CLINICAL AND GENETIC STUDIES OF KERATOCONUS: A CASE CONTROL STUDY

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CLINICAL AND GENETIC STUDIES OF KERATOCONUS: A CASE CONTROL STUDY

By

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A thesis submitted to the Department of Pre-clinical Sciences,

Faculty of Medicine and Health Sciences,

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ABSTRACT

CLINICAL AND GENETIC STUDIES OF KERATOCONUS: A CASE CONTROL STUDY

JENNY PARAMESHVARA DEVA @ nee NG GEK PHENG

Keratoconus (KC) was initially described about 150 years ago, but still remains an enigmatic disease. International research is still pursuing the wide ranging genetic and environmental factors which play an important role in aetiopathogenesis. The complexity of Keratoconus makes it difficult to investigate and conclusively find a solution to treat it clinically and genetically.

The first objective of this study is to analyse the risk factors associated with keratoconus. The main contribution of this study, shows that eye rubbing and severe rubbing in KC were more significantly present (78.6%) as a habit than in the control Family (50%) and Normal (58.3%) groups. Eye rubbing is 4.85 times increased risk compared to normal controls. Eye itchiness seems to be 4.39 times more a risk in KC. Eye wateriness is 19.8 times more associated with KC compared with normal controls.

The second objectives is to assess the clinical keratoconus, with diagnostic methods such as topography, and the use of Crosslinking for the biomechanical stabilization and treatment of KC patients. We find that the visual acuity of post-CXL KC patients improved to above 90%, achieving optimized normal vision. This study has shown, that progression can be halted and the cornea can be

remolded and vision rehabilitated with Rigid Gas Permeable contact lenses or INTACS. Crosslinking definitely improves the corneal biomechanical strength, but does not fully correct, so that some cases need a second crosslinking a few years later.

Research to date has not identified any single major gene associated with the aetiology of KC, though in some families, the inheritance pattern does suggest such a model. It is clear that Keratoconus is a complex disease, and multifactorial issues complicate its aetiology and even its genetic heritage. The third objective of this study is to assess the role of the three candidate genes, *VSX*1exon3, *SOD*1exon2, and *COL*4A3exon17 in the aetiopathogenesis of KC. Results through PCR sequencing and Exome sequencing are encouraging but inconclusive. Multivariate analysis with Odds Ratio, Linkage Disequilibrium and Haploview Software have only partially confirmed the association between the VSX1 gene variants, A182A, P237P and R217H.

Further genomic research using Next Generational Sequencing (NGS) and GWAS possibly and hopefully will uncover the keratoconus mystery!

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APPROVAL SHEET

This thesis entitled **"CLINICAL AND GENETIC STUDIES OF KERATOCONUS: A CASE CONTROL STUDY"** was prepared by JENNY PARAMESHVARA DEVA @ nee NG GEK PHENG and submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy (Medical Sciences) at Universiti Tunku Abdul Rahman.

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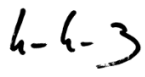

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Date: September 2021

SUBMISSION OF THESIS

It is hereby certified that JENNY PARAMESHVARA DEVA @ nee NG GEK PHENG (ID No: 14UMDO6333) has completed this thesis entitled "CLINICAL AND GENETIC STUDIES OF KERATOCONUS: A CASE CONTROL STUDY" under the supervision of Associate Professor Dr. Thaw Zin from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, and Professor. Dr. Ngeow Yun Fong from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, UTAR.

I understand that University will upload a softcopy of my thesis in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

(JENNY PARAMESHVARA DEVA @ nee NG GEK PHENG)

DECLARATION

I , Jenny Parameshvara Deva @ nee Ng Gek Pheng , hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

JENNY PARAMESHVARA DEVA @ nee NG GEK PHENG

Date: September 2021

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LIST OF SYMBOLS

bp	base pair
D/ Ds	dioptre/ dioptres
g/gm	gram
Kb	Kilobases
l	litre
M	molar
mg	milligram
ml	millilitre
Mm	millimolar
mg	milligram
mL	millilitre
mm	millimetre
OD	Oculus Dexta (*Latin*:Right Eye)
r	Pearson's correlation coefficient
SD	Standard Deviation
U/L	Units per litre
V	Volts
α	alpha
µ	micron

µg	microgram
µl	microlitre
µM	micromolar
p	rho: and Pearson correlation coefficient
r	Kendall's tau
x^2	Chi-square
±	plus or minus
-	negative
x	times or negative
/	per
%	precentage
>	more than
<	less than
^{0} C	Degree Celcius

LIST OF ABBREVIATIONS

Acm	anterior corneal mosaic
ANOVA	Analysis of variance
BLAST	Bsic Local alignment Search Tool
BS	Basement Layer or membrane
BSCVA	Binocular Single Visual Acuity
$CaCl_2$	Calcium Chloride
CCT	Central Corneal Thickness
CH	Corneal Hysteresis
CHISAM	Chloroform-3methyl-1-butanol
Corp	Corporation
CRF	Corneal Resonance Factor
CXL	Crosslinking
ddH_2O	double distilled water
DM	Descemet's membrane
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EPI-OFF	Epithelium removed before the CXL
et al	and others

EtBr	Ethidium bromide
Fig.	Figure
GAGs	Glycosamineglycans (Mucopolysacchaides)
GWAS	Genome Wide Sequencing
HCl	Hydrochloric acid.
HD	homeodomain
HWE	Hardy Weinberg Equilibrium
IBM	International Business Machines
ILIA	Interleuken 1alpha
ILIB	Interleuken1beta
IMHP	2-isopropyl -4methyl -6hydroxypyrimidine
Inc.	Incorporate
INTACS	Intracorneal lens Implant
IOL	Intraocular lens Implant
IOPcc	Corneal Collaborated Intraocular Pressure
IOPg	Intraocular Pressure
K	Keratometry
KC	Keratoconus
K-EDTA	potassium ethylene diamine tetraacetic acid.
Kmax	Maximum Keratometry
Kmin	Minimum Keratometry

Kstructure	Koyabashi Structure
$Mg\ Cl_2$	Magnesium Chloride
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre of Biotechnology Information
NGS	New Generational Sequencing
OCT	Ocular Coherent Tomography
OMIM	Ordinary mendelian Inheritance in Man
ORA	Ocular Resonance Analyser
PCR	Polymerase Chain reaction
PGs	Proteoglycans (Glyconated proteins)
PK	Penetrating Keratoplasty
PostCXL	After Crosslinking
PreCXL	Before Crosslinking
RFLP	restriction fragment length polymorphism
RGP	Rigid Gas permeable lenses
RINX	retinal inner nuclear homeobox
RNA	Ribonucleic Acide
ROS	reactive oxygen species
SE	Spherical Equivalent
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dimutase

SPSS	Statistical Package for the Social Sciences
TBE	TRIS-borate (boric acid)-EDTA
TE	TRIS-EDTA
TRIS	tris-(hydroxy methyl) aminomethane
USA	United States of America
UTAR	Universiti Tunku Abdul Rahman
UV	UltraViolet
VSX	Visual System Homeobox
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WHO	World Health Organisation

CHAPTER 1 INTRODUCTION

1.1 Introduction, history and overview

Keratoconus (KC) has historically been named from the Greek word "Kerato" meaning "cornea' and "conus" meaning a "protrusion". Thus, the term for this corneal disease resulting from thinning of the cornea has become" Keratoconus". This ocular condition was known as early as 1748 by Manchart, and 1766 by Taylor, but it was only identified by Nottingham in 1854 as quite different from other ectatic corneal conditions of the eye. Professor Sir Edward Duke Elder, well renowned author and editor of 15 volumes of Systematic Clinical Ophthalmology, has rightly earned himself the title of "Father of Ophthalmology". His volumes of knowledge are called "Encyclopedia Ophthalmologica". He described KC as a "non-inflammatory ectasia" of the cornea in its axial part, usually becoming manifest in youth or adolescence and resulting in immense visual impairment due to the development of a high degree of irregular myopic astigmatism" (Duke Elder, 1965, pg 964, Vol VIII).

The original text of Campinchini described KC as of "obscure aetiology". Decades later today, after much research, the aetiology still remains an enigma. KC is still known as a complex disease of multifactorial aetiology.

Campinchini and Haye in their original text in1962, described KC aetiology as "A multitude of theories has been put forward to account for its development, most of them relating to relatively few cases, none of them of general applicability and all of them representing inadequate attempts to solve a problem, which in our present state of ignorance is insoluble." Today, we

still find the following possibilities to be contributory in different ways to the development of KC.

Keratoconus (KC) is one of the most complex eye diseases which has been often missed in routine diagnostic procedures by ophthalmic personnel, optometrist, opticians, and even ophthalmologists. It is often mistaken as Amblyopia, and sometimes inadequately treated owing to misdiagnosis. It is a treatable disease when diagnosed early and treated with crosslinking (CXL) which halts the KC progression, and visual rehabilitation with Rigid Gas permeable contact lenses (RGPs).

1.2 Clinical Keratoconus

1.2.1 Clinical picture and progression

KC is the best- known of all ectactic conditions of the cornea. In KC, the eyeball does not have the usual almost spherical shape, as the anterior corneal surface, protrudes due to thinning either in the inferotemporal or inferonasal area, resulting in an unusually eccentric, paracentral coning. Thinning of the superior portion of the cornea is very rare. This thinned cornea, however, further thins with degeneration and this progressive ectasia results in high and irregular myopic astigmatism. As a result, vision is considerably reduced. This progressive corneal thinning process usually begins in adolescence and progresses at different rates in different individuals, irrespective of race, genetics, or environmental risk factors. Universally, the progression of KC usually slows down around the mid-thirties, stabilizing permanently to a condition referred to as “Forme Fruste Keratoconus”, but exceptions occur both with severity and duration of disease progression. It is uncommon for the KC condition to progress beyond 40 years of age (AlShammari *et al.*, 2016).

In most (90%) cases, KC is bilateral, though usually asymmetrical in severity and progression. Li *et al.* (2004) reported that approximately 50% of clinically normal fellow eyes progressed to KC within 16 years. Although a large proportion of KC patients can be managed with contact lenses, an average of about 20% of all KC corneas still require penetrating keratoplasty. Some authors report markedly different surgical indication rates of 6.5 and 12% to 45% (Ihalainen,1986).

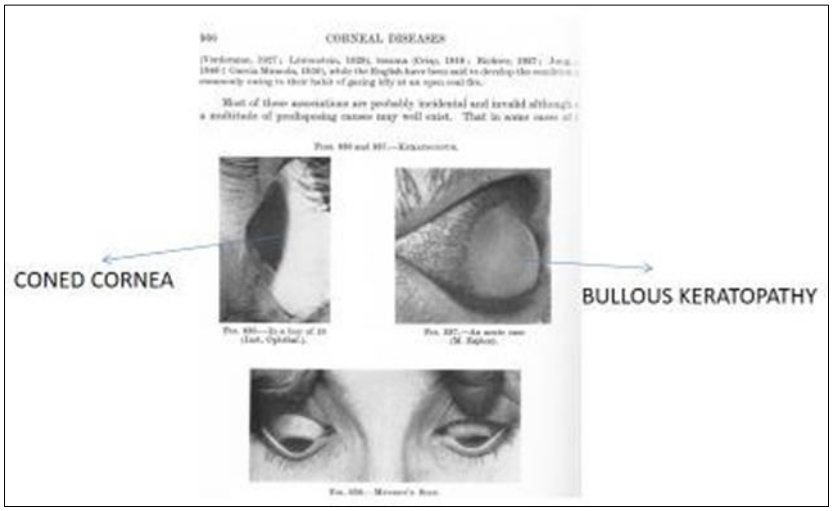


Figure 1. 1:Earliest and old pictures of KC eyes, at different levels of severity.
Source: Duke Elder (1965)

Figure 1. 2: Keratoconus with clear coned cornea
Source JPD

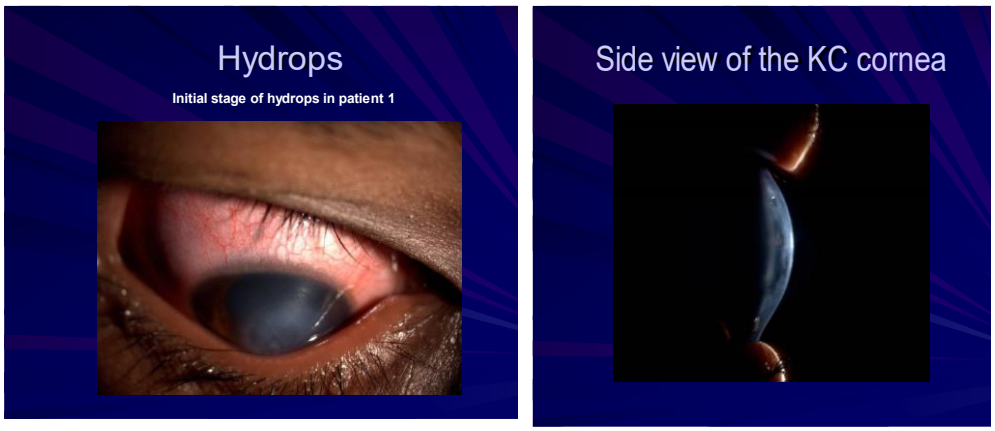


Figure 1. 3: Slit-Lamp view of the Corneal Hydrops
Source: JPD‘

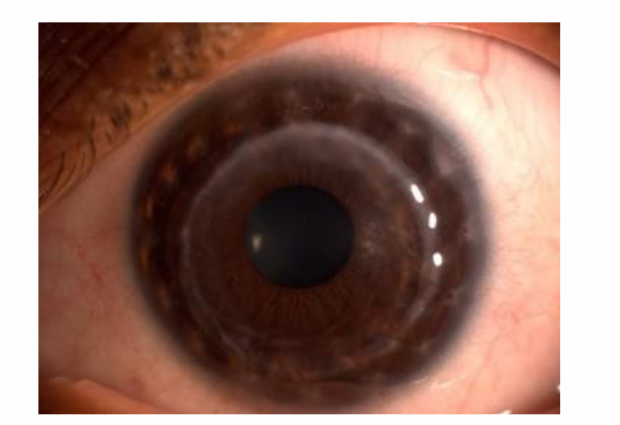


Figure 1. 4: Slit-Lamp view of the Post PK Cornea and recent topographical picture.
(This case was excluded from study, according to exclusion criteria)
Source: JPD

1.2.2 Histopathological Changes in Keratoconus

The normal intact corneal epithelium is 50u thick. Stratified non-keratinised squamous cells make up the epithelium. The Bowman's membrane is the anterior membrane. The substantia propria, the stroma comprises of corneal lamellae arranged parallel to each other, thus giving transparency to the whole cornea. The internal or posterior membrane is the Descemet's membrane which lines the inner surface of the endothelium.

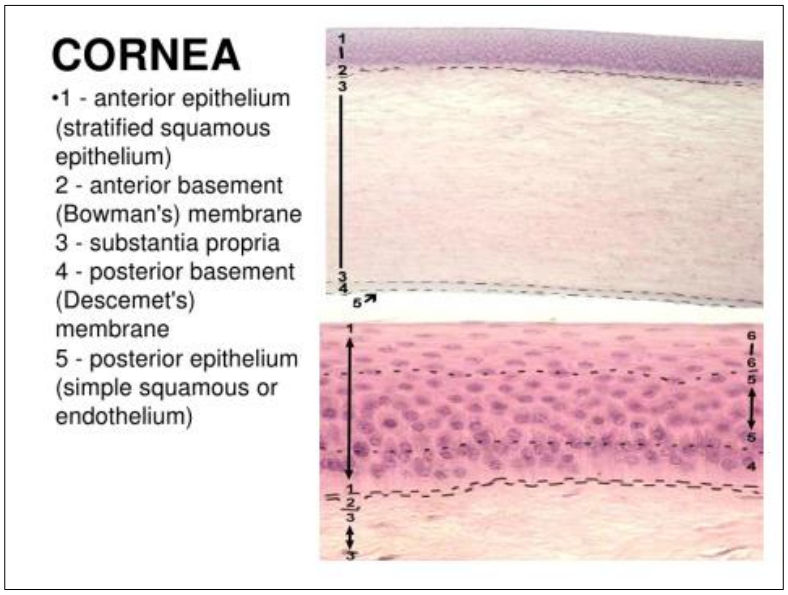


Figure 1. 5: Histopathology of the normal cornea
Souce: Vincent Borderie (2016)

Table 1. 1: Pathogenic Features of Keratoconus

Parts of cornea	Pathogenic features of keratoconus
Epithelium	The corneal epithelium shows elongated superficial cells, arranged in a whorl-like fashion[9-12] Iron particle deposition (Fleischer's ring) [13,14] Nerve fibers are thickened, visible, and less in number[15]
Bowman's membrane	Ruptures/breakages resulting in direct contact between epithelial and stromal cells[16,17]
Stroma	Thinning of stroma due to reduced number of lemellae and keratocytes Vertical lines/striae in the deeper layers of stroma[2,3,9,10,18]
Descemet's membrane	Ruptures and folds in keratoconus results in a porous membrane which leads to loss of endothelial cells[11,17]
Endothelium	Unaffected in keratoconus or may demonstrate pleomorphisms and elongation of cells[2]

Source: Shetty R (2015).

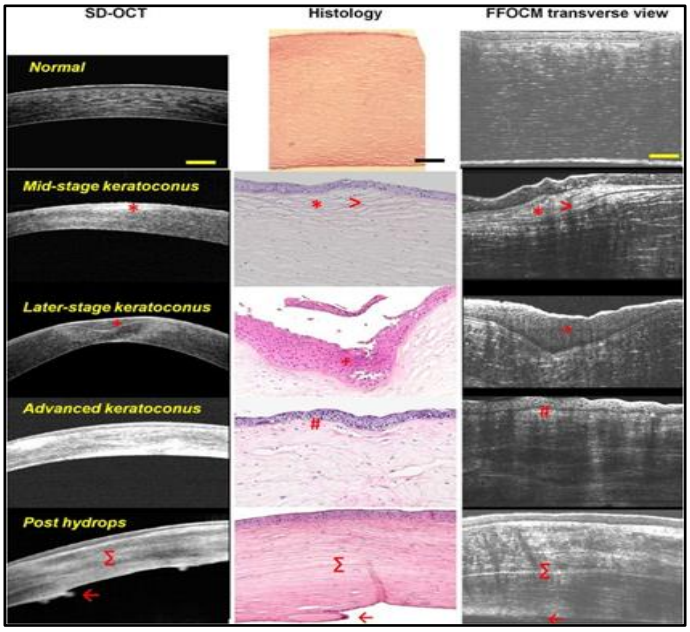


Figure 1. 6: Cross-sectional views of the normal cornea and keratoconus at various stages of development. Scale bars 100 mm in FFOCM and histology, 200 mm in SD-OCT.

From the figure above, the normal cornea and keratoconus in different patients at early to advanced stages (top to bottom rows) as viewed with SD-OCT (left column), histology (center column), and FFOCM in cross-section (right

column panels). Indicators noted are as follows: BL interruption; *fibrotic tissue; +thickened epithelium; #BL absent; P pan stromal scar; Descemet scarring. (Source: Vincent Borderie ,2016)

1.2.3 Clinical diagnosis

In KC, the eyeball does not have the usual normal, almost spherical shape, as the anterior corneal surface, protrudes due to thinning either in the inferotemporal or inferonasal area, resulting in an unusually eccentric, paracentral coning.(Fig. ?) Thinning of the superior portion of the cornea is very rare. This thinned cornea, however, further thins with degeneration and this progressive ectasia results in high and irregular myopic astigmatism. As a result, vision is considerably reduced. This progressive corneal thinning process usually begins in adolescence and progresses at different rates in different individuals, irrespective of race, genetics, or environmental risk factors. Universally, the progression of KC usually slows down around the mid-thirties, stabilizing permanently to a condition referred to as "Forme Fruste Keratoconus", but exceptions occur both with severity and duration of disease progression. It is uncommon for the KC condition to progress beyond 40 years of age [Leibowitz HM, "Keratoconus "pg 100; AlShammari *et al.* (2016)].

In most (90%) cases, KC is bilateral, though usually asymmetrical in severity and progression. Li *et al.* (2004) reported that approximately 50% of clinically normal fellow eyes progressed to KC within 16years. Although a large proportion of KC patients can be managed with contact lenses, an average of about 20% of all KC corneas require penetrating keratoplasty. Some authors report markedly different surgical indication rates of 6.5 and 12% to 45% (Ihalainen,1986).

In early KC disease, there may be no symptoms. With disease progression, vision is hampered by irregular astigmatism, myopia, and corneal scarring. Later, fine iron deposits are seen at the base of the KC cone called Fleischer's ring. Vogt's striae which are vertical lines produced by the compression of Descemet membrane may be seen at or near the apex of the cone. Corneal scarring is also common.

In advanced cases of KC, the ectatic cornea becomes visible when the patient looks downward and the corneal protrusion will push the lower lid into a "v-shaped" dent clinically named Munson's sign. In extremely advanced and severe cases, breaks in the Descemet membrane appear. This gives rise to "hydrops" which is stromal oedema, resulting in vision loss and associated pain.

