are in 'split' locations (e.g. cardiology outpatient clinic and WHRI) and give details of procedures occurring at each location.

NA

10.0 Pharmacy Support

Does the study involve the use of medicinal products in addition to those used for the participants normal routine patient care? Yes ☐ No ☒

A signature from the Pharmacy Clinical Trials Department is required below for ***all*** *studies involving medicinal products.* ***Before*** *this signature can be obtained, the principal investigator must complete a* ***Pharmacy R&D Application Form*** *and send this, together with a copy of the protocol, to the Pharmacy Clinical Trials Department. A minimum of two weeks is required for Pharmacy to process these applications.*

Signature of Clinical Trials Pharmacist..... Date.....

11.0 Estimated demand for other support services

NB *For commercial clinical trials that involve a contract with the Trust, this section will be completed by the Commercial Trials Office.*

It is important to include tests/procedures being carried out in the investigator's <u>own</u> department.

Support Department	**Type of Test**	**Extra tests/ therapy sessions <u>per patient</u>**	**Estimated Total Cost £**	**Department Signatory** (Please sign and print name) **UHW**	**Llandough**	**Dental**	**RES No. where appropriate (*)**
Biochemistry/ Immunology	NA						*
Cardiology	NA						
Dental Orthodontics Laboratory	NA						
Dental Prosthetics Laboratory	NA						
Dental Conservation Laboratory	NA						
Haematology	NA						*
Histopathology/ Cytology	NA						
Medical Genetics	NA						

Medical Photography	NA						
Medical Physics	NA						
Microbiology	NA						
Radiology	NA						
Other (please specify e.g. phlebotomy)	NA						
Therapy Sessions (Please specify)	NA						

N.B. Indicate if any of the above support is being met by an external funder including commercial sponsors
NA

12.0 Estimated costs of additional NHS purchased consumables

Provide information in terms of costs or approximate number of items to be used
NA

13.0 Additional drug/treatment costs (Excess Treatment Costs)

Are there any additional drug/treatment costs (Excess Treatment Costs) compared to routine patient care?
Yes ☐ No ☒

If yes, specify who will be responsible for applying to the Wales Office of Research & Development for reimbursement of these costs.
NA

14.0 Primary care involvement

Does this project have any resource implications for primary care?
Yes ☐ No ☒

If yes, have these been discussed with the Local Medical Committee (LMC)?
Yes ☐ No ☐

For Bro Taf LMC, contact Dr Gareth Hayes, Medical Secretary, Tel: 029 2046 5261: E-mail brotaflmc@breathemail.net

15.0 Confidentiality/Data Protection

Do all members of the research team who deal with research participants recruited through Cardiff and Vale NHS Trust hold either substantive contracts or honorary contracts with this Trust?
Yes ☒ No ☐

Does the research involve the use of personal data?
Yes ☒ No ☐

Is the Trust's Data Protection Officer aware of this research proposal?
Yes ☒ No ☐
If yes, enter the Reference Number provided to you by the Trust's Data Protection Officer.

RD05207

It is the Principal Investigator's responsibility to ensure that the research complies with the Data Protection Act 1998 and that the advice of the Trust's Data Protection Officer has been sought where appropriate prior to submission of this form
Trust Data Protection Officer – Mr Nic Drew, Tel: Cardiff 029 20336175
E-mail: Nic.Drew@cardiffandvale.wales.nhs.uk

Honorary contract request forms should be obtained from the Trust Human Resources Department. Enquiries should be directed to:
Assistant Recruitment Manager – Ms Andrea Page, Tel: Cardiff 029 20742917
E-mail: Andrea.Page@cardiffandvale.wales.nhs.uk

16.0 Consumer Involvement

Indicate how consumers have been or will be involved in the development and execution of this project:
Subjects and their families with keratoconus will be recruited by direct invitation from Eye Clinics, advertisements in The School of Optometry & Vision Sciences and through local optometry practises. Currently no UK patient support group for keratoconus exists, but publicity for recruitment will also be highligted to potential subjects through contact with RNIB, Cardiff Institute for the Blind, Guide Dogs UK and so forth. The scientific requirements of the actual mutation screening and study design do not lend themselves to consumer involvement. However, consumer issues, such as a need for large print leaflets and forms, general information and feedback, will be addressed if needed. It is very unusual for patients with keratoconus to need large print material.

17.0 Duty of Care

Is the Principal Investigator the clinician who has overall responsibility for the care as well as the conduct of the research for all local participants in the study?
Yes ☐ No ☒ Not applicable ☐

Give the name(s) and contact details of all person(s) who will be responsible for the care of local participants in the study, and indicate if the participant's involvement will be recorded in the relevant notes.
Those subjects recruited from UHW will be under the care of Mr Vinod Kumar. When a subject is recruited a note will be made in the notes and a copy of the Consent form filed, although patient care will not be affected at all.

Will this/these person(s) be informed that their patient/client has been invited to participate in this project?
Yes ☒ No ☐ Not applicable ☐

If no, explain why not?

18.0 Indemnity Arrangements

The guidelines document may be of assistance to those experiencing difficulties with this section

What arrangements have been made to provide indemnification and/or compensation in the event of a claim by, or on behalf of, participants for **negligent** harm?
Cardiff University

What arrangements have been made to provide indemnification and/or compensation in the event of a claim by, or on behalf of, participants for **non-negligent** harm?
Cardiff University

19.0 Intellectual Property

Could the research lead to the development of a new product/process or the generation of significant intellectual property for the Trust?
Yes ☐ No ☒ Not applicable ☐

If yes, has this been discussed with the Trust Procurement Department Commercial Manager?
(Mr Colin McMillan Tel: 029 2074 6388, E-mail Colin.McMillan@cardiffandvale.wales.nhs.uk)
Yes ☐ No ☐

Could the research lead to the development of a new product/process or the generation of significant intellectual property for any other non-commercial organisation?
Yes ☐ No ☒ Not applicable ☐

20.0 Plan of dissemination and implementation

Give a brief description of how the research findings will be disseminated both to those participating in the research and to all those who could benefit from them, through publication or other appropriate means. Describe how the findings will inform the progress of service development and practice.
Communication with patient support groups (such as RNIB, Guide Dogs UK) will be established, to include items in newsletters etc. Local sources of publicity will be sought out. The membership the Principal Investigator (D Votruba) of The Cardiff Institute of Tissue Engineering and Repair (CITER) will facilitate dissemination within a wider scientific community and publication in peer review journals, presentation at scientific meetings as papers and posters. Service develoment and practice will be little affected by this basic research in the short term, although in the very long term there is a possibility that screening for mutations in commonly identified genes associated with keartoconus could be offered to patients.

21.0 Benefits to NHS

Give a brief description of the anticipated benefits of the research to the NHS. This may include issues such as staff and skills development as well as the potential use of the research findings by the NHS

The findings will inform the progress of service development and practice, in the very long term, by casting light on the fundamental mechanisms of keratoconus. This understanding may eventually allow earlier diagnosis, testing of at risk subjects and more rational tailored therapies to be evolved, thus potentially leading to an avoidance of surgery in some of these patients.