1.2.4 Clinical Treatment: How crosslinking works?

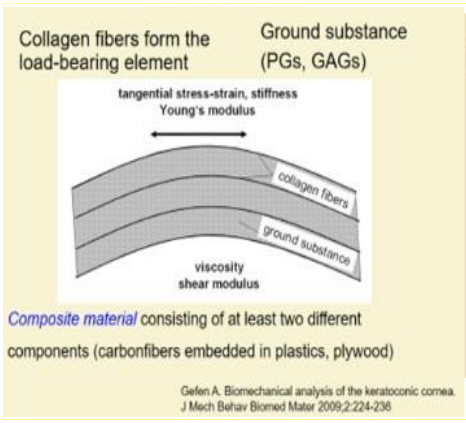
1.2.4.1 Past treatment

Before the twentieth century the treatment of KC consisted of cauterization of the conical area with silver nitrate and the instillation of miotics accompanied by a pressure dressing. In the early months of 1888, an innovative French ophthalmologist Eugene Kalt began work on a crude glass shell designed to "compress the steep conical apex thereby correcting the condition." This was the first known application of a contact lens for the correction of keratoconus (Rowsey, Reynolds and Brown, 1981).

1.2.4.2 Current treatment -Crosslinking (CXL) The Theory behind CXL

Currently, the gold standard of treatment for KC is crosslinking (CXL) to improve the biomechanics of the cornea and halt KC progression.

The cornea comprises of collagen fibres interspersed with the ground substance, made up by the Proteoglycans (PGs) and Glucosaminoglycans (GAGs) (Fig.1.10). KC causes progressive corneal thinning and reduction in the biomechanical strength via progressive interlamellar or interfibrillar slipping, due to reduced inter-layer adhesion (Fig 1.11). The biomechanical structure of the cornea is also influenced by age, the state of hydration of the cornea, the intraocular pressure, smoking, and hormones such as oestrogen, cortisol and low thyroxin. (Elsheikh, 2007).



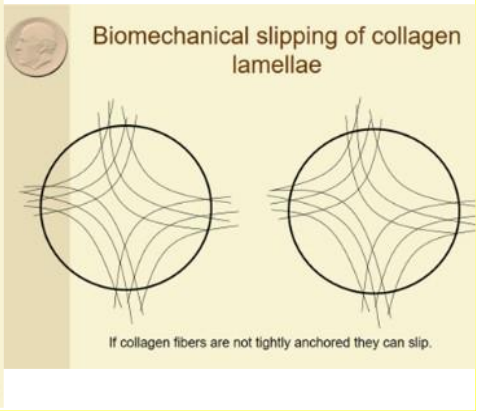


Figure 1. 7: Components of the cornea in KC (Source: Bestawi 2013)

Figure 1. 8: Corneal biomechanical weakening due to the slipping of collagen fibrils

Source: Beshtawi, O'Donnell and Radhakrishnan (2013)

CXL is a photochemical and photosensitive process that includes cornea de-epithelializing (EPI-OFF) with a Tooke's knife, preconditioning of the cornea with Riboflavin-Dextran solution for 20 minutes and exposure to UV (364u) radiation for 30 minutes. Professor Eberhard Spoerl and Professor Wollensak of Dresden University (2005) were responsible for the early research work done on porcine cornea, where they discovered that CXL could increase the rigidity of the porcine cornea by as much as 78%. Later they discovered that the same CXL could increase the human corneal rigidity

by >300 times (Spoerle and Wollensak, 2005). It is now known that CXL produces and restores the cross-links between the collagen fibrils that overtime, restores the biomechanical strength of the cornea (Fig. 1.12).

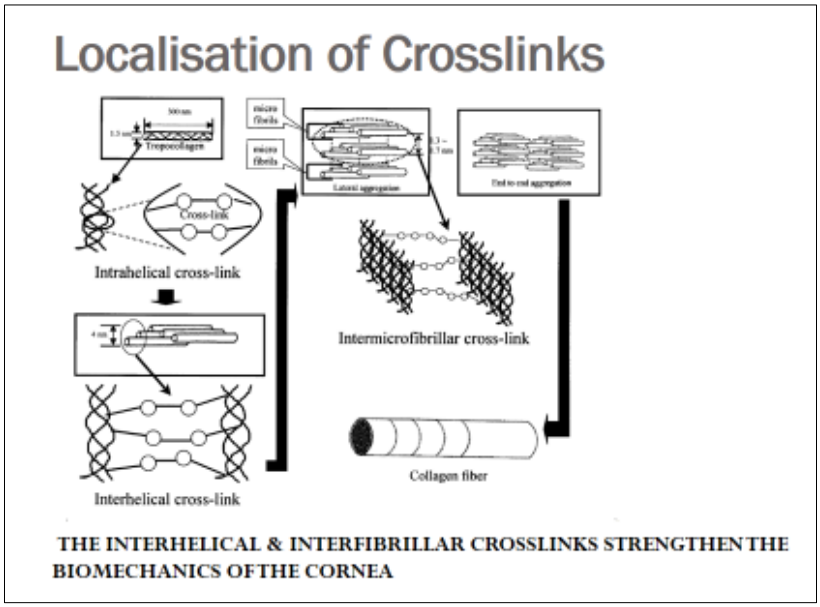


Figure 1. 9: Increased crosslinks after CXL treatment
Source: Beshtawi *et al.* (2013)

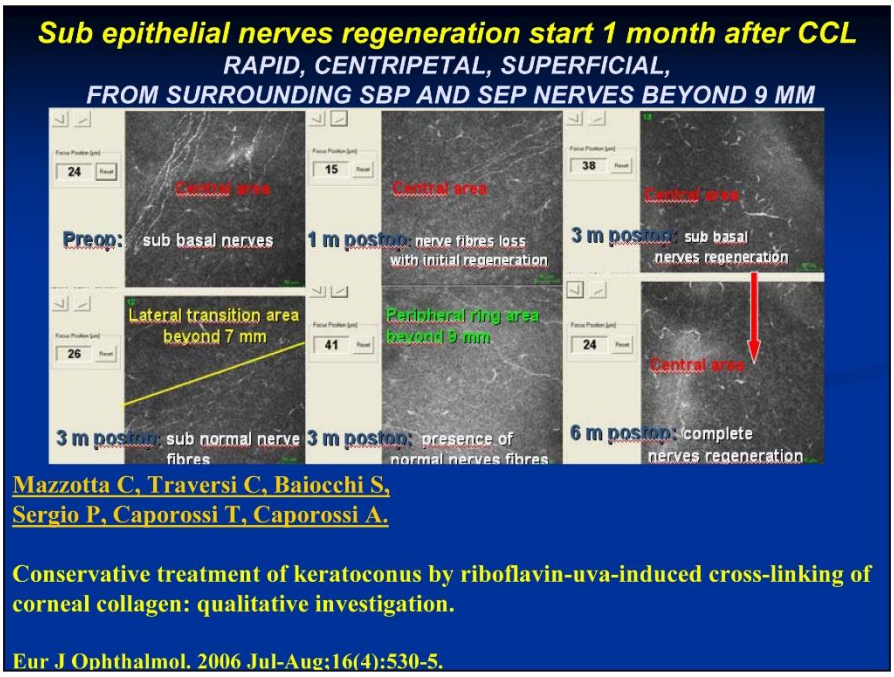


Figure 1. 10: Confocal Microscopyof the crosslinked cornea
Source: Mazzotta, et al 2006

The confocal picture shows that PostCXL, the subepithelial nerves regenerate about one month later. This explains the absence or reduced pain in the initial few days after the procedure. This enables the early fitting of RGPs, as early as two weeks PostCXL.

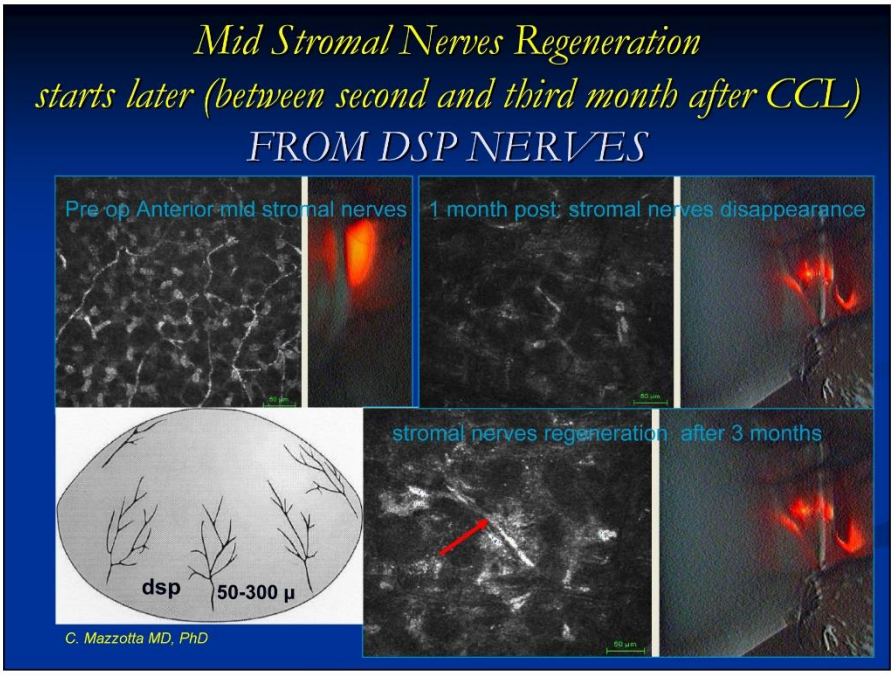


Figure 1. 11. Confocal Microscopyof the crosslinked cornea
Source: Mazzotta, et al 2006

This shows how by the third month almost full and complete regeneration of midstromal subepithlial corneal nerves occur.

1.2.4.3 Post-CXL

Following CXL, visual rehabilitation and optimization is done with Rigid Gas Permeable lenses (RGPs) and other optical devices, spectacles, Intacs and Phakic intra-ocular lenses (IOLs). RGPs enables any remnant Astigmatism to be almost totally removed, because of the anterior regular surface of these contact lenses.

1.2.5 Problem Statement

The complexity of KC makes it difficult to identify factors influencing its development. As there has been no extensive study done on KC in Malaysia, there is scant local data on KC and KC management and there are no guidelines to identify and manage KC high risk individuals in Malaysia.

1.2.6 Hypothesis

Corneal topography is an effective tool for the diagnosis of KC and crosslinking is an appropriate therapeutic option for the treatment of KC.

1.2.7 Objectives of The Study

General objective: This study aims to assess risk factors for KC, the efficacy of clinical diagnostic methods, and the use of CXL for the treatment of KC among patients attending a private ophthalmology clinic in Malaysia.

1.2.7.1 Specific objectives

i. To identify risk factors for KC with the use of a questionnaire on KC cases and controls
ii. To assess the usefulness of corneal topography as a diagnostic tool for KC and KC progression.
iii. To assess the efficacy of CXL followed by using RGPs for the treatment of KC cases and visually rehabilitate to give optimized superior quality of vision.

1.2.8 Epidemiology of Keratoconus

Since the early reports from more than a century ago, KC has become known as the most common ectatic disorder of the cornea. It affects both gender and all ethnicities (Millidot *et al*., 2011; Jonas *et al*., 2009; Assiri *et al*., 2005). Reported

prevalence figures remain largely variable due to different clinical definitions and diagnostic criteria used in different studies and populations. World-wide, the incidence of KC seems to be 1:2000 but the prevalence can be as high as 54.5 per 100,000 (Wang *et al*., 2013). The disease affects a preponderance of Indians, Pakistanis, Arabs, and Polynesians compared with Caucasian populations (Kok, 2012). The reports of two surveys in UK indicated a prevalence of 4.4 and 7.5 times greater for Asian subjects (Indian, Pakistani and Bangladeshi) compared to white Caucasians (Pearson *et al*., 2000; Georgiou *et al*., 2004). In South India, the incidence is closer to 1:500. In European Caucasian populations the incidence is reported to be between 5 and 23 per 100,000 with a mean prevalence of 54 per 100,000 (Romero-Jimenez *et al*., 2005). In the USA, KC reportedly affects approximately one person in 2000, with a total mean incidence of two new cases per 100,000 per year (Kennedy *et al*., 1986). Tanabe *et al*. (1985) reported a prevalence of KC in Japan of less than one-third of that seen in white Caucasians. Although the source of case reports may be biased and affected by disproportional cornea transplants in KC patients, increased incidence of KC has been described in a number of populations. In Malaysia, KC has not been extensively studied. Only two studies alluded to the incidence of KC in the Malaysian population. Reddy *et al.* (2008) reported four (0.3%) KC patients among 1169 eye cases screened. Mohd-Ali B *et al.* (2012) saw 159 (1.2%) KC cases out of 13,000 patients screened.

The world-wide male: female ratio appears to be 2:1 overall. Amsler (1961) reported a greater prevalence (59.2%) in women among 600 cases of KC, but in his last study of 116 KC cases, he also encountered more males than females. Buxton (1973) reported 62% of his 116 KC cases to be male. Tanwar,

in South India, reported that his series showed a higher prevalence among women. (Tanwar, *et al.*, 2010).

World-wide, the incidence of KC seems to be 1:2000 but the prevalence can be as high as 54.5 per 100,000 (Wang *et al*., 2013). The prevalence range can be between 0.2 (Gorskova & Sevos'tianov 1998) and 4790 (TorresNetto et al 2018). per 100,000.

These studies vary greatly in their statistics, due to usage of different technology, methodology, diagnostic tools, geographic locations, and populations with higher prevalence, notably in Asian and Middle Eastern populations compared with Western. A more recent meta-analysis which included 50 million individuals from 50 countries, determined that the global prevalence of keratoconus was 138 per 100,000. (Hashemi et al .2019)

Some more recent reports indicate a higher frequency among female. However, when results are compared together, there is no real difference between the sexes. Further, these conflicting study conclusions might be explained by different age groupings included in each study and a tendency for women to develop keratoconus at a slightly younger age than men. (Hashemi et al 2019)

1.3 Genetics of Keratoconus

1.3.1 Background- Theory of Multifactorial Aetiopathogenesis of Keratoconus

Though most KC cases occur sporadically (Khaled, Abu-Amero, *et al*., 2011;), there is growing evidence of familial disease and the involvement of genetic factors. The evidence for a genetic etiology in KC includes familial inheritance (Karimian, 1998; Amsler and Franschetti, *et al*., 1961), discordance between dizygotic twins, and association with other known genetic disorders (Nowak and Gajecka, 2011). On the other hand, Mendelian inheritance is said to be rare. The mode of hereditary transmission is not clear. Dominant, recessive, and irregular transmission have all been documented as in the case of ectasia in KC cases with a familial incidence. The situation is complicated by the observation that the genetic factor for KC seems to have a rather feeble penetrance and a great variability of expression (Franceschetti *et al.*, 1961) resulting in minimal forms of KC which are often not recognized clinically.

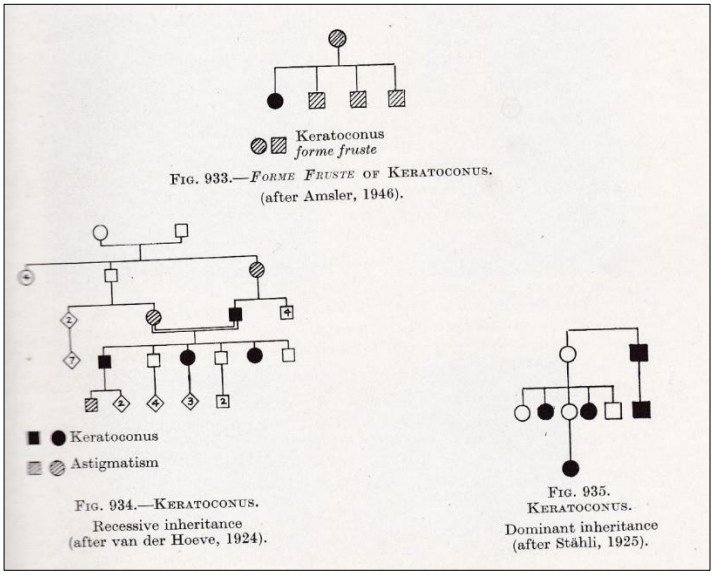


Figure 1. 12: Patterns of recessive and dominant inheritance in KC
Source: Duke Elder,1965)

Population studies have repeatedly found that Indians and Pakistanis make up a significantly greater percentage of patients with KC, suggesting a genetic component to the disease (Cozma *et al*, 2005; Davanger, 1978; Georgiou *et al*, 2004; Pearson *et al*, 2000). Previous prospective studies revealed that the relatives of patients with KC had a high prevalence of undiagnosed KC. If complete slit-lamp examination, refraction, and corneal topography were performed, 11-14% apparently unaffected relatives of patients with KC would be diagnosed with KC, thus changing the KC classification from sporadic to familial. Ninety percent of pedigrees with familial KC display an autosomal dominant inheritance with reduced penetrance. Other modes of inheritance have been described, including the autosomal recessive mode in families with children of consanguineous parents. Several gene loci responsible for the familial form of KC have been mapped, such as the *VSX1* gene (Bonis *et al*., 2011). This latter Iranian study pointed to a significant association between KC patients and *VSX1* genetic alterations p.R166W and p.H244R. However, one South Indian study showed a lack of *VSX1* mutations in many unrelated sporadic KC patients. This seems therefore to point to the possible complementary involvement of other genetic, environmental, cultural or behavioural / habitual factors in the development of this complex disorder. However, still 5- 10% of KC cases do have a positive family history (Rabinowitz *et al*, 2003; Kennedy *et al*., 1986).

Polymorphisms in the interleukin 1 alpha (ILIA) and ILIB gene regions were previously associated with KC in a Korean population study. A similar study done in Japan (Mikami *et al.*, 2013) also indicated that ILIB might play an important role in the development of KC through genetic polymorphisms. A locus for autosomal dominant genes was mapped in Finnish families to 16q22.3-q23.1 (Stabuc-Silih *et al.,* 2010). Another additional locus for KC was identified on 5q14.3-q21.1 (Palliwal *et al.,* 2009). A locus on 2p24 was identified in a heterogenous population of 28 families recruited in France, Spain, and Guadeloupe of European, Arab and Caribbean-African descent. Evidence of linkage to chromosomes 4, 5, 9, 12 and 14 has been obtained from a genome-wide linkage analysis with data from KC sibling-pair families of white or Hispanic origin.

1.3.2 Monozygotic Twin Studies

Monozygotic twin studies constitute an adequate research model to evaluate genetic and environmental factors in the disease pathogenesis. The higher the rate of concordance between the monozygotic twins, the greater the evidence for primary genetic causation rather than environmental etiology. In addition, if the concordance were greater between monozygotic compared to dizygotic twins, genetic factors are more likely to play a key role in the disease phenotype. The concordance supports the evidence of heredity as a genetic factor in the etiology of KC.

In cases of discordance between monozygotic twins, other factors like reading, rubbing of eyes and hormonal influences were suggested as

contributory environmental factors. Further phenotype differences in monozygotic twin pairs could reflect genotypic differences resulting from somatic changes occurring during development.

1.3.3 Association of Keratoconus with Other Known Genetic Disorders

More than two dozen syndromes are associated with KC, including Down syndrome, Leber congenital amaurosis, connective tissue disorders, Osteogenesis Imperfecta, GAPO, and some subtypes of Ehlers-Danlos syndrome. Down Syndrome has a strong association with KC with a reported prevalence ranging from 0.5% to 15% (10 to 300 - fold that of the normal population). The association of KC with mental retardation is common. One study showed that among 212 institutional mentally retarded individuals, there were 16 patients with KC (7.5%), eight of whom had unilateral disease.

1.3.4 The Polygenic Theory

There is increasing evidence that in many diseases, more than one gene is involved. Fig. 1.14 illustrates the numerous genes reported by researchers to be associated with the development of corneal disorders. It is apparent that, for KC alone, at least 18 genes have been linked to the disease. The role of each of these genes, either alone or in combination, is yet to be determined.

This most recent updated study, Molecular Genetics of Keratoconus by YM Wang and CP Pang (2020) says “Occurrence of keratoconus is pan-ethnic with reported prevalence ranging widely from 1:400 to about 1:8000, higher in Asian

than Western populations.". This emphasizes the higher prevalence of KC in Asian societies but does not explain why the prevalence is higher in Asian populations.

1.3.5 Scientific Evidence from Systematic Reviews and Meta-Analysis

Shi Song Rong *et al*. (2017) summarised recent scientific data from systematic reviews and meta- analysis in their report "Genetic associations for Keratoconus: a systematic review and meta- analysis". Although they identified a list of genes and loci associated with KC and certain SNPs as priority candidate biomarkers for functional investigations, their study was biased towards Western White populations. This inadequate representation of Asian populations resulted in a lack of association with almost all SNPs in Asian cohort's data. Hence, more studies need to be performed in non-White, particularly Asian populations, to confirm the genetic associations observed in their study, besides presenting a true reflection of actual prevalence in the Asian context.

Quoting from a review of literature by Patel D & McGhee C. (2013). "Although first described more than 150years ago, Keratoconus is still an enigmatic disease that remains an area of wide-ranging, dynamic, international research". This was from a review paper of data from New Zealand/ Autearoa, "where keratoconus is both relatively common and extensively studied." The review even highlights the amount of research in New Zealand, yielding identification of a higher prevalence of keratoconus, among indigenous Maori and Polynesian populations, compared to other international studies. In addition, over the last 20 years, keratoconus has remained the leading indication for keratoplasty.

1.3.6 Problem Statement -Genes with PCR Sequencing

Research to date has not identified any single major gene, though in some families, the inheritance pattern does suggest such a model. It is clear that Keratoconus is a complex disease, and multifactorial issues complicate its aetiology and even its genetic heritage.

The significance of the candidate genes, *VSX*1 exon3, *SOD*1 exon2 and *COL*4A3 exon17 genes in the aetiopathogenesis of KC is still unclear.

1.3.7 Hypothesis

The gene variants at *VSX*1 exon3, *SOD*1 exon2 and *COL*4A3 exon17 could be related to the aetiopathogenesis of KC

1.3.8 Objectives

The general objective of this study is to assess the role of the three candidate KC genes, *VSX*1exon3, *SOD*1exon2, and *COL*4A3exon17 in the aetiopathogenesis of KC.

1.3.8.1 Specific Objectives

i. To identify variants of the three candidate genes in KC cases and controls
ii. To compare the occurrence of the candidate gene variants in KC cases and controls
iii. To study the association of the candidate gene variants with the KC phenotype.

1.3.9 Background- Exome Sequencing

Historically, prior to 2005, DNA sequencing required a series of steps that included subcloning into a vector, introduction into a host with plating on selective media, growth and picking of selected subclones, DNA isolation and capillary sequencing. With the advent of Next Generation Sequencing (NGS) also known as massively parallel DNA sequencing, this paradigm has changed dramatically, both in terms of the numbers and types of steps required to generate data as well as the scale of data generation. In NGS, DNA is fragmented into multiple pieces to which adapters are ligated. This is followed by sequencing and reassembling the sequences to form a genomic library. In this process, millions of genomic fragments are sequenced in a massively parallel fashion, improving speed and accuracy while reducing the cost of sequencing.

Whole Genome and Exome sequencing have become important diagnostic weapons for the researcher to study molecular defects in patients with suspected genetic or Mendelian diseases (Yang and Muzny *et al*., 2013). While whole genome sequencing (WGS) consists of the capture, sequencing and analyzing of the whole genome of roughly 3 billion base-pairs, whole exome sequencing (WES) focuses only on approximately 30 million base-pairs which are translated to functional proteins in which mutations ae the most likely to have a severe direct phenotype.

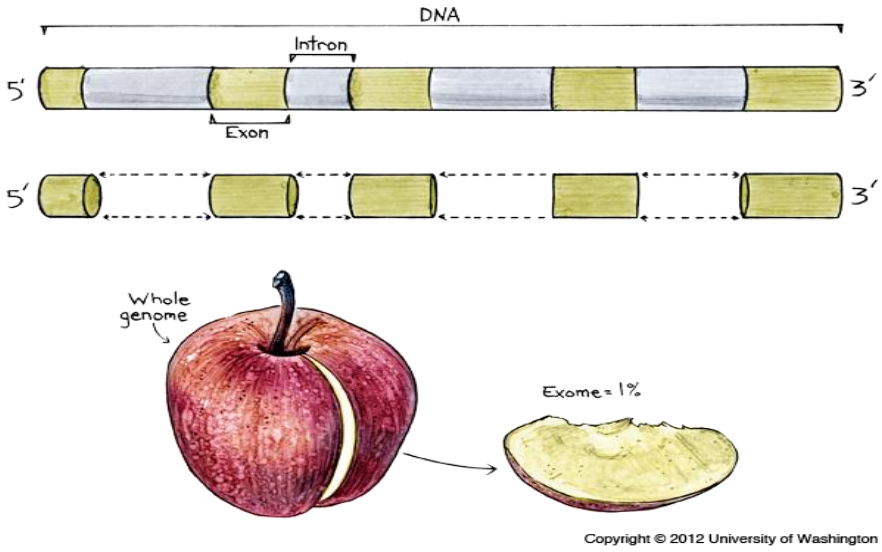


Figure 1. 13*:* The Exome made up of exons which are protein-coding genes, and introns which are the intragenic non-coding sequences

Source: https://mygene2.org/MyGene2/exomesequencingdetails.

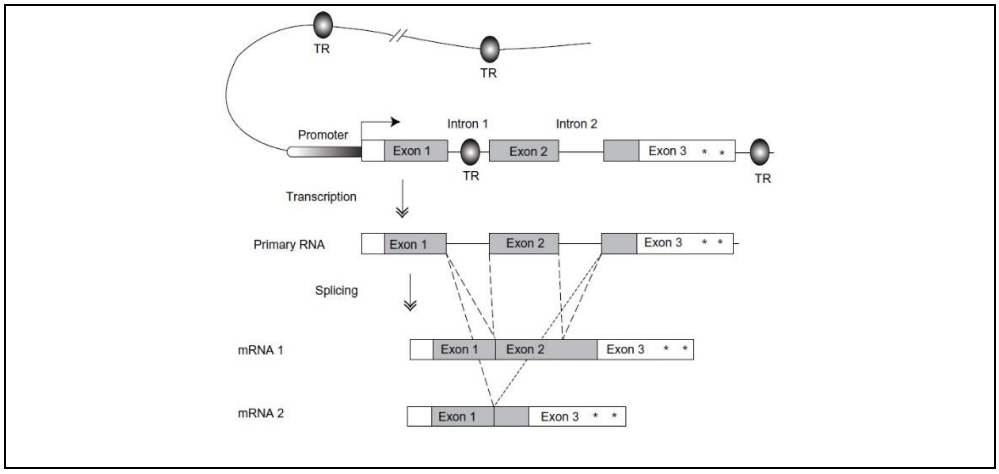


Figure 1. 14: Schematic representation of intron removal by RNA splicing before the mRNA is translated into a protein.
Source: Sastre, Leandro (2014).

1.3.9 Problem Statement: Genes with Exome Sequencing

The significance of the *VSX*1 exon3, *SOD*1 exon2 and *COL*4A3 exon17 genes in the aetiopathogenesis of KC is still unclear.

1.3.10 Hypothesis

Whole exome sequencing can uncover and identify the genetic cause of KC.

1.3.11 Objectives

1.3.11.1 Specific objectives

i. This study aims to search for sequence variations in targeted candidate genes *VSX1, SOD1* and *COL4A3* in patients with KC and controls, and to determine the significance of these variants in the pathogenesis of KC
ii. Exome Sequencing can uncover other gene variants through its filtering process, for all genes in the whole exome.

1.4 Other Risk Factors

The development of new investigational tools made it possible to study KC from different perspectives.

Risk factors like asthma, allergy and eczema appear to be higher prevalence in New Zealand also. Even positive family history rates are higher than international comparative studies. This all points to future studies of keratoconus to focus on risk factors of allergy and genetic studies, and novel management of clinical keratoconus.

Historically, Keratoconus has been reported with a long list of genetic diseases (Rabinowitz 2003) though some case reports with comorbidities are coincidental. Several associations with genetic diseases have been reported. There are also many reports of co-occurrence with connective tissue disorders such as

Ehlers Danlos syndrome, Marfan syndrome, and osteogenesis imperfecta (Rabinowitz 1998).

A recent study on Down's syndrome patients, from Spain and Egypt had 75% Down's syndrome pattern or have keratoconus features (Woodward 2016). Further study of individuals with these congenital diseases is needed to shed light, on whether genetics or environmental factors are the primary cause of the disease.

1.4.1 Biochemical Cause

Biochemical abnormalities have been identified in the cornea in KC cases (Kim *et al.*, 1972; Robert *et al*., 1970). These include decreased levels of glucose-6-phosphate dehydrogenase, relative decreases in hydroxylation of lysine and glycosylation of hydroxylysine, as well as decreased total collagen and relative increased structural glycoprotein. Examination of reducible collagen crosslinks in cornea with keratoconus revealed that lysinon or leucine is present in amounts far greater than that found in normal age-matched corneas (Robert and Schillinger *et al.*, 1970). This was interpreted to imply that collagen synthesis was normal, but the character of the corneal collagen is unusual and different from that in the normal cornea.

To date, no causal relationship has been established between any of these abnormalities and the clinical pathology of KC. Hence, available data do not permit us to define KC as a specific biochemical disorder (Leibowitz HM, "Keratoconus" pg 100).

1.4.2 Endocrine cause

Many researchers have considered an endocrine aetiological factor as very attractive. The ductless glands do exercise a potent influence over the process of growth, and indeed it has been claimed that an upset of their balance may alter the condition of a tissue which is primarily supportive. This latter view is borne out by the usual occurrence of KC about the time of puberty and the apparent higher incidence in females.

Historically, many cases of endocrine imbalance in KC have been recorded in the literature (Strebel and Steiger, 1913; Weil,1927). Seigrist (1912) first advocated such a hypothesis and considered hypothyroidism an important factor. Signs of hypothyroidism were also noted by Arnold Knapp (1929), Sitchevska (1932) and King (1953). Thomas (1955) observed the development of KC in two women after thyroidectomy

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CHAPTER 2 LITERATURE REVIEW

2.1 Clinical Keratoconus

2.1.1 Clinical diagnosis- Clinical picture and progression

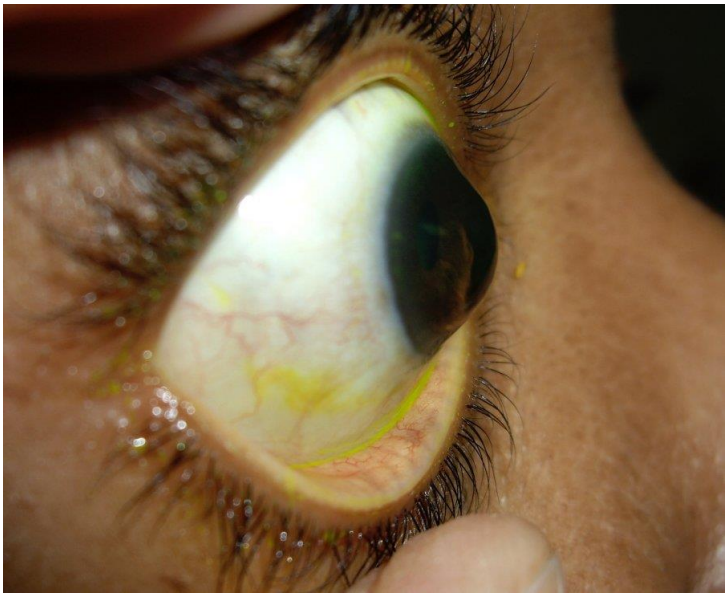


Figure 2. 1:The typical coned cornea in keratoconus
Source: JPD

Keratoconus is clinically a progressive corneal disease which leads to corneal thinning and visual impairment, secondary to development of often high and irregular astigmatism. This can initially be unilateral, but gradually progresses to affect both eyes eventually. The age of onset is in the adolescent years, and progressively worsening till the mid-thirties. It is uncommon for the KC condition to progress beyond 40 years of age [Leibowitz HM, "Keratoconus "pg 100; AlShammari *et al.* (2016)].

Universally, the progression of KC usually slows down around the mid-thirties, stabilizing permanently to a condition referred to as "Forme Fruste

Keratoconus", but exceptions occur both with severity and duration of disease progression.

In most (90%) cases, KC is bilateral, though usually asymmetrical in severity and progression. Li *et al.* (2004) reported that approximately 50% of clinically normal fellow eyes progressed to KC within 16years. Although a large proportion of KC patients can be managed with contact lenses, an average of about 20% of all KC corneas require penetrating keratoplasty. Some authors report markedly different surgical indication rates of 6.5 and 12% to 45% (Ihalainen,1986).

KC prevalence varies in different geographical regions depending on factors such as demography and the environment which play an important role in KC aetioathogenesis.

2.1.2 Corneal Bio-mechanical Features and CXL

WollensakG, E.Spoerle and Theo Seiler (2005) were the pioneer group of researchers to identify and recognize the important role of UV Crosslinking(CXL) to improve the biomechanical strength of the weakened KC cornea. At Dresden University, Wollensak and Spoerle (2005) experimented on pig's cornea, and discovered the stiffening effect of CXL was increased by 76.5 times while that of human cornea was increased by 300 times. Their application to use CXL for treatment for keratoconus was utilized after 2005.

The UV exposure of 30 minutes, according to protocol, increases the formation of interlamellar and interfibrillar covalent bonds by photosensitized

oxidation. Riboflavin, because of its special alkyl-isoalloxazine structure, allows the absorption of a wide range of light spectrum, including an absorption peak in the UV-A range. Thus, it is the standard photoinduction in CXL. (Spaedea *et al*., 2016).

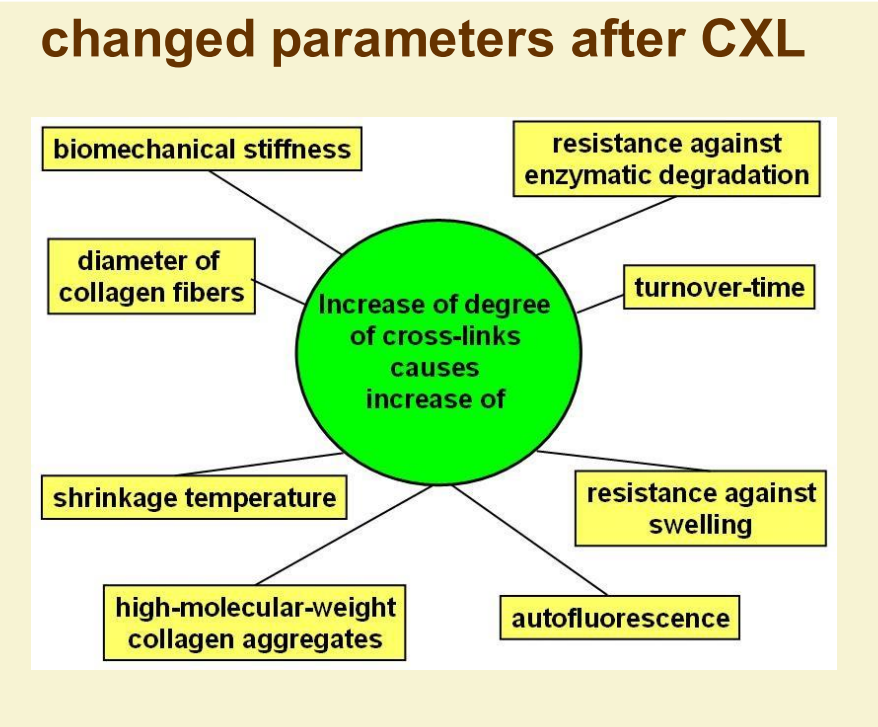


Figure 2. 2 the possible changes and improvement after CXL
Source: E Spoerle (2005).

Spoerle and Wollensak found the biomechanical strength was suitable for the weakened KC cornea, to become stronger and stabilized in order to halt the clinical KC progression. This was attributed to increase in the number of crosslinks, as indicated in the figure above.

The other clinical feature was the cornea became more regularized, and the Apex of the cone, also became more centralized .

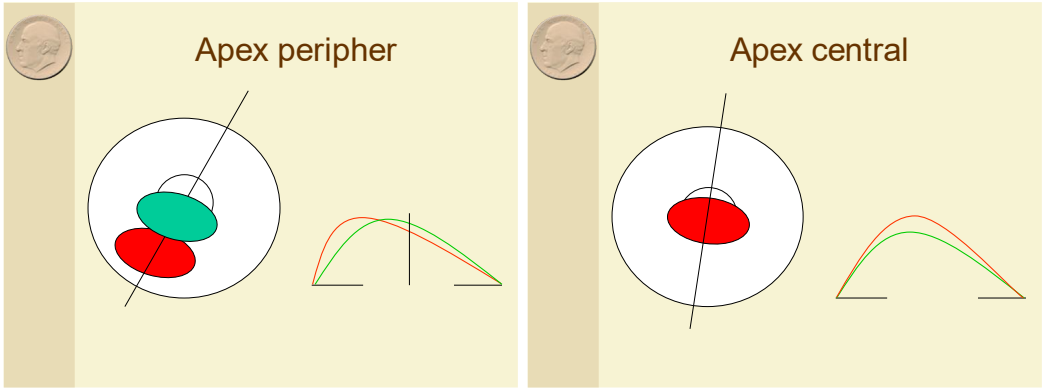


Figure 2. 3: the migration and change in position of the Apex of the cone
Source: E Spoerle 2005

Figures above, illustrate the migration and change in position of the Apex of the cone, from the periphery, due to irregular astigmatism, towards the central cornea after CXL. This regularizes the cornea

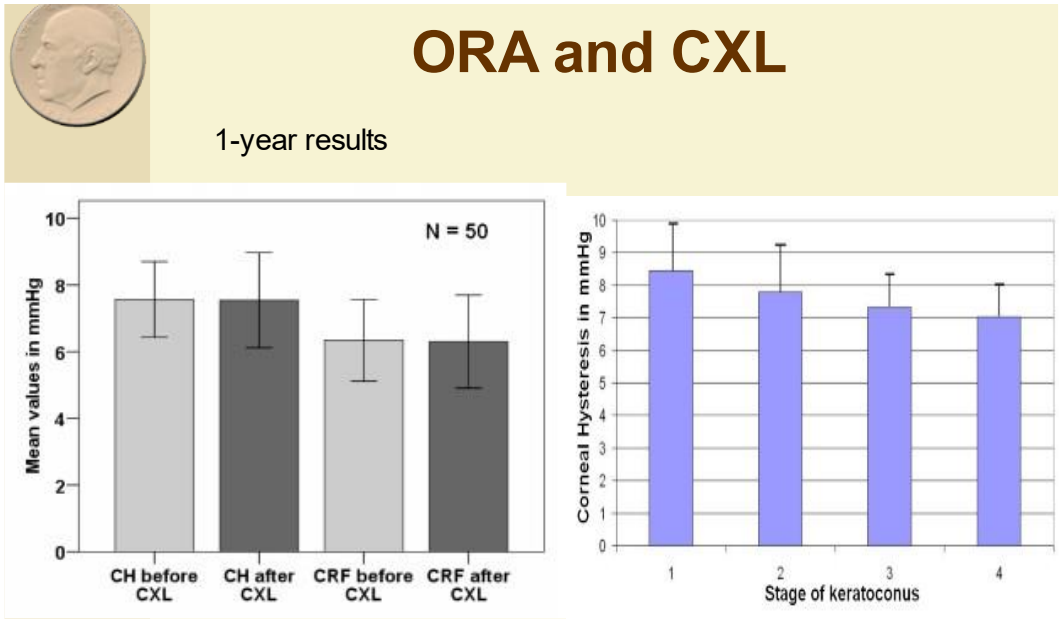


Figure 2. 4: Similar results PreCXL and PostCXL.

In a study by Goldrich, no appreciable changes were seen after one year, as reflected in the graphs above. Source: Goldrich 2009

Tobias Koller (2007) indicated there were complications and limitations to Crosslinking. Complications were however minor like delayed epithelial healing and corneal haze which healed and cleared, with proper management, leaving no corneal scarring.

These minor complications were similar to what was observed in this study. In fact, all cases of corneal haze cleared, and no postCXL scarring was seen in this study.

2.1.2.1 Ocular Response Analyser

The importance and application of the Ocular Response Analysis (ORA) readings lie in its application in helping early detection of suspected KC. This is important, as early detection and early management in KC, determines the final success in maintaining and maximizing vision and stopping KC progression.

At present there are no accepted criteria for categorizing an eye as a KC suspect (Schiegel, et al., 2008) although many studies (Rabinowitz, 1999; Fam, et al., 2006; Saad, et al., 2018) have tried to establish a single index that would distinguish the KC suspect eye from the normal eye. Increased asymmetry on anterior specular topography, inferior and localized steepening, unstable or increasing astigmatism or slight steepening of the posterior corneal surface have been suggested as the first lead for suspecting KC.

However, controversy also surrounds the biomechanical improvement and its maintenance in terms of duration and period, of how long it lasts. Many reports and papers have been written.

2.1.3 Environmental Risk Factors -Eye Rubbing, Atopy Allergy

Eye rubbing, and more recently eye compression due to sleeping position, have been identified to be highly related to the condition, and are present in a high percentage of patients. Today, the predominant model is that these factors can provide the "second hit" or “trigger” necessary, that is the mechanical trauma (i.e. eye rubbing or eye compression at night), is a sine quanon condition for the onset of keratoconus, and quite possibly its only cause. There are various arguments for and against this hypothesis. Indeed, it is possible, as initially suggested around 55 years ago, that the term "keratoconus" include diverse phenotypically similar conditions, which are actually of different etiology, but, generate the condition in a genetically susceptible individual (Yaron S Rabinowitz et al 2021).