22.0 Risk Assessment

Use the section below to identify hazards and indicate if adequate safety control measures are in place:-

NO	The hazard does not apply to this project
Normal Clinical Practice	The hazard is present and risk is controlled through the procedures of normal clinical practice
YES	The hazard is present and risk is controlled through the procedures specified below

	NO	Normal clinical practice	YES		NO	Normal Clinical Practice	YES
Drugs/Chemicals	☒	☐	☐	**Genetically modified micro-organisms**	☒	☐	☐
Ionising radiation	☒	☐	☐	**Solvents and flammable materials**	☒	☐	☐
Non-ionising radiation (e.g. UV, IR etc)	☒	☐	☐	**Medical devices/ electrical equipment**	☒	☐	☐
Lasers	☒	☐	☐	**Manual handling**	☒	☐	☐
Display Screen Equipment	☒	☐	☐	**Other hazards (please specify below)**	☐	☒	☐

Give details of the risk control measures which you consider to be needed in addition to those expected of normal clinical practice. Describe the severity and likelihood of harm arising from manifestation of the hazard(s). Explain who might be at risk (e.g. clinical staff on the project, other clinical staff, visitors to the area, etc) Explain the risk control measures you will implement including the provision of information, instruction, training and supervision.
No additional risk control measures above the normal clinical practice will be needed. Routine risks of phlebotomy will apply and routine measures will be adopted to protect patients and staff.

23.0 Commercial Sponsor Details

Complete this section if the study is a commercially sponsored trial.

Company Name
NA

Name of Study Co-ordinator (CRA)

Tel Number of CRA

DECLARATION

Declaration by the Principal Investigator

- The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
- I undertake to abide by the principles underpinning the Declaration of Helsinki and Good Clinical Practice Guidelines on the proper conduct of research.
- I have read the Trust's guidance document on Research Governance and understand my responsibilities as Principal Investigator.
- If the research is approved I undertake to adhere to the study protocol and comply with any conditions set out by the Research Ethics Committee.
- I undertake to inform the NHS Research Ethics Committee of any changes in the protocol, and to submit annual reports setting out the progress of the research.
- I am aware of my responsibility to be up to date and comply with the requirements of the law and appropriate guidelines relating to security and confidentiality of patient or other personal data, including the need to register with, when appropriate, the Data Protection Officer.
- I understand my responsibility to conform with Health & Safety Regulations.
- I have carried out a risk assessment of the project, identified the hazards involved and assessed the risks arising from those hazards. The risks arising from these hazards will be controlled through normal clinical procedures unless indicated otherwise in Section 22.0.
- I give my consent for information about non-commercial research to be extracted from this application and accompanying protocol for inclusion, where appropriate, in the National Research Register.*
- I understand that personal data about me as a researcher in this application will be held by Cardiff and Vale NHS Trust and that this will be managed according to the principles established in the Data Protection Act.
- I understand that my research records/data may be subject to inspection for audit purposes if required.

Signature of the Principal Investigator: .....
Name (printed): .....
Date: .....

**Unless you have specified in question 3.0 that information should not be submitted to the NRR.*

Declaration by the Directorate R&D Lead

On behalf of the Directorate, I have considered all service and financial considerations and confirm that the Directorate is willing to support this application. Where there are service or financial implications for other Directorates, I can confirm that these have been addressed and that all involved parties are willing to support this application.

Signature of Trust Directorate R&D Lead: .....

Name (printed): ..... Date: .....

Documentation required for Full Application

Non-commercial Trials

- Trust Project Registration Form (electronic and signed hard copy)
- Protocol containing the required elements (electronic copy)
- Local version of Patient Information Sheet (electronic copy)
- Local version of the Consent Form (electronic copy)
- Non-standard questionnaires (all 'in-house' questionnaires and those for which a reference on content/use cannot be provided) (electronic copy)
- Copy of successful grant application for externally funded research (electronic or hard copy)
- For non-commercial grant funded trials, a copy of any study agreements that need to be signed by the Trust and the drug company or University that is funding the work
- Evidence of a current Clinical Trials Authorisation (CTA) where applicable (This may be submitted to the office at a later date)

Applications will not be processed until **all** relevant documentation has been received.

Commercial Trials

- Trust Project Registration Form (electronic and signed hard copy)
- Study protocol (five hard copies)
- Local version of Patient Information Sheet (electronic or hard copy)
- Local version of Consent Form (electronic or hard copy)
- Sponsor indemnity insurance certificate.
- Evidence of a current Clinical Trials Authorisation (CTA)

R&D approval cannot be granted until **all** relevant and fully completed documentation has been received.

R&D Office
Cardiff & Vale NHS Trust,
Ground Floor Radnor House
University Hospital of Wales
Heath Park
Cardiff, CF14 4XW

Tel: 029 2074 3742
Fax: 029 2074 5311
E-mail: Research.Development@CardiffandVale.wales.nhs.uk

APPLICANT'S CHECKLIST

All studies except clinical trials of investigational medicinal products

REC Ref:	06/S0501/61
Short Title of Study:	Genes and keratoconus version 2
CI Name:	Dr Marcela Votruba
Sponsor:	Cardiff University

Please complete this checklist and send it with your application

- Send ONE copy of each document (except where stated)
- ALL accompanying documents must bear version numbers and dates (except where stated)
- When collating please do NOT staple documents as they will need to be photocopied.

Document	Enclosed?	Date	Version	Office use
Covering letter on headed paper	○ Yes ○ No			
NHS REC Application Form, Parts A&B	Mandatory			
NHS REC Application Form, Part C (SSA)	○ Yes ○ No			
Research protocol or project proposal (6 copies)	Mandatory			
Summary C.V. for Chief Investigator (CI)	Mandatory			
Summary C.V. for supervisor (student research)	○ Yes ○ No			
Research participant information sheet (PIS)	○ Yes ○ No			
Research participant consent form	○ Yes ○ No			
Letters of invitation to participants	○ Yes ○ No			
GP/Consultant information sheets or letters	○ Yes ○ No			
Statement of indemnity arrangements	○ Yes ○ No			
Letter from sponsor	○ Yes ○ No			
Letter from statistician	○ Yes ○ No			
Letter from funder	○ Yes ○ No			
Referees' or other scientific critique report	○ Yes ○ No			
Summary, synopsis or diagram (flowchart) of protocol in non–technical language	○ Yes ○ No			
Interview schedules or topic guides for participants	○ Yes ○ No			
Validated questionnaire	○ Yes ○ No			
Non–validated questionnaire	○ Yes ○ No			
Copies of advertisement material for research participants, e.g. posters, newspaper adverts, website. For video or audio cassettes, please also provide the printed script.	○ Yes ○ No			

WELCOME TO THE NHS RESEARCH ETHICS COMMITTEE APPLICATION FORM

An application form specific to your project will be created from the answers you give to the following questions. Please read this guidance carefully before selecting your answers.