Gordon-Shaag et al. (2013) showed that besides eye rubbing, allergy is also significantly associated with KC after adjusting for other predictors. Rabinowitz (2003) did a case control study for allergies associated with KC. He found 83% of KC subjects had severe eye rubbing compared to 58% in standard controls.
Gordon-Shaag et al. (2017) and Hatim Najmi et al. (2019) highlighted the role of chronic eye rubbing in KC development and progression. Hadas ben-Eli (2019) also postulated that eye watering and itchiness could trigger the vigorous and prolonged rubbing that could lead to corneal remodeling and ectatic disorders like KC.

Studies for Identification and recognition of risk factors in the environment has been problematic and inconclusive. The actual triggers for actually causing keratoconus also have been elusive. Many studies actually are just small cohorts.

Larger studies have become accessible more recently, and pointing to a broader range of potential risk factors.

There is increasing evidence from other more recent research that eye rubbing, is a risk factor and significantly related to keratoconus development (Bawazeer et al, 2000; Gordon Shaag A et al, 2015). Bawazeer claimed that the most significant "predictor of keratoconus" was eye rubbing through multivariate analysis, odds Ratio? They even claimed that atopy may just play a secondary role producing eye irritation resulting in eye rubbing.

One of the risk factors often reported in medical literature is eye rubbing. The exact mechanism of KC development from eye rubbing, however, still remains a mystery. Najmi, H et al (2019), in their attempt to correlate KC with risk factors, could only select 11 articles related to eye rubbing, that fitted the criteria of selection in his study. They concluded that "eye rubbing causes thinning of the keratocytes, and the degree of effect of rubbing depends on the period and force of performing eye rubbing". They also gave advice on how to prevent KC: "It is recommended to avoid eye rubbing to prevent keratoconus, this can be achieved by avoiding itching and treating dryness of the eye and avoiding wearing eye lenses." It is also well-known in many studies (Bawazeer, et al., 2000; Gordon-Shaag, et al., 2015) that KC is associated with atopy and eye rubbing as well.

The most recent case-control study by Moran, et al. (2020) includes both the above as significantly important environmental factors, indicated by an OR= 8.29 (95% CI:3.92-18.26, p< aetiopathogenesis of KC.

Eye Severity in Eye Rubbing weakens the eye structure

One of the risk factors often reported in medical literature is eye rubbing. As well as severe eye rubbing. The exact mechanism of KC development from eye rubbing, however, still remains a mystery. Najmi, H et al (2019), in their attempt to correlate KC with risk factors, could only select 11 articles related to eye rubbing, that fitted the criteria of selection in his study. They concluded that "eye rubbing causes thinning of the keratocytes, and the degree of effect of rubbing depends on the period and force of performing eye rubbing" They also gave advice on how to prevent KC: "It is recommended to avoid eye rubbing to prevent keratoconus, this can be achieved by avoiding itching and treating dryness of the eye and avoiding wearing eye lenses." It is also well-known in many studies (Bawazeer, et al., 2000; Gordon-Shaag, et al., 2015) that KC is associated with eye rubbing and atopy as well.

The most recent case-control study by Moran, et al. (2020) includes both these risk factors above as significantly important environmental risk factors, indicated by an OR= 8.29 (95% CI:3.92-18.26, p<00.1) to play a complimentary role in the aetiopathogenesis of KC.

The questionnaire findings in this study support the importance of eye rubbing as a major risk factor in the development of KC among Malaysians. It is known that intense eye rubbing is often practiced in Indian and Asian cultures such as Pakistanis and Iranians, among whom, it occurs spontaneously before sleep, on awakening and through the day, in response to emotional stress or ocular irritation

or fatigue (Shetty, 2017). This response can also be due to dryness and itching or can be psychogenic and compulsive (Hawkes, 2014).

Eye rubbing has been related to higher KC incidence rates in these more Asian cultures. The disease process, presumably disrupts the anterior corneal mosaic (Koyabashi et al.,2017) to cause loss of surface epithelium, disruption of the corneal clarity due to fibrous reactions and weakening of the biomechanical strength of the anterior lamellae of the stroma.

Other studies have also shown relationship between Atopy, Asthma and even food allergy. As early as 1989, Harrison RJ et al recognized the association between keratoconus and atopy. In his study of 67 patients, he found that patients with high IgE levels were more prone for corneal graft rejection, and indirectly indicating more severity of keratoconus. Again, this study seemed to indicate, that "excessive eye rubbing" has been considered a reason for this relationship.

Review literature till as recent as 2013, by Patel D and C McGhee have reemphasized the relevance of asthma, allergy and eczema as "potential risk co-factors" in aetiology of keratconus in New Zealand subjects, compared with estimates from general population.

A much larger study of 886 keratoconus patients, done in Iran by Naderan et al (2017). had a similar association of both allergy and asthma with keratoconus. Even more recently, Burdon K and Vincent AI (2020) stressed that though many small studies have suggested links between these environmental risk factors of, allergy, atopy or asthma and keratoconus, the larger studies are more convincing

and less biased. However, though progress has been made “environmental triggers for keratoconus remain largely elusive” was their conclusion.

Even Rabinowitz et al (2021) in a current eye research publication, has reemphasized the important role of “mechanical micro-trauma” due to eye rubbing. He emphasised this in an earlier paper in 2003, and now in 2021 he again says, “Eye rubbing, and more recently eye compression due to sleeping position, have been identified to be highly related to the condition, and are present in a high percentage of patients. Today, the predominant model is that these factors can provide the "second hit" necessary to generate the condition in a genetically susceptible individual. This reemphasises again and again the multifactorial aetiological factors of keratoconus, as is evident in current eye research. Also, though research progresses and expands, it is seen very clearly, certain basic factors cannot be denied, and instead of becoming obsolete, instead becomes emphasised and important again.

Table 2. 1: Potential risk factors in different studies

ENVIRONMENTAL RISK FACTORS

The environmental triggers for keratoconus remain largely elusive, although progress has been made on identifying risk factors. Many of the studies evaluating risk factors are relatively small, with fewer than 500 cases. Several larger studies, mostly using medical or billing records to assess the association of a broad range of potential risk factors, have emerged in recent years (**Table 1**).

Allergy and Asthma

Multiple small studies have suggested links between allergy, atopy, or asthma and keratoconus; however, the most convincing studies are those on larger numbers of individuals. The largest

Table 1 Studies of more than 500 keratoconus patients assessing risk factors for keratoconus

Reference	Country	Data source	N cases	N controls	Matched?	Allergy	Asthma	Diabetes
Seiler et al. (2000)	Germany	Notes	571	571	Yes	NA	NA	–
Kuo et al. (2006)	United States	Code	2,102	232,548	No	NA	NA	–
Kosker et al. (2014)	United States	Notes	1,377	4,131	Yes	NA	NA	+
Naderan et al. (2014)	Iran	Notes	1,383	1,383	Yes	NA	NA	–
Merdler et al. (2015)	Israel	Code	807	>600,000	No	+	+	NA
Woodward et al. (2016)	United States	Code	16,053	16,053	Yes	+[a]	+	–
Naderan et al. (2017)	Iran	E + Q	885	1,526	No	+	+	NA
Bak-Nielsen et al. (2018)	Denmark	Code	2,679	26,790	Yes	+	+	No association

[a]Significant in univariate but not multivariate analysis.
The column on matching indicates whether the case and control group were matched for relevant demographics. Notes refers to a review of medical records; Codes refers to data extracted from coded billing or claims records; E + Q indicates examination and questionnaire; + indicates a risk for keratoconus; – indicates that a factor is protective for keratoconus.
Abbreviation: NA, not assessed.

Source: Lucas & Burdon (2020)

Lucas and Burdon conducted multiple studies involving larger study groups, with more than 500 participants, using data from medical bills or billing records. The largest study so far (Woodward et al 2016) identifies 16,053coded billing records from 16,053 keratoconus patient records and matched controls with similar demographics to each case. Another study from Israel (Merdler 2015) used data from teenagers from military medical assessment. Yet another study from Denmark (Bak-Nielsen 2018) reported on 2679 keratoconus cases, with matched controls. All these three studies, reported both allergy and asthma as risk factors for keratoconus. Atopy was also associated as a risk factor for the third study.

2.1.5 Confocal Microscopy Study

Koyabashi (2017) described through laser confocal microscopy epithelial changes of the cornea before and after eye rubbing. He showed the presence of Koyabashi structures (or K-structures) in the Bowman and sub-Bowman layers of the cornea before eye rubbing but the absence of these structures in the central cornea after eye rubbing. These observations suggest that eye rubbing, by weakening the corneal biomechanical strength, with loss of these K-structures, contributes to the development and progression of KC.

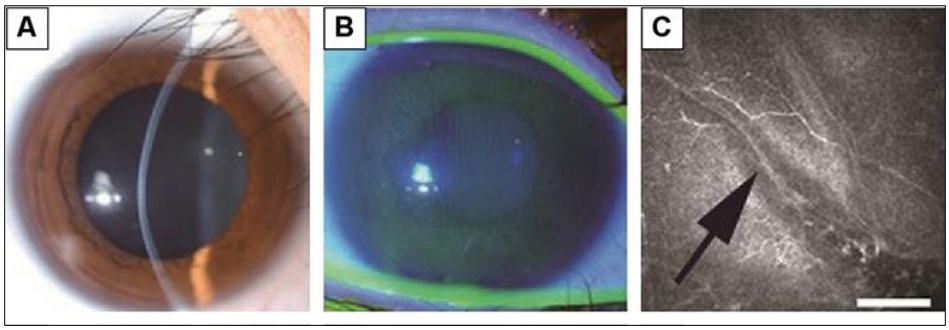


Figure 2. 5: The early keratoconus cornea seen with slit lamp and confocal microscopy

Source: Koyabashi *et al*. (2017)

Notes: Slit-lamp biomicroscopic photo with or without fluorescein staining and in vivo confocal microscopic image at the level of Bowman's layer. (A) slit-lamp biomicroscopic photo of the right eye (B) after rubbing the right eye through the eyelid under fluorescein staining, corneal mosaic was apparent (C) At the level of Bowman's layer, K-structures (sub-Bowman's fibrous structures) were apparent (bar =100 µm).

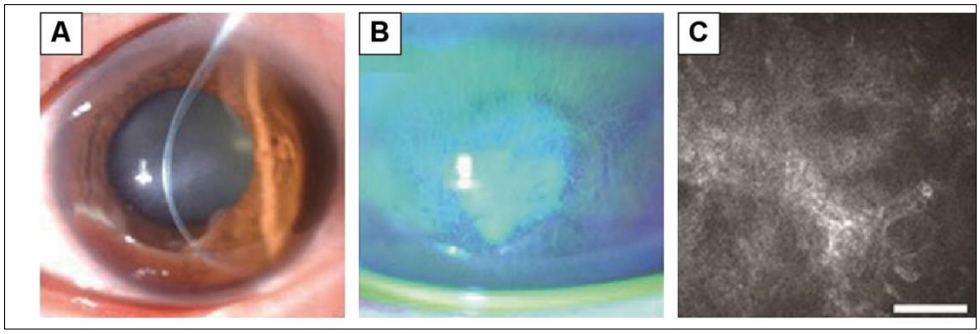


Figure 2. 6: The abnormal corneal structures in advanced keratoconus
Source: Koyabashi *et al*. (2017)

Notes: Slit-lamp biomicroscopic photo with or without fluorescein staining and in vivo confocal microscopic image at the level of Bowman's layer showing absence of aCM (anterior corneal mosaic) after eye rubbing. (A) slit-lamp biomicroscopic photo of the left eye (B) After rubbing the right eye through the eyelid under fluorescein staining, corneal mosaic was not apparent in the central cone area (C) At the level of Bowman's layer, K-structures were absent in the central cone area (bar =100 µm). Abbreviations: aCM, anterior corneal mosaic; K- structure, Kobayashi-structure

Through these confocal studies, Koyabashi illustrates the significance of loss of epithelial structures called, K- structures, present in the anterior mosaic layer. This is proof of how, gradually through repeated, eye rubbing , loss of these , result in stromal weakening, resulting in the biomechanical strength of the cornea , and further posing as an environmental risk factor.

2.2 Candidate GENES

Abu-amero (2014) stressed that the majority of keratoconus cases are sporadic; however, autosomal dominant with reduced penetrance and autosomal recessive mode of inheritance have also been documented. First-degree relatives are at much higher risk of the disease than the general population Monozygotic twins show a high concordance of keratoconus with a greater similarity of phenotypes indicating a strong role of genetic component(s) in the disease phenotype as well. These data provide strong evidence to support the role of heredity in keratoconus.

2.2.1 Familial Clustering of Keratoconus

Evidence of genetic components for keratoconus comes from family studies as well. Evaluation of family histories., range from 5% in Scotland (Weed et al 2008) to 28% in Israel (Shneor 2013). A comprehensive study of a large series, and their nuclear families, found a prevalence 3.34% in first-degree relatives (Wang et al 2000).

Though most keratoconus cases appear to be sporadic, there are also numerous reports of extended pedigrees with apparent autosomal dominance or autosomal recessive keratoconus, indicating Mendelian inheritance and likely presence of major gene effects in those families (Gajecka et al 2009, Nowak et al 2013). Thus genetics do play an important role as a risk for some individuals.

However, research to date has not identified any single major gene, though in some families, the inheritance pattern does suggest such a model. It is clear that

Keratoconus is a complex disease, and multifactorial issues complicate its aetiology and even its genetic heritage.

2.2.2 Genes Identified from Family Studies

Linkage analysis in families emphasise the regions of the genome that are most likely to contain a causative variant. Further detailed analysis of the affected and unaffected relatives, will help focus the mutation specifically for the disease. Several examples were successful gene identification for keratoconus in families. (Lucas S & Burdon K et al 2020).

2.2.3 Candidate Genes: VSX1, SOD1 and COL4A3

VSX1, SOD1 and COL4A3 are three candidate genes targeted in this study.

Genetic risk factors for keratoconus have been difficult to identify because of the complex etiology of the disease. Among other approaches used to identify genetic components in families with suspected dominant forms of keratoconus, candidate gene analysis has also been employed to study keratoconus cohorts. (Abu-amero et al 2014).

Based on the underlying biological traits of the disease, candidate genes are predicted depending on their known biological functions and expression patterns relevant to the disease. Candidate gene approaches are particularly useful in studying complex multifactorial diseases and enables us to identify even small gene effects using large case-control cohorts. An attempt has been made with a similar approach of choice of candidate genes listed for this case control study.

2.2.3.1 VSX1

Heon (2007) was the first to report that VSX1 was the keratoconus gene. Initially this gene was proposed for Posterior Polymorphous Dystrophy (PPCD, OMIM ID 122000)) for the cornea. The gene was located in a linkage area identified in a family, with 21 PPCD cases and known to be expressed in ocular tissues. Based on these findings, the authors concluded the missense variants were the cause of keratoconus in at least 4.7% of cases.

Bisceglia et al (2005) in an Italian keratoconus study, identified missense variants in seven out of 80 (8.7%) cases. It was from this, that Bisceglia concluded that missense variants within VSX1, p.(G160D) and p.(D144E) were rare polymorphisms, compared with relatives and other controls.

Aldave et al (2005) did not support that VSX1 was involved in Keratoconus. His study screened five known exons and intron-exon boundaries of the gene in 100 US keratoconus patients and identified only one missense variant p.(D144E) in one case only.

Liskova et al (2007), screened VSX1 in 85 unrelated probands from UK families with multiple cases of keratoconus, and 50 unrelated controls of mixed ethnicities. This was the first study that fully screened VSX1 in the control cohort. The only potentially disease- causing variant identified in the case cohort was the p(D144E) variant. However, it did not segregate with keratoconus in the pedigree.

The authors conducted a meta-analysis of the p.(D144E) with the results of the three studies, and demonstrated that the variant was not associated with

keratoconus (p= 0.14). Liskova's final conclusion was that coding variants in VSX1 were not involved in keratoconus.

Many other papers have been published with claims that VSX1 is involved with keratoconus. It has been challenging, differentiating between population-specific polymorphisms and potentially disease-associated variants. Conclusions have also been controversial and confusing.

A recent meta-analysis assessed five recurrent variants, identified in multiple cohorts, found no evidence for association with keratoconus (Rong et al 2017). But the author added that his studies had a white population bias mainly. Another metanalysis by Lucas & Burdon (2020) concluded that VSX1 namely as not contributing to keratoconus susceptibility.

2.2.3.2 VSX1 In Asia: Indian, Iranian, Pakistani Studies

Though VSX1 is a frequently chosen as candidate gene conclusions remain inconclusive, conflicting or even negative. (Verma et al., 2005). He suggested studies with genetic screening of *VSX1* from other different ethnic populations could possibly solve part of the confusion.

The lack of *VSX1* pathogenic variations in a large number of unrelated sporadic keratoconus patients tend to omit its role, and corroborate the involvement of other genetic, environmental or behavioural factors in the development of this complex disorder.

Heon et al. (2005) was the first to identify *VSX1* mutations in patients with KC or posterior polymorphous corneal dystrophy (PPCD). This led to the

assumption that mutations in *VSX1* gene may be associated with KC pathogenesis. Indeed, *VSX1* and its variants have repeatedly surfaced as a prominent candidate gene in different studies (Verma et al., 2013). However, because it is also found together with other genes in KC cases, it is suspected that the aetiopathogenesis of KC could involve multiple genes or be polygenic.

In different KC populations, various single nucleotide polymorphisms (SNPs) were found in *VSX1* across different populations (DeBonis *et al*., 2011). However, the findings of these studies revealed discordance in that *VSX1* was pathogenic in certain study populations but non-pathogenic in other populations. Thus, the role of *VSX1* in keratoconus could not be conclusively established (Reddy et al., 2008).

In Malaysia, Ng JB et al 2017 studied all the exons on VSX!. He confirmed that VSX1 has seven exons spanning across 11.5kb in size (Ng et al., 2017), and features the nuclear localisation signal (NLS), homeodomain (HD), octapeptide region, putative transcription activation domains, RV *RINX/VSX1*) domain and CVC (*VSX2/VSX1* and *CEH10*) domain. *VSX1* regulates the function of cone opsin genes, controls activation of the red-green visual pigment in the locus control region and is involved in the retinal and anterior eye development (Hayashi et al., 2005). *VSX1* is also expressed in corneal tissues and thought to play a role in corneal wound healing (Maycock & Marshal, 2014).

In Malaysia, limited studies have been done. Only two different eye disease studies have alluded to the incidence of KC in the Malaysian population. Reddy et al. (2008) in “Prevalence of eye diseases and visual impairment in urban

population" - a study from University of Malaya Medical Centre. *Malaysian Family Physician*. In his study, only four (0.3%) KC patients were found among 1169 eye cases screened. Mohd-Ali B et al. (2012) in another Malaysian study, saw 159 KC cases from the screening of 13,000 patients (1.2%).

The latest study by Ng *et al.*(2020), showed. in his study,that two SNPs namely A182A and P237P were over-represented among KC patients, compared with controls, wheras R217H was more common in controls compared with KC patients. Ng's study seems to show some similarity to this present study, adding to the current repertoire of variation in the *VSX1* gene in the quest for predictors or biomarkers of KC.

2.2.3.3 VSX1, SOD1 and COL4A3 As Candidate Genes for This Study-

For this study, the choice of *VSX1* exon3 as a candidate gene was based on the results of four other Indian researchers namely, Verma et al. (2003) Tanwar et al. (2010), Palliwal et al. (2011) and Shetty et al. (2015).

Tanwar's paper identified 4 *VSX1* variants, namely p.A182A, pR217H, p.P237P and c.64-24 C>T as having significance in KC aetiology. His conclusion was that c.64-24 C>T which was in intron 2, but very near to exon3, was a novel variant. His other findings were that A182A and P237P were present in both cases and controls. However, according to Tanwar, R217H and c.64-24 C>T could be of significance, as they were present only in patients . His result is the opposite to this study's findings, as R217H was more common in Normal controls., and not in the keratoconus cases . Shetty *et al.* (2015) identified two other novel missense substitutions in VSX1 variants, namely (p.Leu268His)*X1* gene. and p.Ser251.

Much earlier research studies in different ethnic groups, showed the presence of *VSX*1 variants in KC patients of different populations (Kennedy *et al*., 1986; Dash *et al*., 2011). Both exogenic and intragenic polymorphisms were found to be associated with KC in a few studies (Mok *et al*., 2008). Several variants were found in highly conserved residues of *VSX*1 and were predicted to be pathogenic by bioinformatics tools. However, the same variants were also found to be not pathogenic in other studies (Liskova *et al*., 2007). Thus, the role of *VSX*1 in the aetiopathogenesis of KC remains ambiguous and enigmatic.

2.2.3.4 SOD1 Candidate Gene

The superoxide dismutase 1 (SOD1) was first proposed as a candidate gene

for keratoconus, based on observation that individuals with Down's syndrome (trisomy 21) have higher prevalence of keratoconus compared to the general population, as well as the gene's location on chromosome 21.