1. Is your project an audit or service evaluation?

○ Yes ◉ No

2. Select one research category from the list below:

○ Clinical trials of investigational medicinal products (including phase 1 drug development)
○ Clinical investigations or other studies of medical devices
○ Other clinical trial or clinical investigation
○ Research administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
○ Research involving qualitative methods only
◉ Research limited to working with human tissue samples and/or data

If your work does not fit any of these categories, select the option below:

○ Other research

2a. Please answer the following questions:

a) Will you be taking new human tissue samples?	◉ Yes	○ No
b) Will you be using existing human tissue samples identifiable to the researcher?	○ Yes	◉ No
c) Will you be using only existing human tissue samples anonymous to the researcher?	○ Yes	◉ No
d) Will you be using identifiable data?	◉ Yes	○ No
e) Will you be using only anonymous or pseudonymised data?	○ Yes	◉ No

3. Is your research confined to one site?

○ Yes ◉ No

4. Does your research involve work with prisoners?

○ Yes ◉ No

5. Does your research involve adults unable to consent for themselves through physical or mental incapacity?

○ Yes ◉ No

6. Is the study, or any part of the study, being undertaken as an educational project?

◉ Yes ○ No

NHS Research Ethics Committee NHS
Application form for research limited to working with human tissue samples and/or data

This form should be completed by the Chief Investigator, after reading the guidance notes. See glossary for clarification of different terms in the application form.

Short title and version number: (maximum 70 characters – this will be inserted as header on all forms)
Genes and keratoconus version 2

Name of NHS Research Ethics Committee to which application for ethical review is being made:
Fife & Forth Valley LREC

Project reference number from above REC: 06/S0501/61
Submission date: 09/06/2006

PART A: Introduction

A1. Title of the research

Full title: Investigating genes that may cause keratoconus.

Key words: keratoconus, genes, VSX1

A2. Chief Investigator

Title:	Dr
Forename/Initials:	Marcela
Surname:	Votruba
Post:	MRC Clinician Scientist, Hon Consultant Senior Lecturer
Qualifications:	MA PhD FRCOphth
Organisation:	Cardiff University
Address:	Cardiff University, Redwood Building
	King Edward VII Avenue
	Cardiff
Post Code:	CF10 3NB
E-mail:	votrubam@cardiff.ac.uk
Telephone:	44 (0)29 2087 0134
Fax:	44 (0)29 2087 4859

A copy of a current CV (maximum 2 pages of A4) for the Chief Investigator must be submitted with the application

A3. Proposed study dates and duration

Start date: 01/07/2006
End date: 01/07/2008
Duration: Years: 2 ; Months:

A4. Primary purpose of the research: *(Tick as appropriate)*

- ☐ Commercial product development and/or licensing
- ☑ Publicly funded trial or scientific investigation
- ☑ Educational qualification
- ☐ Establishing a database/data storage facility
- ☐ Other

A6. Does this research require site-specific assessment (SSA)? (Advice can be found in the guidance notes on this topic.)

○ Yes ◉ No

If No, please justify:
The procedures involved are routine and only involve the study patient giving a DNA sample via mouthwash.

If Yes, Part C of the form will need to be completed for each research site and submitted for SSA to the relevant Local Research Ethics Committee. Do not submit Part Cs for other sites until the application has been booked for review and validated by the main Research Ethics Committee.

Management approval to proceed with the research will be required from the R&D Department for each NHS care organisation in which research procedures are undertaken. This applies whether or not the research is exempt from SSA.

PART A: Section 1

A7. What is the principal research question/objective? *(Must be in language comprehensible to a lay person.)*

What is the role and significance of mutations in single genes in the development of keratoconus in a UK population. This question aims to address the ongoing debate whether a gene called Vsx1 is responsible in causing keratoconus by screening a UK population of patients. This is the first study to screen this gene in Wales.

A8. What are the secondary research questions/objectives? *(If applicable, must be in language comprehensible to a lay person.)*

A9. What is the scientific justification for the research? What is the background? Why is this an area of importance? *(Must be in language comprehensible to a lay person.)*

Keratoconus is a major eye disease in the western world and the main candidate for corneal surgery. It is a disease in which the shape of the cornea is altered, thus changing its optical properties, and leading to blurred vision. The disease progresses until the third or fourth decade of life, when it usually stops. However there is a possibility that it may commence again at a later stage. Keratoconus has been associated with a vast number of other conditions, including Down's syndrome. At the moment early stages of keratoconus can be treated with hard contact lenses but as sight worsens only a surgery can fully restore sight. As yet there is no known cure for keratoconus other than corneal grafting; however numerous studies are being carried out into the structure of affected corneas and its developmental mechanisms.
There is evidence that keratoconus is inherited in some patients and families and that the cause in all patients could have a genetic contribution. There is an increasing amount of evidence that this is the case and a number of potential locations within a persons DNA have been found that might have a mutation leading to keratoconus. Current research suggests a number of possible genetic causes for keratoconus; on the one hand keratoconus may have multiple mutations causing it or on the other hand it may be primarily a single gene defect in some families and individuals. To date one candidate gene, VSX1, has been associated with keratoconus and mutations in this gene have been determined to be disease causing. A total of eight different mutations have been found so far. Mutation in VSX1 has also shown to lead to craniofacial anomalies in addition to keratoconus in one family. Mutations in VSX1 have also been linked with another corneal disorder, posterior polymorphous corneal dystrophy (PPCD).
There are a number of other candidate genes that may, by virtue of their position on a persons DNA or by their proposed function, be implicated in the genetic cause of keratoconus. We plan to screen for mutations firstly in the VSX1 gene as this gene is the foremost linked with keratoconus. We then will screen the other genes dependent on availability of primer sequences and reported functions in the literature.
The principle hypothesis that we are investigating is that keratoconus is a disease in which mutations in single genes may cause disease. Our rationale for this is that in the gene, VSX1, eight mutations have been found segregating in families and have been shown to cause keratoconus or posterior polymorphous corneal dystrophy in individuals in these families. The mutations were also shown to be inherited within these families. We believe that there may be other genes like VSX1. By gathering a cohort of keratoconus patients we hope to find novel mutations as well as confirm existing mutations in a population within the UK. This research is necessary and important because in the long term, this information may enable us to identify people who will develop KC in later life at an earlier stage than is now possible, and thus provide more targeted follow-up and appropriate treatment.

A10. Give a full summary of the purpose, design and methodology of the planned research, including a brief explanation of the theoretical framework that informs it. It should be clear exactly what will happen to the research participant, how many times and in what order. Describe any involvement of research participants, patient groups or communities in the design of the research.

This section must be completed in language comprehensible to the lay person. It must also be self-standing as it will be replicated in any applications for site-specific assessment on Part C. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.

The design of the investigation does not lend itself to scrutiny in the public domain as subject involvement is minimal. However the methodology of this investigation is very similar to other research studies that have been carried out in the past. Once ethical approval has been granted invitation letters will be sent to prospective volunteers who are existing patients seeing participating ophthalmologists in the study. If they agree to the study or request more information an information leaflet along with a consent form will be sent to them. Upon written consent being obtained a 'sample kit' containing instructions and a small tube containing salt water (saline) will be sent to them. This would theoretically be returned as soon

as possible to Cardiff University Similarly if patients are seeing their ophthalmologist they could be informed of the study and give consent followed by the sample at their visit. All samples would go to Cardiff University for testing and analysis. That would be the extent of subject contact providing a mutation is not found.
Once samples have been delivered they will be analysed using molecular genetic techniques. DNA will be extracted and amplified up using a known short sequence of single stranded DNA called primers. Once the DNA has been amplified it will be sequenced using the BIG DYE sequencing protocol. The sequences will then be analysed using bioinformatical techniques and the EMBOSS software suite. This will provide data on any changes in sequence present within the samples. Throughout the process samples will be coded and only if something is found within a sequence will the sample be traced back to the subject and that subject informed. The subject will be provided with any information they wish and counselling should the need arise. The subject will then be asked if they can recruit any of their relatives. This would be the only second time that the subject is consulted by us. We will of course provide any additional information at any time should the subject request it. The theory behind this investigation is that changes in a subjects DNA can lead to the presentation of the corneal disease keratoconus and that these changes can be inherited and can be seen as localising to certain families, though this can only be found provided relatives are also screened.

A12. Give details of any clinical intervention(s) or procedure(s) to be received by research participants over and above those which would normally be considered a part of routine clinical care. *(These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material.)*

Additional Intervention	Average number per participant		Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.
	Routine Care	Research		
Other tissue/bodily sample		1	1/0/0	A mouthwash of 15ml 0.9% sterile saline solution will be given by the subject under the supervision of either the PI, researcher, consultant or GP. No training is necessary.

A13. Give details of any non-clinical research-related intervention(s) or procedure(s). *(These include interviews, non-clinical observations and use of questionnaires.)*

Additional Intervention	Average number per participant	Average time taken (mins/hours/days)	Details of additional intervention or procedure, who will undertake it, and what training they have received.

A15. What is the expected total duration of participation in the study for each participant?

5 minutes

A17. What is the potential for pain, discomfort, distress, inconvenience or changes to lifestyle for research participants?

Very minimal, in the short term a visit to participate in research in a participating centre near the volunteer, or a short mouthwash routine for 1 morning. In the long term if the patient has a mutation they will be informed and any extra information or counselling will be provided. However there should be minimal distress if any.

If a particular mutation happens to be found in a control subject or subjects then this change would be considered non disease causing as it appears in a patient that does not have keratoconus, in which case there is no need to take any particular action to do with the affected patient as it has no bearing on the affected individual. The purpose of the study is to discover what changes occur in the target patient group compared to the control group.

A18. What is the potential for benefit to research participants?

Participants will be more informed about their condition if this applies.

A19. What is the potential for adverse effects, risks or hazards, pain, discomfort, distress, or inconvenience to the researchers themselves? *(if any)*

none

A20. How will potential participants in the study be (i) identified, (ii) approached and (iii) recruited?
Give details for cases and controls separately if appropriate:

Patients will be initially recruited if they have clinically diagnosed keratoconus. A letter of invitation of participation will be sent to existing patients of ophthalmologists and the Cardiff University Eye Clinic upon ethical approval. The age of onset of keratoconus is generally from the mid teens to early twenties. We hope to recruit individuals for this study who are 16 years or above Patients will be identified from patient records within the Optometry clinic in Cardiff and from consultant ophthamlmologists who are participating in the study. We aim to recruit 100+ individuals for the study from a number of different centres around the country. If this initial study is successful then additional family members will also be recruited and all who give consent will have samples taken.
A control population will be recruited initially form spouses and partners of affected individuals. These individuals are unrelated and so do not have the same genetic background. More control individuals shall be recruited through advertisements placed in participating institutions and centres and information will be provided about the study upon contact with the principle investigator.

A21. Where research participants will be recruited via advertisement, give specific details.

☐ Not Applicable

Posters explaining the nature of the study, patient involvement and contact details will be placed in participating institutions. Care will be taken to make sure that the advertisement material is open and clear and able to be read by all.

If applicable, enclose a copy of the advertisement/radio script/website/video for television (with a version number and date).

A22. What are the principal inclusion criteria? *(Please justify)*

Individuals for the study will be included if they have clinically diagnosed keratoconus. Consenting individuals will also be included if they are related to an affected individual.

A23. What are the principal exclusion criteria? *(Please justify)*

Exclusion will be based on non–compliance.

A24. Will the participants be from any of the following groups? *(Tick as appropriate)*

- ☐ Children under 16
- ☐ Adults with learning disabilities
- ☐ Adults who are unconscious or very severely ill
- ☐ Adults who have a terminal illness
- ☐ Adults in emergency situations
- ☐ Adults with mental illness (particularly if detained under Mental Health Legislation)
- ☐ Adults with dementia
- ☐ Prisoners

- ☐ Young Offenders
- ☐ Adults in Scotland who are unable to consent for themselves
- ☑ Healthy Volunteers
- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, e.g. those in care homes, medical students
- ☐ Other vulnerable groups

Justify their inclusion.

Healthy volunteers will be recruited to provide an age and background matched controls.

No other group will be included

A25. Will any research participants be recruited who are involved in existing research or have recently been involved in any research prior to recruitment?

○ Yes ○ No ◉ Not Known

If Yes, give details and justify their inclusion. If Not Known, what steps will you take to find out?

Patients will be asked upon their given consent.

A26. Will informed consent be obtained from the research participants?

◉ Yes ○ No

If Yes, give details of who will take consent and how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material.

If participants are to be recruited from any of the potentially vulnerable groups listed in A24, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.

If consent is not to be obtained, please explain why not.

Consent shall be obtained from a consent form given to the prospective volunteer upon initial interest by the volunteer and some form of agreement. i.e. verbal, written agreement and if they ask for more information. Information shall be provided in an information leaflet and additional information can be provided by the recruiting ophthalmologists, principle investigator or researcher upon request.

The same consent form shall be used for both the study group and control group

Copies of the written information and all other explanatory material should accompany this application.

A27. Will a signed record of consent be obtained?

◉ Yes ○ No

If Yes, attach a copy of the information sheet to be used, with a version number and date.

A28. How long will the participant have to decide whether to take part in the research?

Subjects will have a period of between 1 week to 3 months to decide if they wish to participate.

A29. What arrangements have been made for participants who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? *(e.g. translation, use of interpreters etc.)*

Translations will be provided and copies of all information will be made available in a different language upon request. As the study will be conducted in Wales, welsh and english forms will be provided.