(Udar et al 2006)

SOD1 encodes an enzyme that is responsible for detoxifying superoxide radicals, a form of reactive oxygen species, in the cytoplasm of cells. throughout the whole body, including the cornea. Oxidative stress from free radicals has been proposed as a mechanism involved in keratoconus pathogenesis.

Udar et al (2006) screened the exons and intron-exon junctions of SOD1 by direct sequencing in 15 probands with keratoconus from the United States.

Superoxide dismutase 1(SOD1) on chromosome 21 was researched as a possible candidate gene for familial keratoconus. A heterogenous genomic 7-base deletion

in Intron2 was identified in two KC families.

Most other studies have not observed this variant in their cohorts. Much larger case-control cohorts are required to determine the true frequency of the variant in keratoconus cases. studies. To date gene-screen studies have not identified any potentially disease -causing SOD1 variants. (All Muammar et al 2015, Stabuc-Silih et al 2010.)

2.2.3.5 COL4A3 Candidate Gene

The choice of *COL4A3* exon 2 for this study was based on Rabinowitz's publications (1998, 2016). In 1998, Rabinowitz described Collagen types I, III, V, VI, VII and VIII as scattered throughout different layers of the cornea below and identified the chromosomal locations of the genes (*COL*) for these collagen variants (Table 2.2).

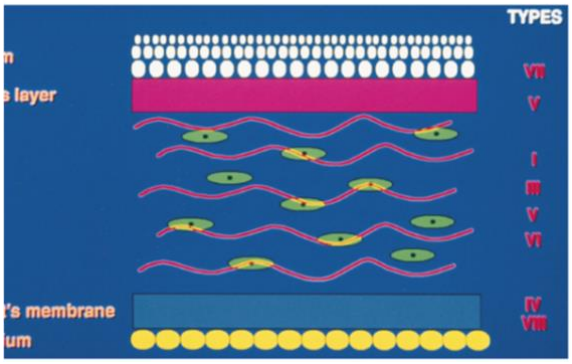


Figure 2. 7: Distribution of Collagen Types in different levels of the cornea. (Rabinowitz 1998)

Table 2. 2: Collagens in the cornea and their chromosomal locations

Collagens in the Cornea and Their Chromosomal Location

Collagen Type	Chain	Gene	Chromosomal Location
I	alpha 1 (I)	*COL1A1*	17q21-q22
	alpha 2 (I)	*COL1A2*	7q21-q22
III	alpha 1 (III)	*COL3A1*	2q31-q32
IV	alpha 1 (IV)	*COL4A1*	13q33-q34
	alpha 2 (IV)	*COL4A2*	13q33-q34
	alpha 3 (IV)	*COL4A3*	2
	alpha 4 (IV)	*COL4A4*	2
	alpha 5 (IV)	*COL4A5*	X
V	alpha 1 (V)	*COL5A1*	2q31-q32
	alpha 2 (V)	*COL5A2*	9p
	alpha 3 (V)	*COL5A3*	2q31-q32
VI	alpha 1 (VI)	*COL6A1*	22q
	alpha 2 (VI)	*COL6A2*	22q
	alpha 3 (VI)	*COL6A3*	6
VII	alpha (VII)	*COL7A1*	3q
VIII	alpha 1 (VIII)	*COL8A1*	3
	alpha 2 (VIII)	*COL8A2*	1

Source: Rabinowitz (1998)

Preliminary studies at Rabinowitz's institution using molecular genetic approaches excluded several collagen types, leaving *COL*1A1 and *COL*1A2 as possible candidate genes for KC (Rabinowitz, 1998, 2016.). He continued to elaborate new markers distal to *COL*6A1 and *COL*6A2, on the telomere of chromosome 21. However, no definite conclusions were made, and the importance of this group of collagens and collagen genes for KC remains inconclusive.

However, Mirna Stabuc-Silih et al (2009) further researched on Collagen genes COL4A3 and COL4A4, believing that they may be the responsible genes for reducing collagen in Collagen Types I and III. They detected 8 eight polymorphisms in COL4A3. For this study, COL4A3Exon 17 on chromosome 2, was selected based on the latter's results.

The major protein in the cornea is collagen., and several types of collagen have been identified in the degraded collagen products in tear film, of keratoconus patients.by biochemical and immunochemical method. (Abalain JH et al 2000)

Corneas from KC patients contain reduced amounts of collagen proteins and alterations of the extracellular matrix and basement membrane. Type IV collagen is only found in basement membrane where it is the major structural component. Mariyama et al ((1992) mapped the collagen type IV, alpha-3 (COL4A3) and collagen type IV, alpha-4(COL4A40 genes to the same region, 2q35-q37, but on opposite strands and transcribed in opposite directions (Mirna Stabuc-Silih et al 2009).

Yani Wang and CC Pang (2020) just illustrated how Keratoconus Genes are actually grouped into subgroups functionally as "corneal associated Genes", according to the biomechanics of cornea, corneal curvature, corneal dystrophies, and those with othe ocular syndromes (Fig 2.4). This also showed the relationship between genes and various corneal phenotypes and disorders. The genes involved (COL5A1, RAB3GAP1, IL1RN, IL1A, and IL1B) were different from those commonly associated with KC. All these genes are involved in the development of corneal thickening. Perhaps this seems to support a multigene theory for the development of KC and to eventually understand how they work together or against each other.

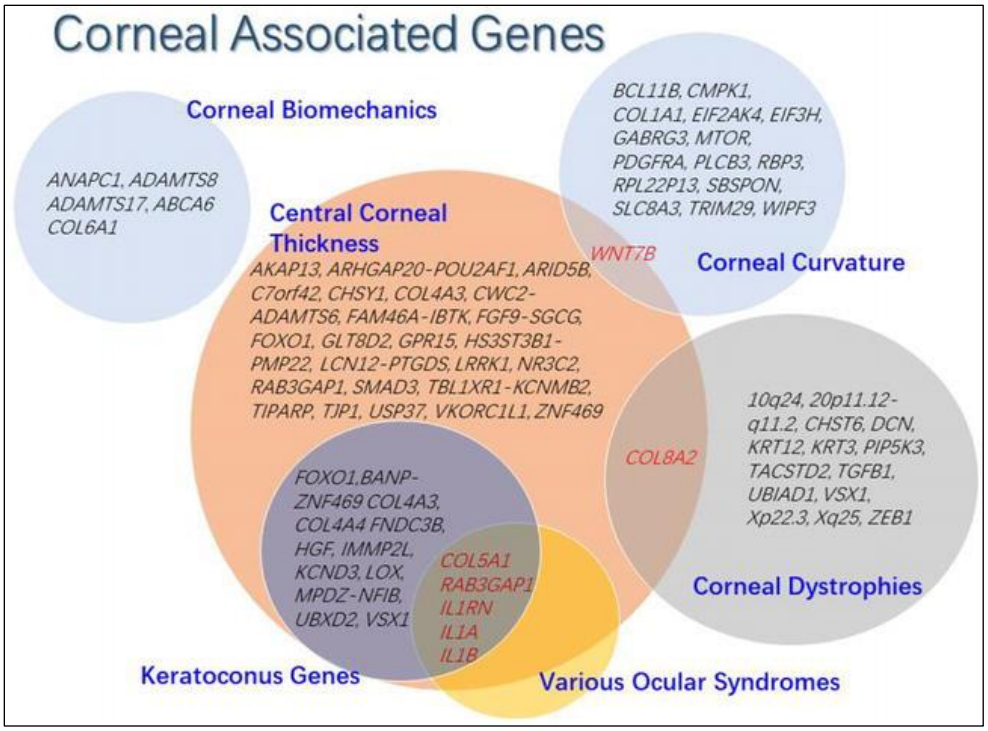


Figure 2. 8: Corneal Associated Genes
Source: Y Wang & CC Pang (2019)

2.2.4 Exome Studies on Keratoconus

2.2.4.1 Introduction

Exome and genome sequencing are relatively new clinical diagnostic genetic testing platforms for identifying a genetic aetiology among individuals with congenital anomalies or diseases having complex, multifactorial even enigmatic causation.

Exome sequencing is a cost-effective approach when whole-genome sequencing is not practical or necessary. Sequencing only the coding regions of the genome enables researchers to focus their resources on the genes most likely to affect the phenotype and offers an accessible combination of turnaround time and price. Exome sequencing detects variants in coding exons, with the capability to expand targeted content to include untranslated regions (UTRs). DNA libraries can be prepared in as little as 1 day and require only 4–5 Gb of sequencing per exome.

Whole Mendelian diseases are rare, yet genetic disorders occur at a rate of 40 to 82 per 1000 live births (Global report on birth defects: the hidden toll of dying and disabled children.2006. White Plains, NY: March of Dimes Birth Defects Foundation). Many patients with a genetic disease are not given a diagnosis. The standard of practice involves recognition of specific phenotypes or radiographic features or biopsy findings in addition to the analysis of metabolites, genomic tests such as karyotyping or array-based comparative genomic hybridization, (Baird *et al*., 1988, Cheung, 2005) or selection of candidate gene tests, including single-gene analysis and gene-panel tests. Most patients remain without a diagnosis (Boone *et al.*, 2010) This lack of diagnosis

can have considerable adverse effects for patients and their families, including failure to identify potential treatments, failure to recognize risk of recurrence in subsequent pregnancies, and failure to provide anticipatory guidance and prognosis. Genome sequencing with the use of massively parallel next-generation sequencing technologies has proven to be an effective alternative to locus-specific and gene-panel tests in a research setting for establishing a new genetic basis for disease (Gahl *et al.,* 2012). In clinical practice, however, the applications of WGS are complicated because of the difficulties in determining which of the thousands of variants of unknown significance (Yang and Muzny *et al*., 2013; Need *et al*., 2012) are relevant to the individual patient's presenting signs and symptoms. It is expected with great anticipation that WES in which 1% of the genome that codes for proteins is sequenced (Dixon-Salazar *et al.,* 2012, de Light *et al.,* Enns *et al.,* 2014) will improve diagnostic approaches in genetic diseases.

There are significant advantages and limitations of both WGS and WES. Whereas WGS allows more uniform and reliable coverage and reveals wider and broader variations across diverse human populations, WES is more cost- and time-effective WGS.

Much of the promise of genome sequencing relies on our ability to associate genotypes to physical and disease traits. Understanding genetic variations, such as single nucleotide polymorphisms (SNPs), small insertion-deletions (InDels), multi-nucleotide polymorphism (MNPs), and copy number variants (CNVs) helps to show the connection between genotype and phenotype (Might et al., 2014; Li et al., 2009)

Targeted capture studies can have a variety of approaches, but is particularly well suited to specific gene lists (or whole exomes) and massively parallel sequencing. Several group researchers have managed to generate PCR products that are targeted towards genes found under GWAS peaks, eventually successfully finding variants in causative genes.

Many patients with a genetic disease are not given a diagnosis. The standard of practice involves recognition of specific phenotypes or radiographic features or biopsy findings in addition to the analysis of metabolites, genomic tests such as karyotyping or array-based comparative genomic hybridization, (Baird PA et al 1988, Cheung SW 2005) or selection of candidate gene tests,, including single-gene analysis and gene-panel tests. However, the majority of patients remain without a diagnosis(Boone PM et al 2010) and this lack of a confirmed diagnosis can have considerable adverse effects for the patient and their families. This includes failure to identify potential treatments, failure to recognize risk of recurrence, and failure to provide anticipatory guidance and prognosis.

Genome sequencing with use of massively parallel next-generation sequencing technologies has proven to be an effective alternative to locus-specific and gene-panel tests in a research setting for establishing a new genetic basis for disease. (Gahl WA et al 2012),

Next- generation sequencing (NGS) in which the whole genome , or portion of it, is sequenced has proven extraordinarily useful for identifying new causes of genetic diseases, especially those with Mendelian disorders.

However, the applications of NGS directly in the clinic is complicated because of the difficulties in determining which of the thousands of variants of unknown significance (Yang Y, Muzny DM et al 2013, Need AC et al 2012) are relevant to the individual patient's presenting signs and symptoms. Yet it is expected with great anticipation that NGS especially WES in which 1% of the genome that codes for proteins is sequenced (Dixon-Salazar TJet al 2012, de Light J et al, Enns GM et al 2014) will improve diagnostic approaches in genetic disease.

Wheras WGS gives a more comprehensive picture, as it covers coding and non-coding genomic regions, WES only covers Protein-coding regions, more in detail, but producing less raw data. WGS thus differs, as it reveals wider and broader variations across diverse human populations. There are significant advantages and limitations of both techniques, namely WGS and WES. However, balancing cost- and time-effectiveness against the desired results, enables choosing the optimal sequencing approach. WGS allows more uniform and reliable coverage, thus has become a more universal method than WES.

Understanding genetic variations, such as SNPs, small insertion-deletions (InDels), multi-nucleotide polymorphism (MNPs), and copy number variants (CNVs) helps again to show the connection between genotype and phenotype (Might M et al 2014, Li H et al 2009,)

Much of the promise of genome sequencing relies on our ability to associate genotypes to physical and disease traits. Understanding genetic variations, Single Nucleotide polymorphisms (SNPs), helps to reveal the relationship, if any, between Genotype and Phenotype and reveals variations

across diverse human populations (De Pristo MA et al 2011, Cingolani P et al 2012, Li H et al 2009).

2.2.5 Case Control and Cohort Studies

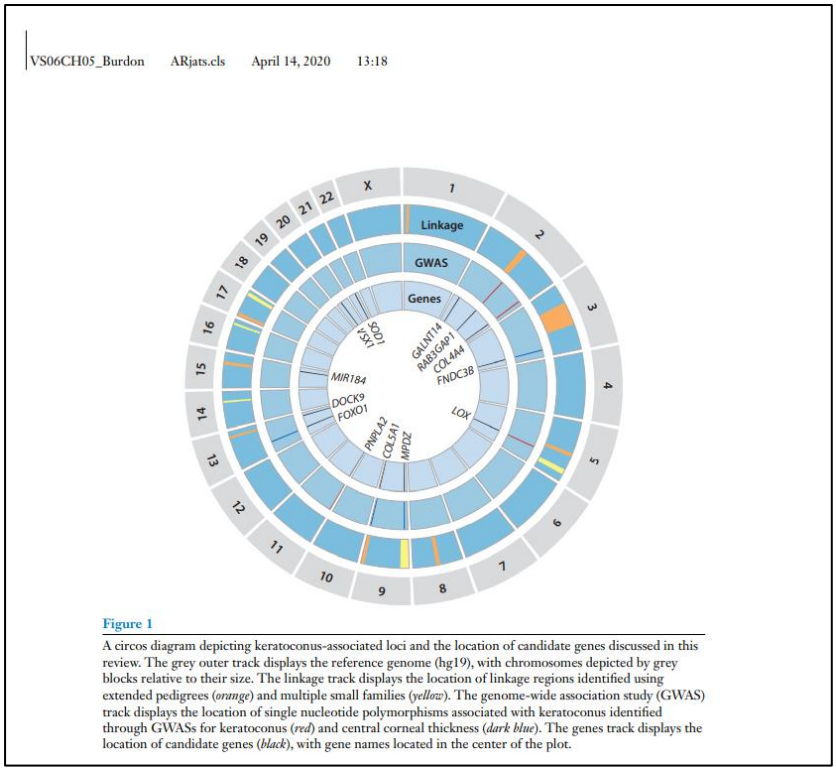


Figure 1

A circos diagram depicting keratoconus-associated loci and the location of candidate genes discussed in this review. The grey outer track displays the reference genome (hg19), with chromosomes depicted by grey blocks relative to their size. The linkage track displays the location of linkage regions identified using extended pedigrees (*orange*) and multiple small families (*yellow*). The genome-wide association study (GWAS) track displays the location of single nucleotide polymorphisms associated with keratoconus identified through GWASs for keratoconus (*red*) and central corneal thickness (*dark blue*). The genes track displays the location of candidate genes (*black*), with gene names located in the center of the plot.

Figure 2. 9: Case control and cohort study
Source: Lucas & Burdon (2020)

Figure 2.9 depicts keratoconus-associated loci and location of candidate genes discussed in a linkage studies annual review, by Lucas FM and Burdon KP (2020). This genome-wide association study (GWAS) track, displays the location of SNPs associated with keratoconus(red) and central corneal thickness (dark blue). The genes track displays the location of candidate genes(black) and gene names located in the centre of the plot. This circus figure, explains and highlights, the genetic heterogeneity of keratoconus, and clarifies to some extent the significant linkage regions throughout the genome. The conclusion highlights again, the complexity of keratoconus genetics.

However again, the conclusion derived in this enormous linkage studies, and GWAS reported, are in predominantly white population.

Despite dozens of genes having been hypothesized to play a role in keratoconus susceptibility, the collective results of candidate gene studies in keratoconus have not been successful. Most of these studies screened candidate genes to identify variants present or absent in cases and controls or differing significantly in allele frequency.

Disadvantages exist in these studies, as they are usually small sample size, which do not allow accurate estimation, and may lead to false or biased conclusions. Many studies did not assess the controls in full, leading to a lack of the true variability of several key genes in the general population.

This may be because of ignorance that “benign rare missense variants are present in healthy individuals”. Furthermore, very few follow up studies have been conducted to determine whether the specific identified variants actually have a role to play in keratoconus susceptibility or are biologically relevant. As a result, these factors have given limited success to perpetuate the focus on candidate genes.

CHAPTER 3 RESEARCH METHODOLOGY

3.1 Clinical Keratoconus

3.1.1 Study Population

Participants recruitment

KC patients were recruited after their diagnosis was confirmed at 2 specialist eye centres in the Klang Valley. Their family members who turned up for screening were recruited as family controls. College student controls were those who volunteered to be screened for eye disorders at a college function and school children who participated in a school eye-sight screening campaign were recruited as controls, with parental consent.

Each participant or participant's parent in the case of children, had to sign an informed consent form and answer a questionnaire.

3.1.1.1 Inclusion Criteria

i. KC Cases:

- All races (Malay, Chinese, and Indian).
- Aged 15 – 40 years old.
- Both male and female.
- Patients with Corneal Thickness >400u, both eyes fitted with trial KC RGP lenses before the CXL treatment and power reconfirmed about one-month PostCXL treatment.
- Patients with Vogts lines and eccentric scarring were accepted.
- Early hydrops cases were accepted, as CXL treats it also.
- Residing in Klang Valley.

ii. Controls

The Controls are comprised of 3 groups:

- Close family members of KC patients who turned up at the clinic for screening after their relatives have been diagnosed as KC.
- Age matched College controls
- School children who agreed to or had parental permission for KC screening and examination of their eyes (Age 9-12 yrs)
- Aged 15 to 40 years old.
- Both male and female
- All races (Malay, Chinese, and Indian).
- Residing in Klang Valley.

3.1.1.2 Exclusion Criteria

Exclusion criteria involves participants that have:

i. Patient with history of ocular surgery, or other significant eye disease.
ii. Advanced Keratoconus with central corneal scarring, corneal oedema or corneal thickness of <300u, were also excluded.
iii. Patients or controls who were unwilling or unable to give informed consent

3.1.1.3 Case Control Study: Conceptual Framework

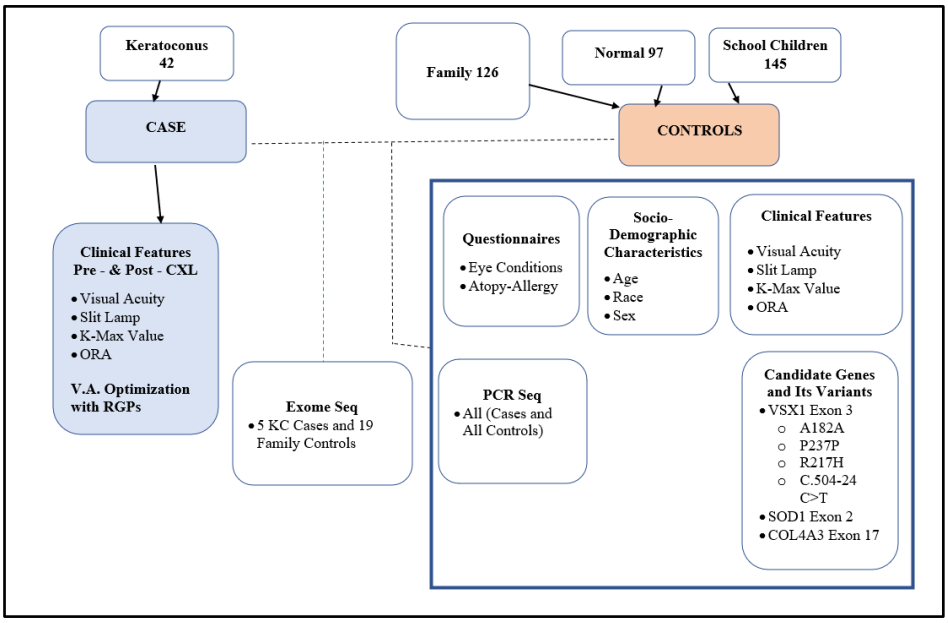


Figure 3. 1: Conceptual frameworks

3.1.1.4 Study Sample Size

KC is a complex, genetically heterogeneous, multifactorial degenerative disorder (Rabinowitz *et al* 1998) with a sporadic distribution (Kennedy RH et al., 1986) Reports indicate that proportions ranging from 5–10% to 8–10% ((Rabinowitz *et al* 1998) or 14% (Zadnik K et al.,1998), and even up to 23% of KC patients have a family history of KC. It follows an autosomal recessive or dominant pattern of inheritance.