A31. Does this study have or require approval of the Patient Information Advisory Group (PIAG) or other bodies with a similar remit? *(see the guidance notes)*

○ Yes ◉ No

A32a. Will the research participants' General Practitioner be informed that they are taking part in the study?

◉ Yes ○ No

If Yes, enclose a copy of the information sheet/letter for the GP with a version number and date.

A32b. Will permission be sought from the research participants to inform their GP before this is done?

◉ Yes ○ No

If No to either question, explain why not

It should be made clear in the patient information sheet if the research participant's GP will be informed.

A33. Will individual research participants receive any payments for taking part in this research?

○ Yes ◉ No

A34. Will individual research participants receive *reimbursement of expenses* or any other *incentives or benefits* for taking part in this research?

○ Yes ◉ No

A35. What arrangements have been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, participants for *negligent* harm?

NHS indemnity applies

Please forward copies of the relevant documents

A36. What arrangements have been made to provide indemnity and/or compensation in the event of a claim by, or on behalf of, participants for *non-negligent* harm?

No compensation arrangements are available under NHS indemnity

Please forward copies of the relevant documents.

A37. How is it intended the results of the study will be reported and disseminated? *(Tick as appropriate)*

- [x] Peer reviewed scientific journals
- [x] Internal report
- [x] Conference presentation
- [ ] Other publication
- [ ] Submission to regulatory authorities
- [ ] Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- [x] Written feedback to research participants
- [ ] Presentation to participants or relevant community groups
- [ ] Other/none e.g. Cochrane Review, University Library

A38. How will the results of research be made available to research participants and communities from which they are drawn?

Peer reviewed journal papers will be made available to participants if they wish.
A short report detailing the findings that are relevant to the subject will be sent to them upon completion of their involvement in the study.

A39. Will the research involve any of the following activities at any stage (including identification of potential research participants)? *(Tick as appropriate)*

- [x] Examination of medical records by those outside the NHS, or within the NHS by those who would not normally have access
- [ ] Electronic transfer by magnetic or optical media, e-mail or computer networks
- [ ] Sharing of data with other organisations
- [ ] Export of data outside the European Union
- [x] Use of personal addresses, postcodes, faxes, e-mails or telephone numbers
- [ ] Publication of direct quotations from respondents
- [ ] Publication of data that might allow identification of individuals
- [ ] Use of audio/visual recording devices
- [x] Storage of personal data on any of the following:
  - [ ] Manual files including X-rays
  - [ ] NHS computers
  - [ ] Home or other personal computers
  - [x] University computers
  - [ ] Private company computers
  - [ ] Laptop computers

Further details:

A40. What measures have been put in place to ensure confidentiality of personal data? Give details of whether any encryption or other anonymisation procedures have been used and at what stage:

Samples gathered will be anonymised to ensure confidentiality. Patients will be not be referred to directly and only the principle investigator and researcher will have access to the real data.

A41. Where will the analysis of the data from the study take place and by whom will it be undertaken?

Analysis will take place within the School of Optometry and Vision Sciences at Cardiff University and will be carried out by the principle investigators research student.

A42. Who will have control of and act as the custodian for the data generated by the study?

Data will belong to Cardiff University

A43. Who will have access to the data generated by the study?

Data will be available to the priciple investigator and the researcher.

A44. For how long will data from the study be stored?

10 Years Months

Give details of where they will be stored, who will have access and the custodial arrangements for the data:
Data will be stored on optical media at Cardiff University and data will belong to Cardiff University and be made available to the PI and researcher in the School of Optometry and Vision Sciences.

A45–1. How has the scientific quality of the research been assessed? *(Tick as appropriate)*

- ☐ Independent external review
- ☐ Review within a company
- ☐ Review within a multi–centre research group
- ☑ Internal review (e.g. involving colleagues, academic supervisor)
- ☐ None external to the investigator
- ☐ Other, e.g. methodological guidelines *(give details below)*

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review:
Local ethical approval and R&D approval has been granted by Cardiff University and the Cardiff and Vale/ Glamorgan Vale NHS Trust

If you are in possession of any referees' comments or other scientific critique reports relevant to the proposed research, these must be enclosed with the application.

A45–2. Has the protocol submitted with this application been the subject of review by a statistician independent of the research team? *(Select one of the following)*

- ○ Yes – copy of review enclosed
- ○ Yes – details of review available from the following individual or organisation (give contact details below)
- ◉ No – justify below

The research being conducted does not rely on finding a statistical difference and relies instead on finding actual mutations within a gene that goes not need statistical analysis

A48. What is the primary outcome measure for the study?

If our hypothesis is correct then the study would show that keratoconus in some individuals develops as a result of single mutation in a candidate gene. The frequency of these mutations in the study population will be a measurable outcome however the primary outcome will be the actual changes found within the gene.

A49. What are the secondary outcome measures?*(if any)*

A50. How many participants will be recruited?
If there is more than one group, state how many participants will be recruited in each group. For international studies, say how many participants will be recruited in the UK and in total.

We will aim to recruit 100+ affected individuals with a control group to be consisting of several hundred individuals. Age and background matched controls shall be sought from the local population through advertisement.

A51. How was the number of participants decided upon?

A snowballing approach to recruitment will be used to gather research participants. As we intend to look for mutations the chance of finding them is increased with a larger population size. Participants are being recruited through local opthalomogists and corneal specialists at the local hospital. Once ethical approval has been gained contact shall be made with willing collaborators who have existing contact with affected individuals.

If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.

A52. Will participants be allocated to groups at random?

○ Yes ◉ No

A53. Describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Mutation frequency will be analysed using T Test, Chi-square, and simple parametric statistics comparing frequency of mutation or DNA change detected in affected subjects versus unaffected subjects.

A54. Where will the research take place?*(Tick as appropriate)*

- ☑ UK
- ☐ Other states in European Union
- ☐ Other countries in European Economic Area
- ☐ Other

If Other, give details:

A55. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK, the European Union or the European Economic Area?

○ Yes ◉ No

A56. In how many and what type of host organisations (NHS or other) in the UK is it intended the proposed study will take place?

Indicate the type of organisation by ticking the box and give approximate numbers if known:

	Number of organisations
☑ Acute teaching NHS Trusts	4
☐ Acute NHS Trusts	
☐ NHS Primary Care Trusts or Local Health Boards in Wales	
☐ NHS Trusts providing mental healthcare	
☐ NHS Health Boards in Scotland	
☐ HPSS Trusts in Northern Ireland	
☐ GP Practices	
☐ NHS Care Trusts	
☐ Social care organisations	
☐ Prisons	
☐ Independent hospitals	
☐ Educational establishments	
☐ Independent research units	
☐ Other (give details)	

Other:

A57. What arrangements are in place for monitoring and auditing the conduct of the research?

The audit of the research and the monitoring of how it is performed is under the control of the Chief investigator, who will adhere to all clinicial governance guidlines and research governance guidelines.

Will a data monitoring committee be convened?

○ Yes ◉ No

If Yes, details of membership of the data monitoring committee (DMC), its standard operating procedures and summaries of reports of interim analyses to the DMC must be forwarded to the NHS Research Ethics Committee which gives a favourable opinion of the study.

What are the criteria for electively stopping the trial or other research prematurely?

The research would be stopped once enough patients have been gathered.

A58. Has external funding for the research been secured?

○ Yes ◉ No

If No, what arrangements are being made to cover any costs of the research? If no external funding is being sought, please say so:

Funding is available from within the School of Optmetry & Vision Sciences, Cardiff University and within the Investigators

research funds.

A59. Has the funder of the research agreed to act as sponsor as set out in the Research Governance Framework?

○ Yes ◉ No

Has the employer of the Chief Investigator agreed to act as sponsor of the research?

◉ Yes ○ No

Sponsor *(must be completed in all cases)*

Name of organisation which will act as sponsor for the research:

Cardiff University

Status:

○ NHS or HPSS care organisation ◉ Academic ○ Pharmaceutical industry ○ Medical device industry ○ Other

If Other, please specify:

Address:	Cardiff University, Redwood Building King Edward VII Avenue Cardiff
Post Code:	CF10 3NB
Telephone:	Fax:
E-mail:	

The responsibilities of the sponsor may be shared between co-sponsors. If this applies, name the lead sponsor for the REC application in this box and enclose a letter giving further details of co-sponsors and their responsibilities.

Sponsor's UK contact point for correspondence with the main REC

Title: Mr Forename/Initials: Chris Surname: Shaw

Address:	Cardiff University, Redwood Building King Edward VII Avenue Cardiff
Post Code:	CF10 3NB
Telephone:	02920 879130 Fax:
E-mail:	ShawC3@cardiff.ac.uk

A60. Has any responsibility for the research been delegated to a subcontractor?

○ Yes ◉ No

A61. Will individual *researchers* receive any personal payment over and above normal salary for undertaking this research?

○ Yes ◉ No

A62. Will individual *researchers* receive any other benefits or incentives for taking part in this research?

○ Yes ◉ No

A63. Will the host organisation or the researcher's department(s) or institution(s) receive any payment or benefits in excess of the costs of undertaking the research?

○ Yes ◉ No

A64. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share-holding, personal relationship etc.) in the organisation sponsoring or funding the research that may give rise to a possible conflict of interest?

○ Yes ◉ No

A65. Other relevant reference numbers if known*(give details and version numbers as appropriate):*

Applicant's/organisation's own reference number, e.g. R&D (if available):	05/IBD/3361
Sponsor's/protocol number:	SPON CU 128
Funder's reference number:	NA
International Standard Randomised Controlled Trial Number (ISRCTN):	NA
European Clinical Trials Database (EudraCT) number:	NA
Project website: NA	

A66. Other key investigators/collaborators*(all grant co-applicants should be listed)*

Title:	Mr	
	Forename/Initials: Jack	Surname: Sheppard
Post:	PhD Student	
Qualifications:	BSc (Hons)	
Organisation:		
Address:	Cardiff University	
	Redwood Building	Telephone: 02920 876471
	King Ecward VII Avenue	Fax:
Postcode:	CF24 3NB	
E-mail:	sheppardj@cf.ac.uk	

PART A: Summary of Ethical Issues

A68. What do you consider to be the main ethical issues which may arise with the proposed study and what steps will be taken to address these?

The main ethical issues involved are storage of samples and subject confidentiality. This will be dealt with by an annonymisation process to be carried out during the investigation where the samples will be coded by a number system so that at no time can a sample be linked back to a subject. The only time a sample would need to be identified is when there

is a finding and the subject will be informed. In this case the sample code will be decoded and the subject information obtained.

PART A: Student Page

A70. Give details of the educational course or degree for which this research is being undertaken:

Name and level of course/degree:
The Genetic and Structural Pathogenesis of Keratoconus. Doctorate of Philosohpy (PhD).

Name of educational establishment:
Cardiff University

Name and contact details of educational supervisor:
Dr M Votruba MA PhD FRCOphth
MRC Clinician Scientist
Hon Consultant Senior Lecturer
School of Optometry & Vision Sciences
Cardiff University, Redwood Building
King Edward VII Avenue
Cardiff CF10 3NB

A71. Declaration of supervisor

I have read and approved both the research proposal and this application for the ethical review. I undertake to fulfil the responsibilities of a supervisor as set out in the Research Governance Framework for Health and Social Care.

Signature: .....

Print Name:

Date: (dd/mm/yyyy)

A one-page summary of the supervisor's CV should be submitted with the application

A72. Declaration by academic sponsor

To be completed by an authorised person on behalf of the academic institution acting as sponsor for student research.

I can confirm on behalf of my academic institution that any necessary indemnity or insurance arrangements will be in place before this research starts, as required by the Research Governance Framework for Health and Social Care.

Signature: .....

Print Name:

Post:

Institution:

Date: (dd/mm/yyyy)

PART B: Section 1 – Conduct of the research at local sites

From the answer given to question A6, it is assumed that:

- *Local Principal Investigators will not be appointed at each research site participating in this study.*
- *Applications for site-specific assessment by local Research Ethics Committees on Part C of the form will not be required.*
- *There will be no requirement for individual research sites to be approved by the main REC as part of the ethical review.*

The following general information should be provided to the main REC about the local conduct of the study.

1. What research procedures will be carried out at individual research sites?

A mouthwash sample will be given. Samples will then be collected and transported to the main research site.

2. Are any ethical issues likely to arise at individual sites that are not covered in the protocol for the study and if so how will these be addressed?
For example, a need for particular facilities, or to notify local clinicians or departments about the research, or to arrange additional local support for participants.

All ehtical issues are the same as the main site. All concerns and queries at local sites will be directed to the main principal investigator at the main research site.

3. How will the Chief Investigator and his/her team supervise the conduct of the research at individual sites? What responsibilities will be delegated to local collaborators?

Local collaborators will only supervise the collection of mouthwash samples. All analysis and investigation of samples shall be conducted by the chief investigator and her team.

Management approval to proceed with the research will be required from the R D Department for each NHS care organisation in which research procedures are undertaken.

PART B: Section 5 – Use of newly obtained human biological materials

1. What samples will be collected and/or analysed and by whom will they be collected?

Samples will be collected by post by the principle investigtor's research student. All samples collected at participating institutions will be collected by their respective participating consultants in clinics then posted to Cardiff. Samples sent via post comply with laws regulating shipping and packaging of biological materials.

2. Are the samples taken solely for research purposes (or are they a by–product of those taken primarily for clinical purposes, i.e. surplus to clinical requirements)?

Samples taken are solely for research purposes.

3. How will samples be labelled/identified?
Indicate if samples can be considered to be "identified", "coded", "de–identified", "anonymised" or "anonymous" and at what stage identifiers are removed.

Samples will be anonymised during the course of the study except when the results indicate that there is a finding in which case they will be identified and the relevant individual will be consulted.