Why is 23% chosen as proportion because it is from Loukovitis 2018, a more recent study. Further evidence is shown below in bold. Evidence of a genetic component for keratoconus comes from studies evaluating family history of the disease. The proportion of keratoconus patients who report a family history of the disease ranges from 5% in Scotland (Weed et al. 2008) to 28% in Israel (Shneor et al. 2013), with most studies falling between 10% and 25% (Bawazeer et al. 2000; Gordon-Shaag et al. 2013, 2015a; Ihalainen 1986;

Naderan et al. 2015; Owens & Gamble 2003; Zadnik et al. 1998). This is a much higher rate than that seen in matched controls in the few studies that did recruit and evaluate controls (Bawazeer et al. 2000; Gordon-Shaag et al. 2013, 2015a; Naderan et al. 2015).

A comprehensive study of a large series of keratoconus cases and their nuclear families found a prevalence of 3.34% in first-degree relatives (Wang et al. 2000), which was at least 15-fold higher than the reported prevalence in that population. A similar study found an even higher rate of 7.6% (Kriszt et al. 2014).

Therefore, calculating 3.34/15=0.22%**.** Sample size calculation will be calculated using the following formulas (Fleiss *et al* 1981).

$$m' = \frac{\left[c_{\alpha/2}\sqrt{(r+1)\bar{P}\bar{Q}} - c_{1-\beta}\sqrt{rP_1Q_1 + P_2Q_2} \right]^2}{r(P_2 - P_1)^2}$$

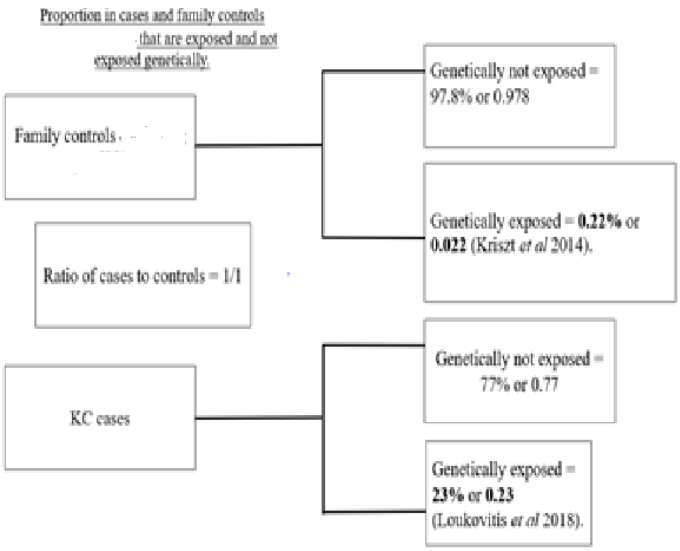
$$m = \frac{m'}{4}\left[1 + \sqrt{1 + \frac{2(r+1)}{m'r|P_2 - P_1|}} \right]^2$$

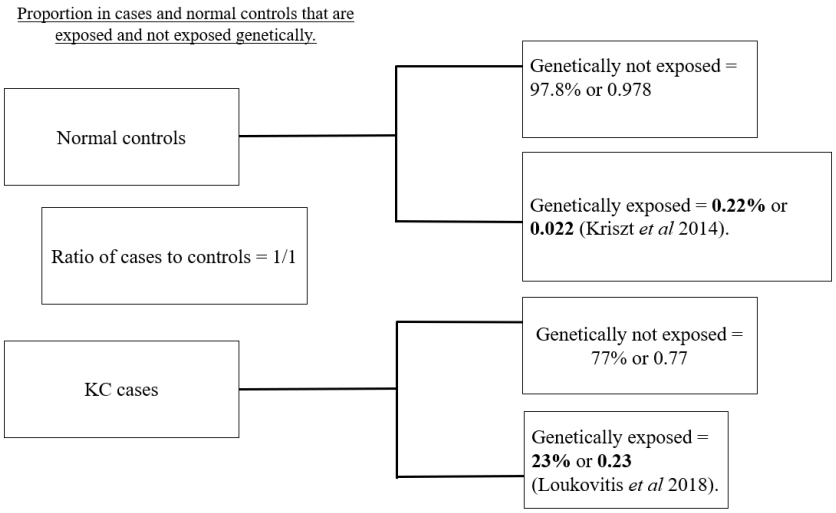
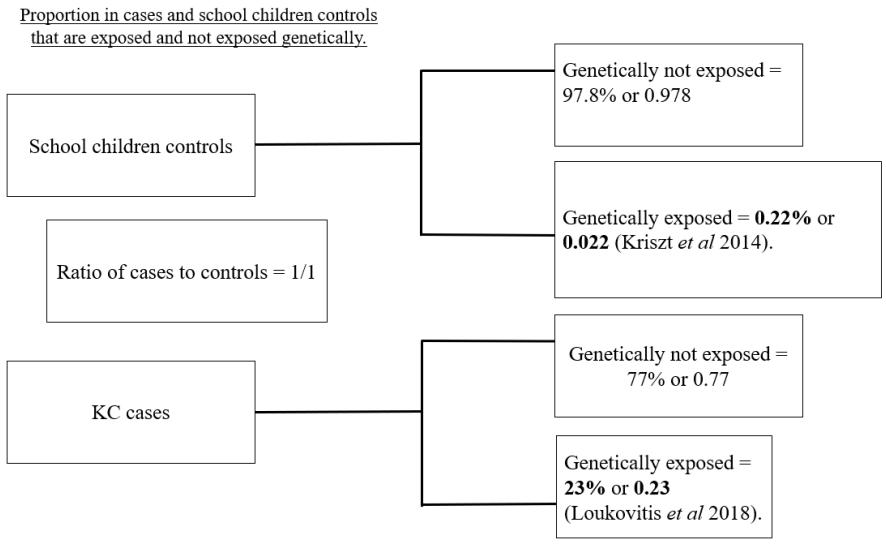
Figure 3. 2: Fleiss' formula to calculate sample size in cases and in controls.

Where,

m = n1 = size of sample from population 1
n2 = size of sample from population 2
P1 = proportion of disease in population 1
P2 = proportion of disease in population 2
α = "Significance" = 0.05
β = chance of not detecting a difference = 0.2
1 – β = Power of detection = 0.8
r = n2/n1 = ratio of cases to control
P = (P1 + P2) / (r + 1)
Q = 1-P
n1 = m
n2 = rm
Q1 = 1 – P1
Q2 = 1 – P2
From table A.2 in Fleiss:
If 1 α is 0.95, then Cα/2 is 1.960.
If 1 – β is 0.80, then C1- β is -0.842.

Calculations Of Sample Size for Each Study Group

Sample size and proportion	**Calculation of sample size**
1. Sample Size for KC and Family Control  Up to 23% of KC patients have a family history of KC (Loukovitis *et al* 2018) which is a more recent reference. A comprehensive study of a large series of keratoconus cases and their nuclear families found a prevalence of 3.34% in first-degree relatives (Wang et al. 2000), which was at least 15-fold higher than the reported prevalence in that population. A similar study found an even higher rate of 7.6% (Kriszt et al. 2014). Therefore, **3.34/15=0.22%** is the proportion for every group of controls that are exposed genetically as they do not have KC	m = n1 **(Control)** = **47.8** n2 **(Cases)** = r x m = 1 x 47.8 = **47.8** n1 + n2 **(Total sample size)** = 47.8 + 47.8 = **95.6**

2. Sample Size for KC and Control (Normal)  *Similar explanation as above as same proportion applies**	m = n1 **(Control)** = **47.8** n2 **(Cases)** = r x m = 1 x 47.8 = **47.8** n1 + n2 **(Total sample size)** = 47.8 + 47.8 = **95.6**
3. Sample Size for KC and Control (School Children).  *Similar explanation as above as same proportion applies**	m = n1 **(Control)** = **47.8** n2 **(Cases)** = r x m = 1 x 47.8 = **47.8** n1 + n2 **(Total sample size)** = 47.8 + 47.8 = **95.6**

SUMMARY AND JUSTIFICATION OF SELECTED SAMPLE SIZE

Based on the results of sample size calculations as listed above, We have concluded that 47.8 for cases and 47.8 for controls is the highest. Attrition rates for each group, **55** cases and **55** controls are applied for the following reasons:

i. Participants may withdraw from the study mid-way.

ii. Participants may provide incomplete data.

iii. Participants may not be keen to provide blood by finger prick sample.

Thus, the total samples for each control group is > 55 except for the KC cases being <55, that is 42 KC cases only,

However, 42 KC cases already make up 80% of the original required sample size of 55. So in this case, the sample size of 42KC cases is adequate and acceptable for this Case Control Study.

3.1.1.5 Study Flow Chart

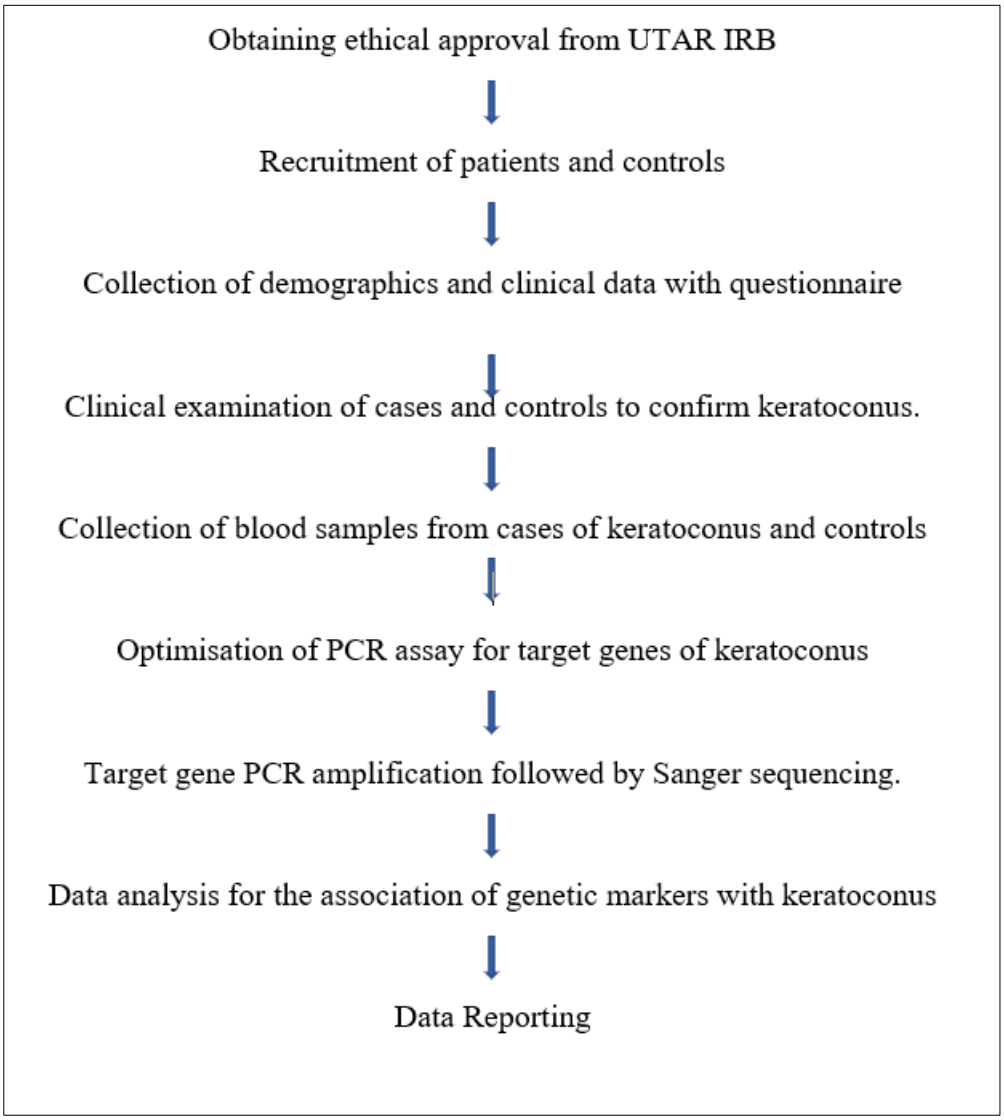


Figure 3. 3: Study flow chart

3.1.1.6 Variables

Table 3. 1: List of variables

Variables	**Type of Data**	**Conceptual Definition**	**Operational Definition**
Cases	Dependent	The number of participants which have been confirmed as KC cases	Continuous data. KC Cases: No. of cases.
Controls	Dependent	The number of participants which are controls (family, normal, and school children).	Categorical Nominal Data. Controls: Family: n% Normal: n% School Children: n%
Age	Independent	The period in which participants are considered adolescence, young adults, and adults.	Ordinal data: 15 – 20 y: Adolescence 21 – 30 y: Young adults 31 – 40 y: Adults
Race	Independent	The three major races in Klang Valley, Malaysia includes, Malay, Chinese, and Indian.	Categorical nominal data. Malay: n% Chinese: n% Indian: n%
Sex	Independent	The gender of the participants includes either male or female.	Categorical nominal data. Male: n% Female: n%
Eye-rubbing	Independent	The number of cases affected by eye-rubbing compared to controls.	Ordinal data. Mild - moderate: Slightly more than normal eye-rubbing habit. Severe: Rubbing the eye much more frequently than usual.
Eye Itch	Independent	The number of cases affected by eye itch compared to controls.	Nominal Data. Yes: Itchy more frequently than normal No: Not itchy or seldom
Eye Redness	Independent	The number of cases affected by eye redness compared to controls.	Nominal Data. Yes: Eye redness more often than normal No: No eye redness or seldom

Watery Eyes	Independent	The number of cases affected by watery eyes compared to controls.	Nominal Data. Yes: Watery eyes more often than normal No: No watery eyes or seldom
Nose Blockage	Independent	The number of cases affected by nose blockage compared to controls.	Nominal Data. Yes: There is nose blockage more often than normal No: No nose blockage or seldom
Skin Allergy	Independent	The number of cases affected by skin allergy compared to controls.	Nominal Data. Yes: There is skin allergy No: No skin allergy
Asthma	Independent	The number of cases affected by asthma compared to controls.	Nominal Data. Yes: There is asthma No: No asthma
Food Allergy	Independent	The number of cases affected by food allergy compared to controls.	Nominal Data. Yes: There is food allergy No: No food allergy
Slit-lamp Findings of Cases	Independent	The number of cases associated with KC clinical findings in both eyes as compared to controls.	Categorical Nominal Data. Munson's RE: n% LE: n% Vogt's RE: n% LE: n% Hydrops RE: n% LE: n% Scarring RE: n% LE: n%
K-max Value	Independent	The proportion of K-max value in cases and controls according to Krumeich Classification in both eyes.	Ordinal Data. RE: < 45 Kmax: n% (Mild) 45 – 50 Kmax: n% (Moderate). 51- 60 Kmax: n% (Advanced). 61-70 Kmax: n% (Severe) LE: < 45 Kmax: n% (Mild) 45 – 50 Kmax: n% (Moderate).

			51- 60 Kmax: n% (Advanced). 61-70 Kmax: n% (Severe)
Intraocular Pressure	Independent	The proportion of intraocular pressure readings in both eyes between cases and controls	Categorical Nominal Data. Cases: n%, mean, SD Controls: n%, mean, SD
Corneal Readings	Independent	The proportion of corneal readings in both eyes between cases and controls	Categorical Nominal Data. Cases: n%, mean, SD Controls: n%, mean, SD
Clinical features of KC pre- and post- CXL	Independent	The comparison of clinical features in cases pre-CXL and post CXL	Categorical Nominal Data. Pre-CXL: Visual Acuity: n%, mean, SD Slit Lamp: n%, mean, SD K-Max Value: n%, mean, SD ORA: n%, mean, SD Post-CXL: Visual Acuity: n%, mean, SD Slit Lamp: n%, mean, SD K-Max Value: n%, mean, SD ORA: n%, mean, SD
Candidate Genes (VSX1, SOD1, COL4A3)	Independent	The comparison of proportion in cases affected by mutations in selected candidate genes as compared to controls.	Categorical Nominal Data. VSX1: n%, mean, SD SOD1: n%, mean, SD COL4A3: n%, mean, SD
VSX1 Variants (A182A, P237P, R217H, c.504 – 24 C>T)	Independent	The proportion of cases affected by VSX1 variants as compared to controls.	Categorical Nominal Data. A182A: n%, mean, SD P237P: n%, mean, SD R217H: n%, mean, SD

			c.504 – 24 C>T: n%, mean, SD
Alleles and genotypes of VSX1 Variants (A182A, P237P, R217H, c.504 – 24 C>T)	Independent	The proportion of alleles and genotypes between cases and controls.	Categorical nominal Data.
Variants found in various candidate genes	Independent	The proportion of various candidate genes variants found in cases and controls.	Categorical Nominal Data. VSX1 A182A: n% P237P: n% ….. ……. and other candidate genes and its variants.

3.1.2 Ethical Approval and Informed Consent

This study was conducted in accordance with the Declaration of Helsinki and approved by the Universiti Tunku Abdul Rahman (UTAR) Ethical Research Committee.

3.1.3 Questionnaire Survey

With informed consent, a questionnaire (Fig. 2.1 A-D) was administered to each patient and control in this study, to obtain information on related epidemiological, cultural and allergy status of the participants.

Screening Project for Keratoconus

3.13 Questionaire Survey

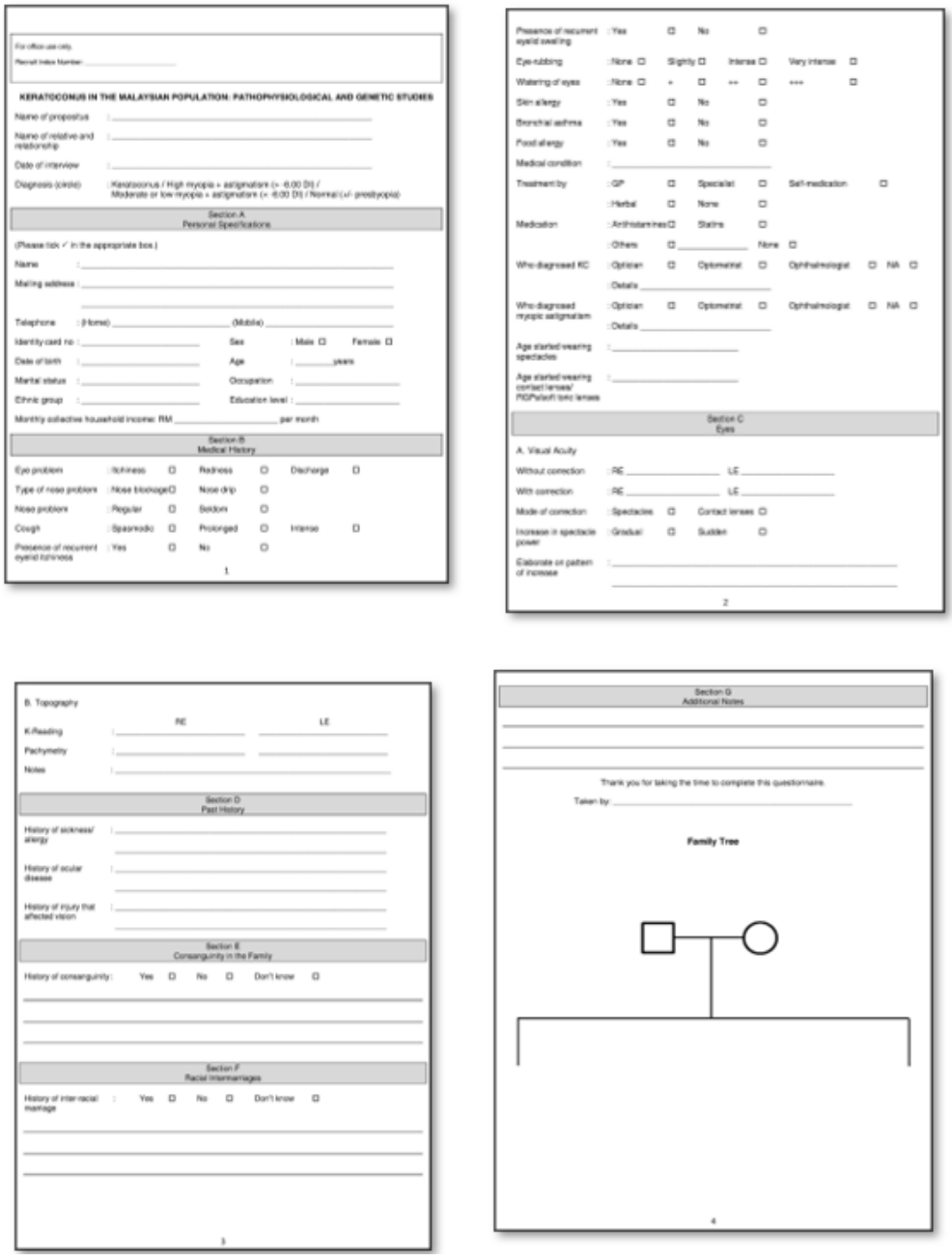


Figure 3. 4: Questionnaire pages (Enlarged versions are in Appendix)

Q1 Patients were asked how they rubbed their eyes and how severely also?