4. Give details of where the sample(s) will be stored, for how long, who will have access and the custodial arrangements.

Samples will be stored at –80 degrees celcius at the School of Optometry and Vision Sciences. Samples will be stored for the duration of the study and then transferred to another freezer for long term storage for no more than 5 years. Cardiff University will have all custodial rights.

5. Will the research participant retain any rights to the sample(s)?

◉ Yes ○ No

If Yes, give details. If the sample is a gift, this must be clear in the information sheet. What will happen to samples if a participant withdraws from the study?
If the participant withdraws from the study then their sample will be destroyed

6. Is it known how the samples will be used in the future?

◉ Yes ○ No

If Yes, give details and indicate if consent will be obtained for the future use of samples:
There wil be no oher use other than in this study.

7. Does the research involve the analysis or use of genetic material from human biological materials?

◉ Yes ○ No

8. Would it be possible to link the results of any genetic analysis back to individuals?

◉ Yes ○ No

9. Is it intended to link the results of any genetic analysis back to individuals?

(●) Yes ( ) No

If Yes, give details of what support or counselling service will be available to individuals:
If a finding presents itself from any of the samples then the subject concerned will be contacted and asked if they wish to know, and then informed about the finding, what it means and what implications there are for the future. Since all subject all ready know they have a disease– ie keratoconus– the knowledge that a specific mutation in a specific gene has been identified will not impact on their routine management or prognosis. Routine genetic counselling will be made available to the subject.

PART B: Section 7 – Declaration

– The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

– I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

– If the research is approved I undertake to adhere to the study protocol, the terms of the full application of which the main REC has given a favourable opinion and any conditions set out by the main REC in giving its favourable opinion.

– I undertake to seek an ethical opinion from the main REC before implementing substantial amendments to the protocol or to the terms of the full application of which the main REC has given a favourable opinion.

– I undertake to submit annual progress reports setting out the progress of the research.

– I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.

– I understand that research records/data may be subject to inspection for audit purposes if required in future.

– I understand that personal data about me as a researcher in this application will be held by the relevant RECs and their operational managers and that this will be managed according to the principles established in the Data Protection Act.

– I understand that the information contained in this application, any supporting documentation and all correspondence with NHS Research Ethics Committees or their operational managers relating to the application, will be subject to the provisions of the Freedom of Information Acts. The information may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

Signature: .....

Date: 05/05/2006 (dd/mm/yyyy)

Print Name: Marcela Votruba

Patient Invitation. Version 1. 22/05/2006

Dear Sir/ Madam,
Research Study: Investigating genes that may cause keratoconus.

We are carrying out a research project to try and discover more about the genes that may cause the eye condition, keratoconus. In order to do this we are collecting DNA via a simple mouthwash from affected and unaffected members of families. The genetic material, called DNA, will be extracted and analysed in order to try and determine the faulty gene. This is important in helping us to understand the exact nature of the condition and will be a starting point in finding future forms of treatment. We would be very grateful if you would be willing to have a mouth wash sample taken for this research

If you are interested in taking part, or getting more information, we include an Information Leaflet and Consent Form. **If you decide to take part in the study you can sign and return the Consent Form.**

If you have any questions please contact **Dr Votruba on tel: 029 2087 0134**. You should be aware that you are under no obligation to take part in this research. Your decision to participate in this study does not affect any treatment you may or may not have. Your GP will be notified of your participation in this study unless you would not like this.

Yours sincerely,
Dr Marcela Votruba PhD FRCOphth
MRC Clinician Scientist, Honorary Consultant Ophthalmologist and Senior Lecturer

Jack Sheppard

Version 2 Information Leaflet 30/05/2006

Direct dial: 029 2087 0134

Dear Sir/Madam,

Re: Investigating genes that may cause keratoconus

My name is Jack Sheppard and I am a research student at Cardiff University in the Ocular Genetics Research Group in the School of Optometry and Vision Sciences. Together with Dr Marcela Votruba, who is my Supervisor, we are investigating potential genes that can cause the eye condition, keratoconus. Keratoconus is a disorder of the cornea, which is the clear window at the front of the eye. This window is normally smoothly curved but it becomes gradually more cone shape as time progresses in people with keratoconus, resulting in visual problems.

Part 1 on this information sheet will tell you about the purpose of the study and what you will have to do if you decide to take part.
Part 2 will give you more information on the conduct of the study

Part 1

Purpose of the study
We are planning to investigate the role of some genes that may be involved in keratoconus. One of the genes we will look at is a gene called *VSX1*. It is known to be linked to keratoconus and another corneal problem, posterior polymorphous corneal dystrophy (*PPCD*), in some patients. Other genes will also be investigated as they become known. As part of this study we are planning to screen the DNA of people with keratoconus to see if we can find any changes in the genes, known as mutations. This will improve our understanding of how keratoconus can begin and what genetic factors can cause keratoconus. This work may also provide us with ways to identify patients with suspected keratoconus quicker and earlier in the future and thus provide a better service and for keratoconus sufferers. The work being carried out is part of a doctorate into the genetics of keratoconus.

Why do we need volunteers?
We are very keen to recruit as many people as we can to make sure that our study is a scientific success and help us to better understand the condition.

We need to collect DNA from subjects with keratoconus and from people who do not have the condition to act as a control group. If there is a family history of keratoconus we will ask you to speak to your relatives. If they then wish to get more information about taking part we will contact them as well with your permission.

Do I have to take part?
If you wish to take part we ask that you complete the consent form attached. Your participation is completely voluntary and you can withdraw from the study at any time for any reason. A decision to withdraw at any time will not affect the standard of care you receive.

What will the study involve?

- The study involves 5 minutes of your time.
- We will ask you to take a mouthwash of very mild salty water, which will allow us to extract your DNA.
- You will be given clear instructions how to do this.
- The main location for this research is the Cardiff University Eye Clinic, which is part of the School of Optometry and Vision Sciences. If you are unable to attend the clinic, or have been approached through a local optician or consultant, we can arrange for you to send us a mouth wash sample by post.

Expenses and payments
All postage costs and packaging will be provided should you choose to send a sample by post. No payments or expenses will be covered for visits or participation.

What happens when the research stops?
When the study has finished the genetic sequence of the gene being studied will be compared to that of control subjects to see if there are any changes that occur, if we find that there is a change in your sequence that does not appear in the control population you will be informed with a short report explaining what this means. Unfortunately the finding will not provide any short term benefit to you just a greater understanding to your condition that could be used to improve the level of care you receive.

Further Information
If you wish further information of the study you can contact us via the details provided

Part 2

Do we have Ethical Approval?
We have Ethical Approval for this Study from the Central Office of Research and Ethics Committees and approval from the Cardiff & Vale NHS Trust Research and Development Office.

Where will the study be carried out?
The laboratory part of the study will take place in the School of Optometry and Vision in Cardiff University. The clinic is situated in the Redwood Building, King Edward VIIth Avenue, which is in the centre of Cardiff. This study is also taking place in centres around the UK and recruitment will be carried out locally to each centre.