RUBBING EYES WITH FINGERS OR KNUCKLES?

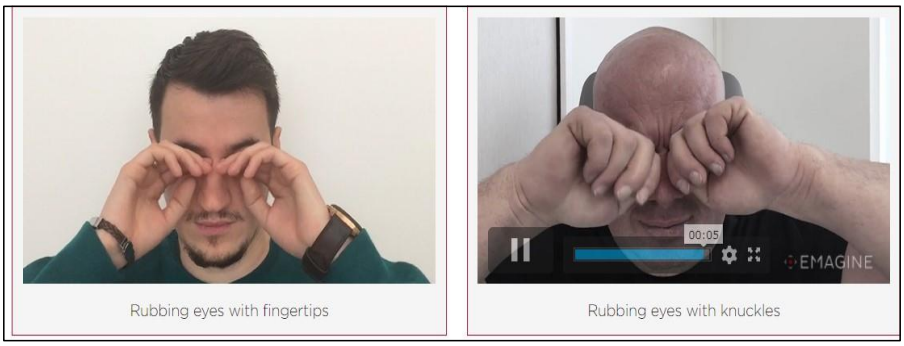


Figure 3. 5 Rubbing eyes with fingers or knuckles.
Source : info@elza-institute.com (2020)

This figure clarifies how the eyes can be severely rubbed with the fingers or the knuckles, to begin the aetiopathogenesis of keratoconus development.

Q2. Is this a habit or allergy?

When the cornea is rubbed , it becomes mechanically weakened. A clear connection between heavy eye rubbing and progression of keratoconus has been known for several years. Eye rubbing can be a habit, for example when a contact lens wearer removes the lenses in the evening and then rubs his eyes extensively.But there may also be an allergy (hay fever, house dust), in which the eyelids itch and redden. (ELZA Institute, 2020)

Figure 3. 6: Ways of rubbing eyes.
Source : info@elza-institute.com (2020)

This figure clarifies how the eyes can be severely rubbed, to begin the aetiopathogenesis of keratoconus development.Source ELZA Institute

Figure 3. 7: Public participants for screening programme

Figure 3.7 above shows participants of the Screening Program, with prior permission given to researcher to utilize picture for illustration. No names are given.

3.1.4 Clinical Examination

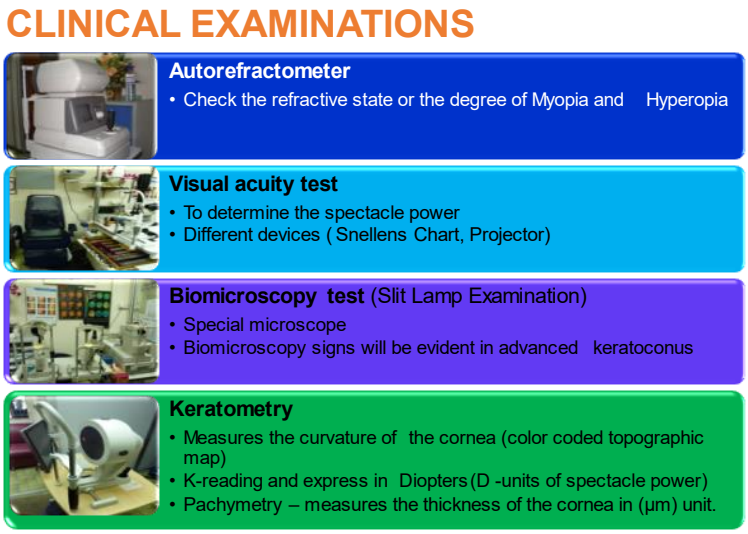


Figure 3. 8: Equipment used for investigating study population and confirming diagnosis of Keratoconus.
SOURCE: JPD

All KC patients underwent complete ophthalmoscopic examination, best spectacle visual acuity (BSCVA), slit-lamp biomicroscopy and corneal topography (Tomey TMS4 or PENTACAM), ocular response analysis (ORA, Reichert) and ultrasonic pachymetry (Tomey). Ocular coherence tomography (OCT, Visante) was selectively used for more detailed analysis of the anterior segment of the eye. All other study groups underwent the same clinical tests and investigations.

KC was classified by its history, early, late, or advanced stages and the accompanying symptoms (Fig.3.6). It was also classified by the The Amsler-Krumeich Clinical Classification to divide the symptomatology, corneal thickness and myopic astigmatism into low, medium and high, corresponding to KMax minimum and maximum readings (Table 3.2)

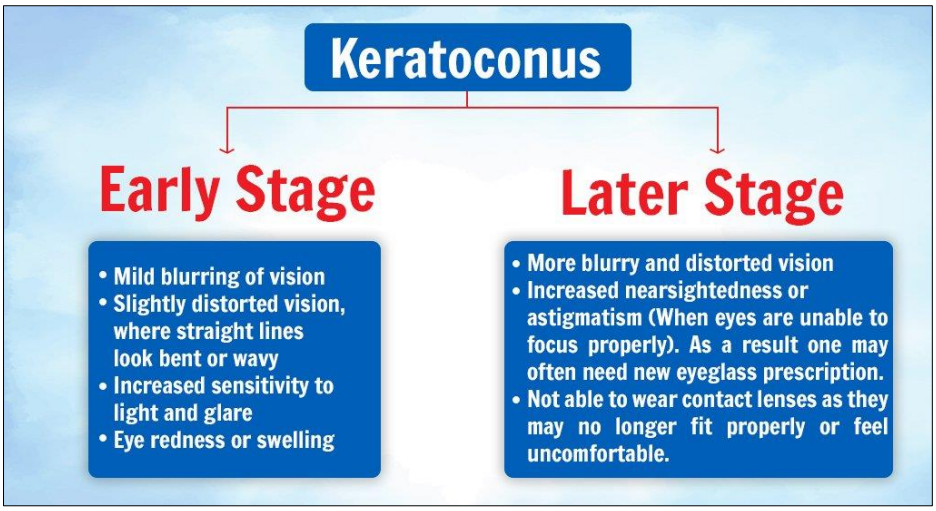


Figure 3. 9: Clinical appearance of Keratoconus in early and late stages.
Source: Rabinowitz (1998)

Table 3. 2: The Amsler-Krumeich Classification Grades Characteristics

Stage 1	Eccentric steepening Myopia and astigmatism CCT400 µm
Stage 2	Myopia and astigmatism from 5.00 to 8.00 D Mean central K 47D, CCT 400
Stage 3	Myopia and astigmatism from 8.00 to 10.00 D Mean central K readings >53.00 D Absence of scarring Minimum CCT from 300 to 400 µm
Stage 4	Refraction not measurable Mean central K >55.00 D Central corneal scarring Minimum CCT 200 µm

3.1.4.1 Visual acuity

For all participants, both right eye (RE) and left eye (LE) were examined with the following methods: Snellen Visual acuity for Distance and Near and correction of refractive errors was done. Later this was correlated with LogMAR equivalent readings.

3.1.4.2 Slit Lamp Examination

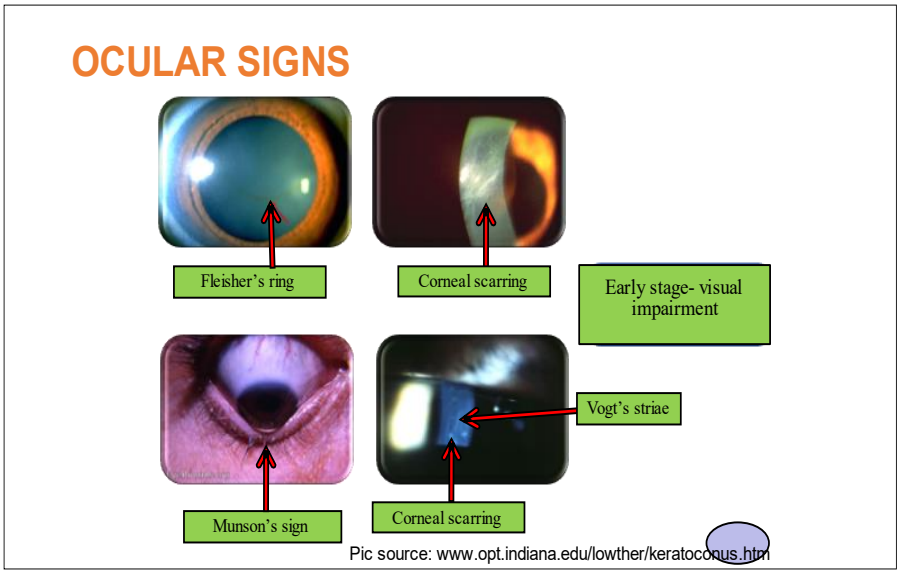


Figure 3. 10: Most common ocular signs of advanced keratoconus
Source: www.opt. Indiana.eduflowlther/keratoconus_html

Slit lamp examination was done for the cornea and anterior segment of the eye. and the diagnosis of KC was based on this examination, Important clinical features such as Munson's sign.(Fig.3.10) The cornea was examined for signs of any advancement in KC progression, like Vogt's lines, Hydrops and Scarring. and additional Fleischer ring, and prominent corneal nerves .

In early KC disease, there may be no symptoms. With disease progression, vision is hampered by irregular astigmatism, myopia, and corneal scarring. Later, fine iron deposits are seen at the base of the KC cone called Fleischer's ring. Vogt's striae which are vertical lines produced by the compression of Descemet membrane may be seen at or near the apex of the cone. Corneal scarring is also common.

In advanced cases of KC, the ectatic cornea becomes visible when the patient looks downward and the corneal protrusion will push the lower lid into a "v-shaped" dent clinically named Munson's sign. In extremely advanced and

severe cases, breaks in the Descemet membrane appear. This gives rise to "hydrops" which is stromal oedema, resulting in vision loss and associated pain.

3.1.4.3 Video-Topography

This was done for the whole corneal surface, both central and peripheral. The topograms of KC usually shows an inferior elevation or steepness, with high Kmax measured in dioptres.

Topography parameters, such as the steep axis and flatter axis, the presence of the cone usually in the infero- temporal or infero-nasal position and the anterior float, posterior float as well.

Orbscan, and Tomey Video-Topographers were used to measure the keratometry and the pachymetry, and readings of the cornea were taken for the final diagnosis.

Corneal imaging has been difficult in KC cases as the cornea is aspheric, irregular, and asymmetric, limiting the usage and reliability of simple measuring methods. Despite the numerous advances in corneal imaging over the past few decades, it is still difficult to assess corneal shape qualitatively and quantitatively. The keratometer or ophthalmometer is used to provide quantitative measurements while the videokeratoscope (videotographer) provides qualitative information. It was the union of rapid computer analysis and digital video by Klyce (1984) that led to the color-coded maps of corneal curvature by multiple commercially available computerised videokeratoscopes. Unlike the keratometer which localizes measurements to data in the central 3-6mm, the videokeratoscopes are capable of digitizing information from thousands of points on the whole corneal surface to produce detailed color-

coded maps depicting corneal curvature. This information is translated into colour coded topographic map by the video-topographer (Fig.3.12)

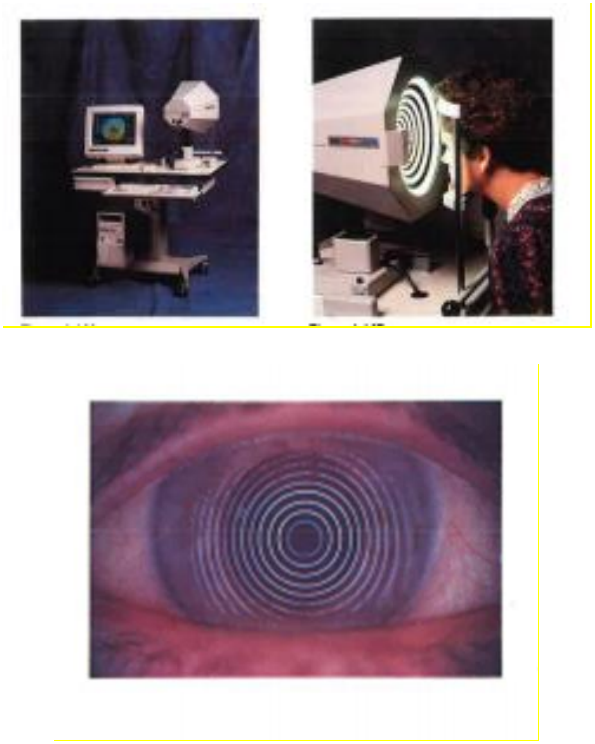


Figure 3. 11: Videotopographer machine (Tomey); Patient being tested; Placido's Rings reflected on the cornea from the videotopographer
Source: Donald Sanders *et al.* (1993)

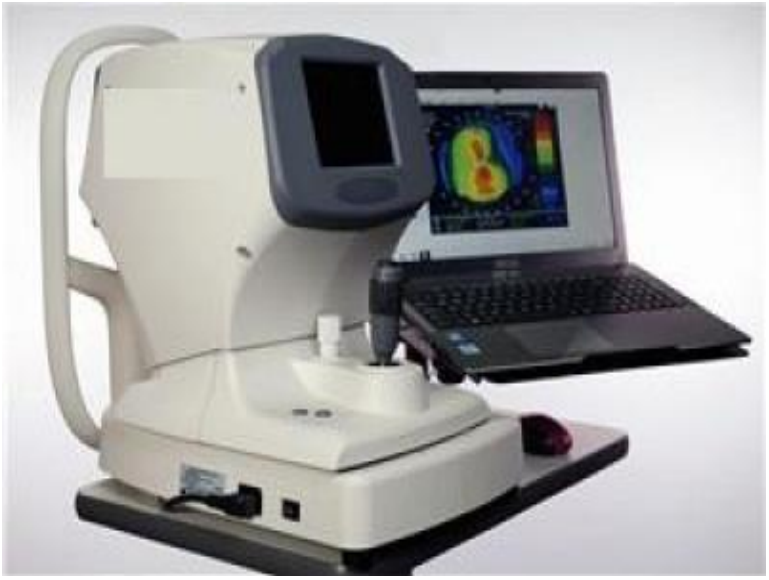


Figure 3. 12: Tomey TMS-4N Topographer w/ Laptop
Source: Tomey TMS-4N Topographer

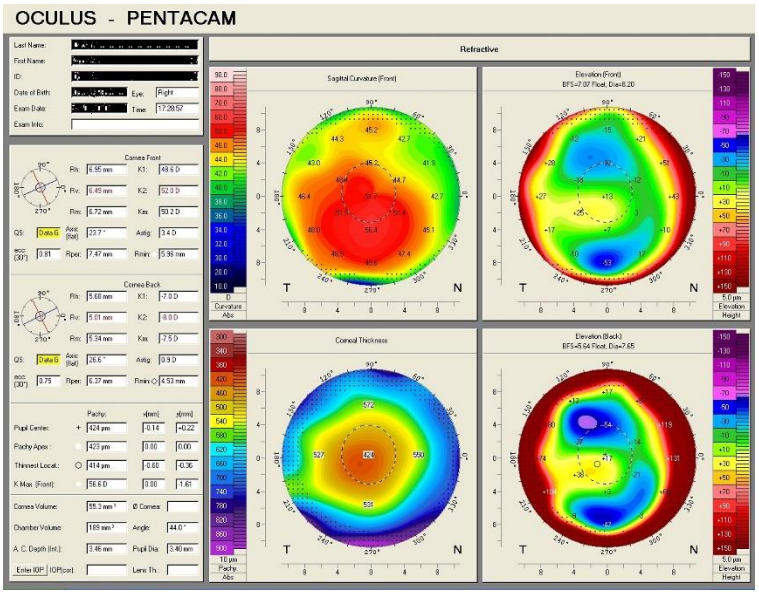


Figure 3. 13: Typical Keratoconus Topographic Map.
Source: Oculus- Pentacam Topographer

Currently, computerised assisted-videokeratoscopes that generate colour–coded maps and topographical indices, provide the most sensitive system to confirm the diagnosis of KC. Several numeric summaries of videokeratographic data can facilitate quantitative analysis, but there is no generally accepted classification system for KC. The classifications proposed either classify KC as mild, moderate or advanced, based on Cone Apex conical degree, or as central or paracentral, based on the location of the cone, or as round, nipple, oval, sagging or globus based on the shape of the cornea (Fig.3.14).

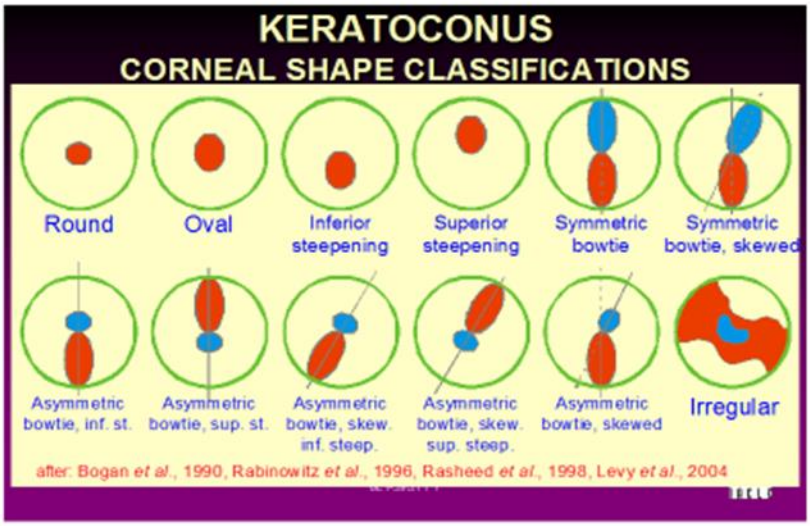


Figure 3. 14:Classification of keratoconus by corneal cone shape

Rabinowitz and McDonnell (1989) similarly established three diagnostic features of KC for classification. These are 1) Central corneal power > 47dioptres, 2) A difference of 3 dioptres or more in corneal power comparing points 3mm.inferior to the center to points superior to the center (I – S), and 3) Asymmetry between central corneal power of fellow eyes in excess of 1 dioptre (Rabinowitz 1989).

Videokeratography is most important in the evaluation of KC progression (Macquire *et al*. 1991), in contact lens fitting and in the monitoring of their effect in unusual forms of KC (Fig 3.15)

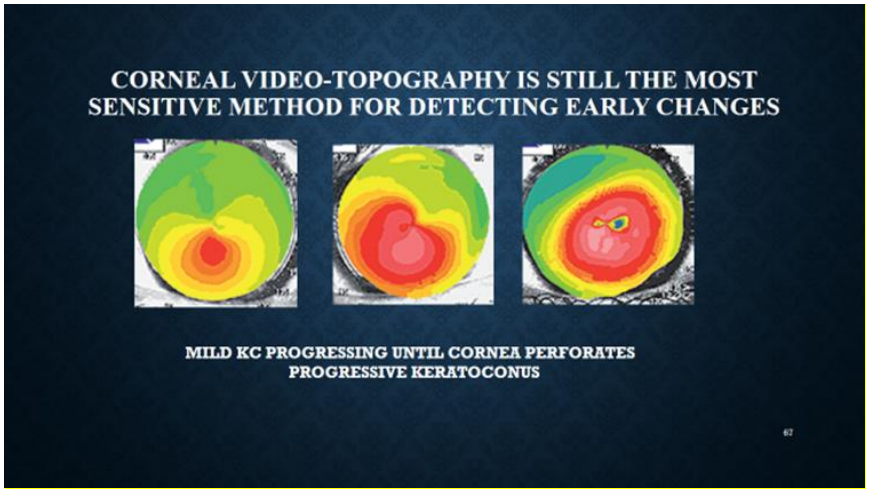


Figure 3. 15: Corneal topography showing progression of keratoconus
Source: Macquire *et al.* (1991)

The techniques of corneal imaging are continuously evolving to become better and more accurate, but misinterpretation still occurs through user inexperience.

3.1.4.4 Pachymetry**:** Usage to measure thickness of cornea in microns

Figure 3. 16: Application of the tip to measure corneal thickness
Source: Peschke

Procedure: The cornea ia anaesthesised with one to two drops of local topical anaesthetic drops like, Alcaine or Tetracaine 1%. Then the tip of the pachymeter is gently placed on the cornea, just touching it , without any pressure, to produce a reading on the inbuilt miniature screen.

This Pachymeter can be paired electronically with other machines like Topographer, and Corneal thickness can be read from the meter installed on it simultaneously.