What if new information becomes available?
During the course of this study it is possible that new genes of interest connected to keratoconus would be brought to light and will be investigated. This would not require any further action on your part.

What If I don't want to carry on with the study?
If you no longer wish to participate then your sample will be destroyed but any data collected from your sample will be included in the research.

Will my participation in this study be kept confidential?
Your sample and any data collected will be assigned a number which only the researchers will know. Any information you give will be kept confidential and secure. This may be looked at by other authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality as you as a research participant and nothing tat could reveal your identity will be disclosed outside the research site.

Will my GP be notified?
If you wish your general practitioner to be notified about your participation in the study then we will inform him with your permission.

What will happen to any samples I give?
Samples will be stored at the research facility and only the researchers will have access. Samples will be numbered and anonymised so that only the researchers will be able to identify which sample belongs to which patient. After the study has finished samples will be stored for 5 years after which they will be destroyed.

What will happen to the data collected?
The data collected will be analysed to see if there are any sequence changes between control clinically normal patient samples and those affected with keratoconus. This data will be used to further the understanding into what causes keratoconus and will be eventually published in peer reviewed journals.

Who is organising and funding the research?
The study is being organised by the School of Optometry and Vision Sciences at Cardiff University and is being funded by the Medical Research Council (MRC).

What you should do if you'd like to participate.
If you would like to participate in this study please fill in and return the consent form enclosed. If you have any questions please do not hesitate to contact us at the above address.

Once you have returned it we will either take the mouth wash sample there and then or contact you to arrange to send you a small stamped and addressed tube for the mouth wash, which you will return by post.

We would like to thank you in advance for your interest and co-operation.
Yours Sincerely,
Dr Marcela Votruba PhD FRCOphth, Honorary Consultant Senior Lecturer
and Mr Jack Sheppard, PhD Student.

CONSENT FORM

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Investigating genes that may cause keratoconus

Declaration

1. I agree to take part in the above study and have a DNA sample taken by mouth wash. ☐

2. I confirm that I have read and understood the information sheet dated 31/03/05 (version 1) for the above study and have had the opportunity to ask questions. ☐

3. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

4. I understand that sections of my medical notes may be looked at by Dr Marcela Votruba and regulatory authorities, including the Ethics Committee, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

5. I AM/ AM NOT happy for my GP to be informed (delete as required).

6. Name, address and telephone of GP if you are happy for them to be informed:

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……
……

Full name of person participating in the study:……

Contact Details:
Address.....
...
.....
.....
.....
.....
.....
.....

Telephone number.....

Signed .....Date.....

Witness' Signature .....Date.....
Print Witness Name.....

Travel expenses will be reimbursed if I attend for an extra appointment necessary for this research.

Research to be carried out by Dr Marcela Votruba (Principal Investigator) and Jack Sheppard.

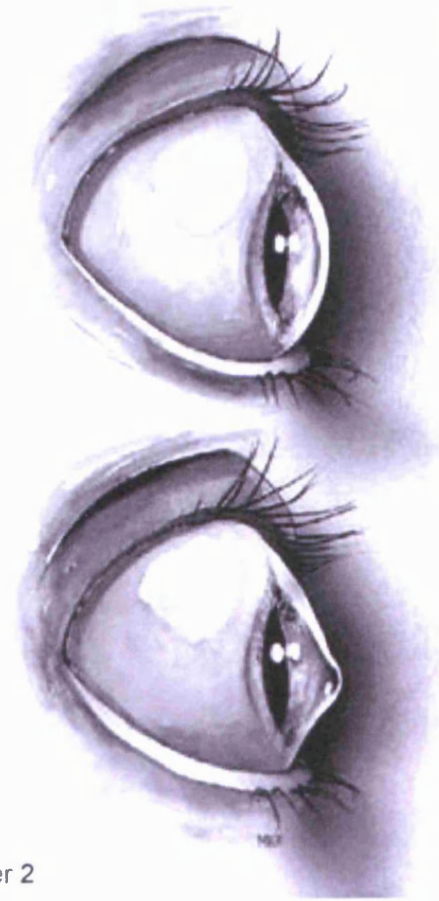
Contact details: Dr M Votruba, PhD FRCOphth. Hon. Consultant Ophthalmologist. Direct dial: 029 2087 0134.

(1 for patient, 1 for researcher, 1 to be kept with hospital notes if subject recruited in a hospital setting.)

Volunteers Needed!

CARDIFF UNIVERSITY
PRIFYSGOL CAERDYDD

Keratoconus Study



We are currently looking for **volunteers** to take part in our study into the genetics of Keratoconus.

We are looking for **volunteers** of 16 years of age or over, who have been diagnosed with keratoconus to take part.

All you have to do is provide a mouthwash sample and we will examine your DNA/genetic material.

The study will only take 5 minutes of your time and will offer you the opportunity to take part in exciting scientific research!

If you wish to take part or want more information do not hesitate to contact Jack on 02920 876471, sheppardj@cf.ac.uk or ask at the Eye Clinic for a leaflet.

This research is being conducted by the Ocular Genetics Research Group at Cardiff University and is part of a multicenter study across the UK. Department of Optometry and Vision sciences, Cardiff University, Redwood Building, King Edward VII avenue, Cardiff CF10 3NB

Ver 2

School of Optometry and Vision Sciences
Ysgol Optometreg a Gwyddorau Golygol
Head of School *Pennaeth Yr Ysgol* Professor *Yr Athro* Tim Wess

Cardiff University
Redwood Building
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http://www.cf.ac.uk/

Prifysgol Caerdydd
Adeilad Redwood
Rhodfa Edward VII Parc Cathays
Caerdydd CF10 3NB
Cymru, Y Deyrnas Gyfunol

Mouthwash Instructions
version 1 22/05/2006

Thank you for your help with the study on keratoconus so far. We are now in the process of collecting mouthwashes and would be grateful if you would please follow the instructions below to provide your samples.

Mouthwash Instructions

The mouthwash is quick and painless, although it does taste quite salty. If you swallow any mouthwash it will not harm you in any way.

You should have received one plastic container with your name written on the side, containing sterile mouthwash solution.

We suggest that you do the mouthwash before breakfast and before brushing your teeth.

PLEASE FOLLOW THESE INSTRUCTIONS

1. Pour the mouthwash solution from the container into your mouth.
2. Vigorously swish the solution around your mouth for *at least* 20 seconds.
3. Carefully spit the solution back into the plastic container.

4. Please post your mouthwash sample to us, *as soon as possible*, in the freepost addressed bubble wrap envelope provided. (Please screw the cap on tightly)

Usually one mouthwash sample is sufficient for our analyses. However each individual is different, therefore if we find that there is not enough DNA in the samples we may contact you again to provide some more.

If you have any questions please contact Jack Sheppard or Marcela Votruba on (029) 20 876471

Thank you for your time.