3.1.4.5 Ocular Response Analyser (ORA, Reichert)

The ORA enables clinicians to examine and classify different corneal conditions according to biomechanical tissue properties instead of geometrical measurements. This advance in technology has enabled users to identify subjects who may not have traditional symptoms at presentation but over time, may be at risk of developing corneal pathologies.

ORA measurements for Non-contact Tonometry (IOPg and IOPcc) and the measurement of Corneal Hysteresis (CH), and Corneal Resistance Factor (CRF) were taken.

Intraocular Pressure (IOP) was measured together with biomechanical properties, Corneal Hysteresis (CH), Corneal Resistance Frequency. (CRF) and with the Ocular Resonance Tomography (OCT).

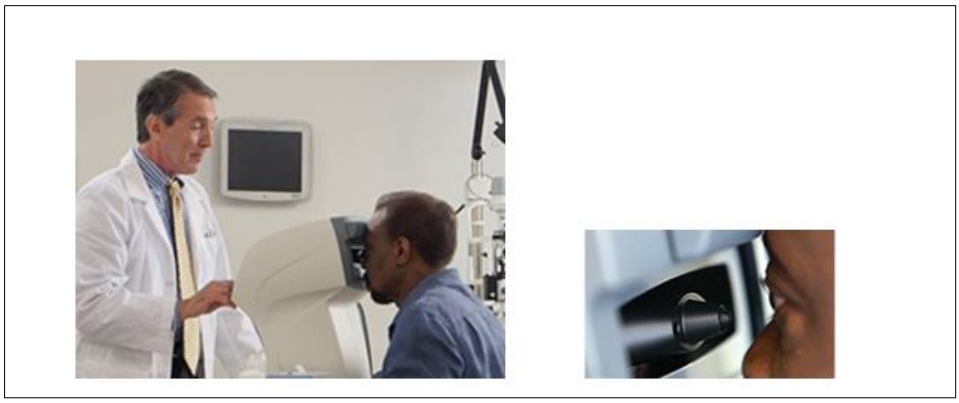


Figure 3. 17: Keratoconus progression detection.
Source: Reichert machine manual, 2006

The biomechanical strength of the cornea can be assessed by special machines such as the Ocular Resonance Analyser (ORA). Positioning of patient's eye to test corneal biomechanics and reassuring patient that the prism scanner will not injure or touch his eye.

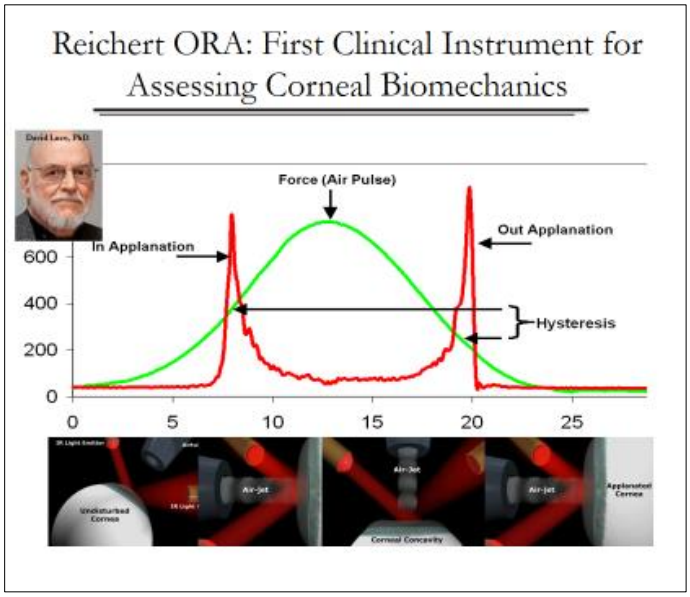


Figure 3. 18: ORA Normal readings and spikes
Source: Reichert Manual 2006

In ORA, the air impulse produced applanates the cornea, to cause the first spike (IN applanation), and the rebound impulse from the cornea causes the second

spike (OUT applanation) (Fig.3.18). The difference between the two readings gives a measure of the hysteresis or elasticity of the cornea. Four different readings can be obtained within one application, namely Corneal Collaborated Intraocular Pressure (IOPcc), Intraocular Pressure (IOPg) mm Hg, Corneal Hysteresis (CH) and Corneal Resistance Factor (CRF).

Corneal Hysteresis is the difference in the inward and outward pressure values obtained during the patented dynamic bi-directional applanation process utilized by the Ocular Response Analyzer. It is a characterization of the cornea's ability to absorb and dissipate energy, which is a function of visco-elastic biomechanical properties of the cornea.

3.1.5 Crosslinking Treatment

CXL followed by RGPs Fitting was done for the final optimisation of visual acuity. This procedure was adopted for all 42 KC cases bilaterally (84 eyes). The UV radiation unit is the prototype Peschke or the Platinum 330 model.

Methodology: The treatment consisted of 1) CXL with UV radiation and 2) Visual rehabilitation with RGPs and other optical devices, spectacles, Intacs, Phakic IOLs.

Figures below illustrate the Steps. Step I: EPI-OFF Crosslinking, was done with the Dresden protocol i.e., the corneal epithelium was removed gently with a Tooke's knife. StepII: The cornea was then pre-soaked in Riboflavin –Dextran 2% for 20mins. The corneal soaking was maintained consistently, with a drop of the R-D solution every 2mins. for the whole 20minutes. Steps III and IV: The CXL Peschke UV machine was then switched on, and UV light 364u wavelength was

delivered over 30 mins. Automatically the UV light switched off at 30minutes as programmed.

Post CXL, RGP contact lenses were fitted for patients to maximise and have best-fit and superior quality of vision.

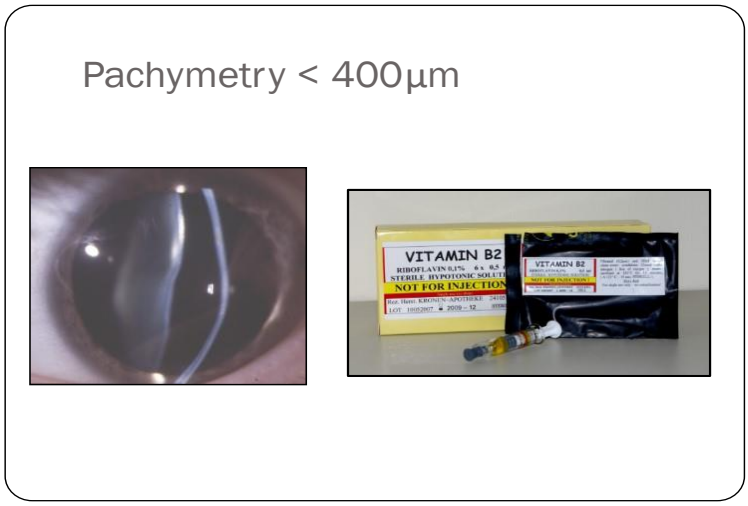


Figure 3. 19:Crosslinking solution of RiboflavinDextran 1-2%, is instilled. Source: JPD

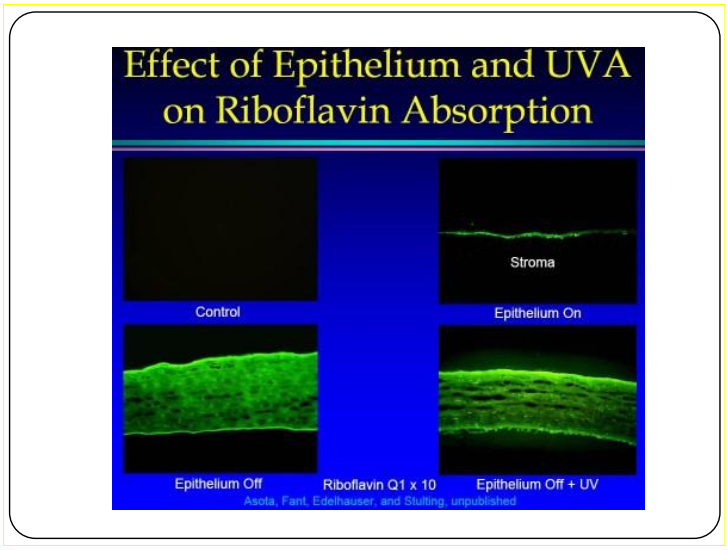


Figure 3. 20: Diffusion of the Riboflavin Dextran solution. Source: Asota Edelhauser Stulting (E Spoerle et al 2005)

Diffusion of the Riboflavin Dextran solution is facilitated by removal of epithelium, and enable the cornea to swell and thicken, as in the Corneal thickness < 400µ.

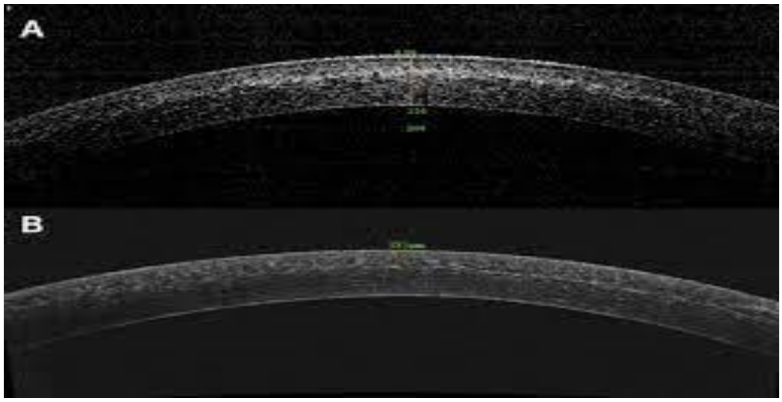


Figure 3. 21: AS-OCT of corneal stromal demarcation line (DL)
Source: Spaeda et al (2011)

AS-OCT of corneal stromal demarcation line at postoperative month 1 in after pulsed-light-accelerated CXL with 30 mW/cm 2 (A) and 45 mW/cm 2 (B) i Spaedea *et al*. (2016) in a review article, stressed the demarcation line (DL) in the stroma as the depth of crosslinking effect.

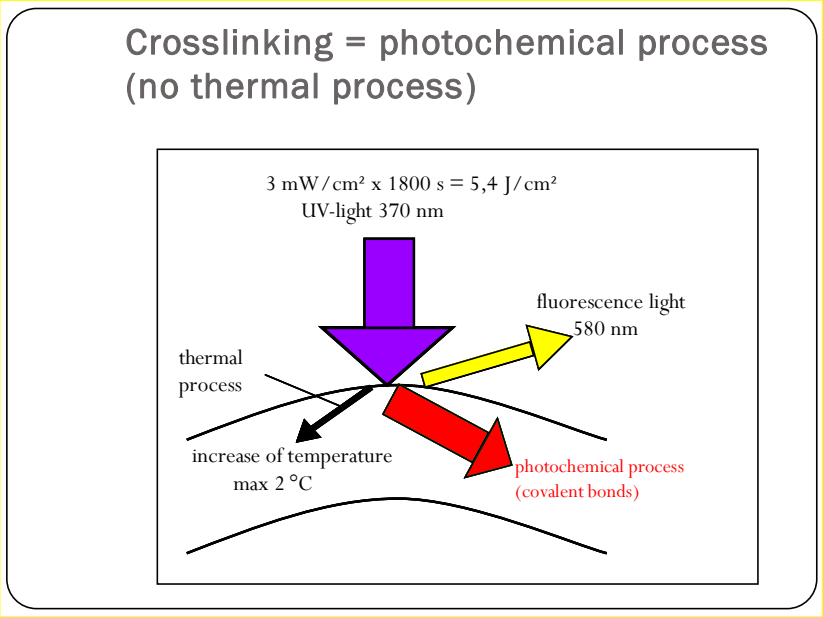


Figure 3. 22 Crosslinking is a photochemical process and not thermal process.
Source: E Spoerle (2005)

The above figure illustrates that CXL is totally a photochemical process, without any thermal (heat) production. It is thus not damaging to the corneal tissue.

CXL PROCEDURE: EPI-OFF TECHNIQUE

The STEPs I-IV are described below.(Figs. 3.23 – 3.26)

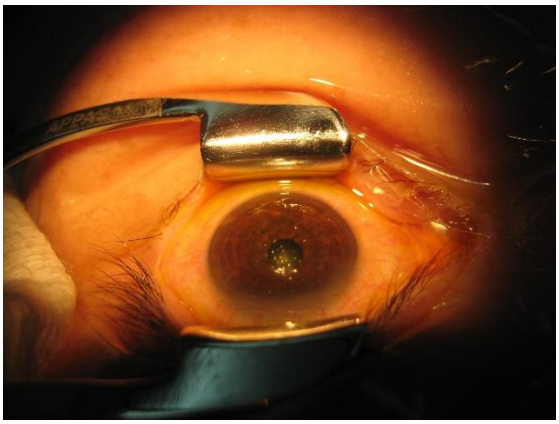


Figure 3. 23:Step I. EPI-OFF Technique. Corneal Epithelium is removed over a diameter of 6-8mm.

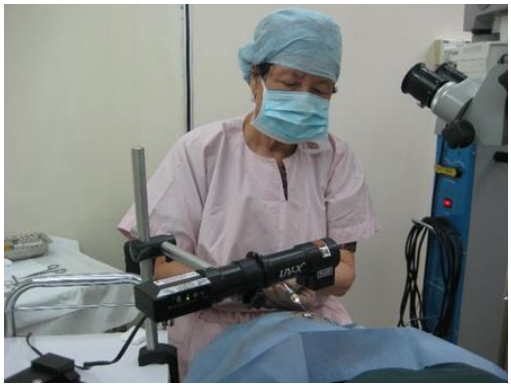


Figure 3. 24: StepII. Pre-sensitization of cornea for crosslinking with Riboflavin-Dextran solution drops at 2min. Intervals for 20minutes.

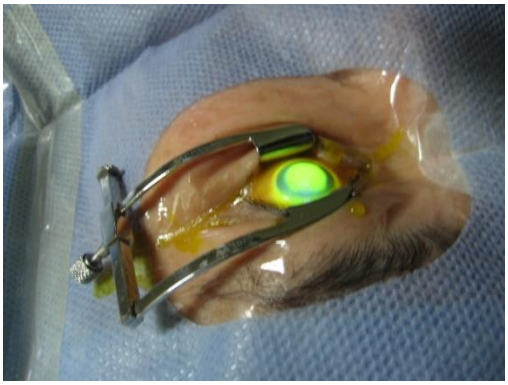


Figure 3. 25: Step III. UV exposure at 364u wavelength. CXL was done with the prototype CXL Peschke UV machine.

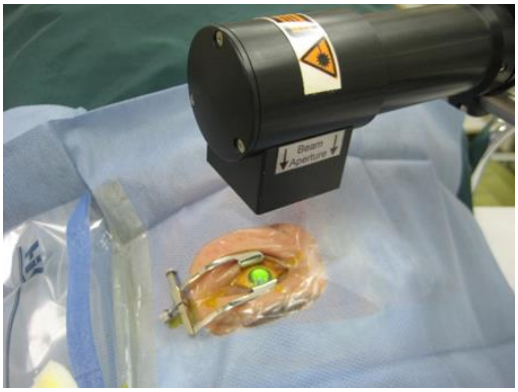


Figure 3. 26: Step IV. The UV Radiation source is adjusted to be 5cms. from the corneal surface. UV exposure was preprogrammed for 30mins. Total energy UV exposue was 90millijoules. . The machine automatically stops at 30mins.

3.1.6 RGP Fitting Protocol For KC

The Trial RGP contact lens was selected based on the Flatter K reading on corneal topography. The Slit Lamp microscope was used to assess the fit of the lens, the centration and movement, to ensure it is neither too tight nor too loose. Flourescein stain was used in cobalt blue illumination, to assess whether there were adequate tears beneath the RGP. This technique was also used for checking the adequacy of the 3-point touch of RGP fitting. In this study, the 3-point touch pattern was the preferred, whenever achievable (Fig.2.8). Whenever, not achievable, apical bearing pattern with a moderate amount of touch on the apex was acceptable. IGEL Boston dK 18 lenses were used in 95% of patients. Over-refraction was done over the fitted RGP on the eye. Residual Astigmatism was measured with the Autorefractometer (Tomey). RGP Visual acuities were recorded and subjective refraction were performed with Snellen Chart at 6 meters distance.

Figure 3. 27:Acceptable lens to cornea fitting relationship.
Source: Leung KK *et al* (1999)

The figure above shows the optimum fit with the central thin pooling of fluorescein stain (2), a free clear zone (1), and a peripheral free edge (3).

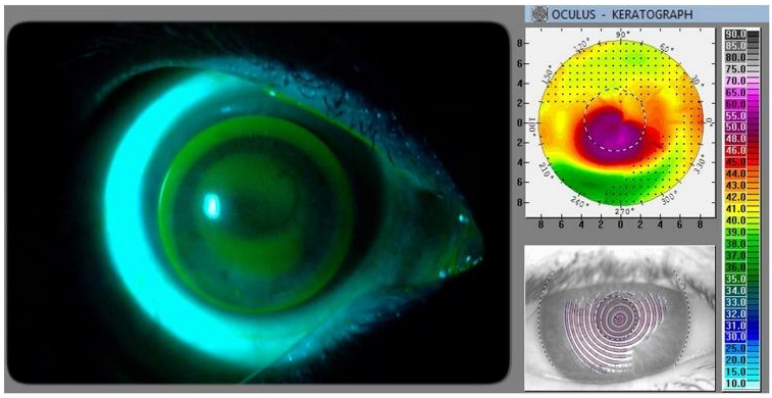


Figure 3. 28:Optimum fit with RGP lens
Source:.Leung et al., (1999)

The central flattening of the KC cone is seen in the upper topogram. The lower videoscope picture of Placido's rings, show how well the RGP produces flattening, and resulting in symmetrical rings. This effectively neutralizes the astigmatism, and results in superior quality of vision. (Leung *et al.*, 1999).

3.1.6.1 Other Treatment Alternatives

In addition to CXL and RGP fitting, where there was RGP intolerance, Semiscleral RGPs, INTACS or Phakic IOLs were considered for maximising management and vision.

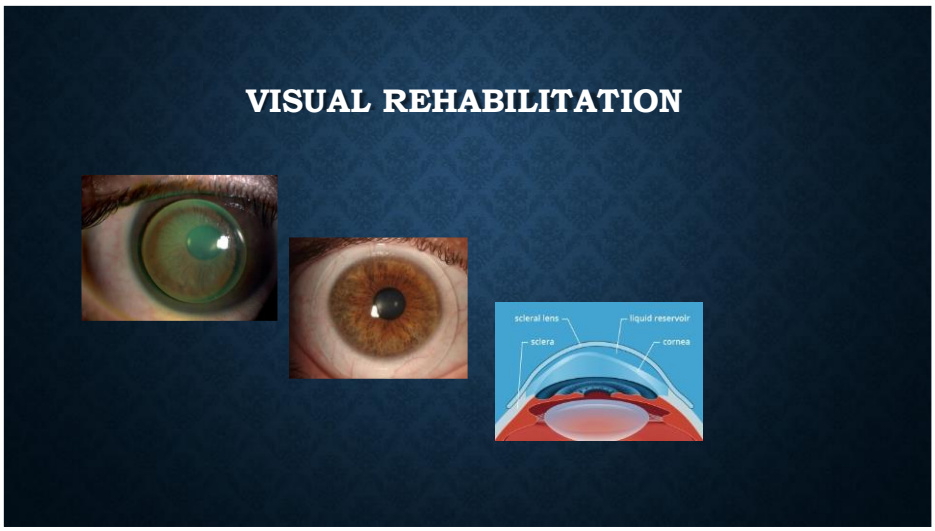


Figure 3. 29:The Semiscleral contact lens
Source: Leung *et al.*, (1999) and JPD

This semiscleral lens is much bigger in diameter than the corneal diameter unlike the corneal RGPs. The range of the semisclerals diameter is 10.3-10.7mm , unlike the RGPs whose diameter range from 8.6 – 8.9mm. The semisclerals have less direct corneal contact, especially at the apex of the conical cornea. As a result of this, patients feel more comfortable and have less corneal irritation or pain. It is thus a better and well-tolerated RGP.

3.1.6.2 Other Treatment Options- Intacs in Keratoconus

Only one case had Intacs Ring (Case Study 6) insertion done in one eye, as his astigmatism was high, and his corneal thickness was >450 u. This thickness is essential to prevent the possibility of corneal perforation in thinner corneas during implantation surgery. The resultant effect was very good. As reported in Case Study 6. Intacs especially also neutralizes the Astigmatism, by flattening the steeper axis. All these different applications aim at optimizing and maximizing PostCXL vision.

Due to the irregularity of the corneal surface and its often highly steep readings, RGP contact lens fitting is often difficult and time consuming. But the RGPs offer the best solution for optimized vision Pre-CXL or Post-CXL, as the anterior regular aspheric surface of the lenses eliminates and compensates for the irregularity and high astigmatism in KC.

Fig 3.30 below is just an illustration of the procedure. The case study report in this study is Case Study 6.

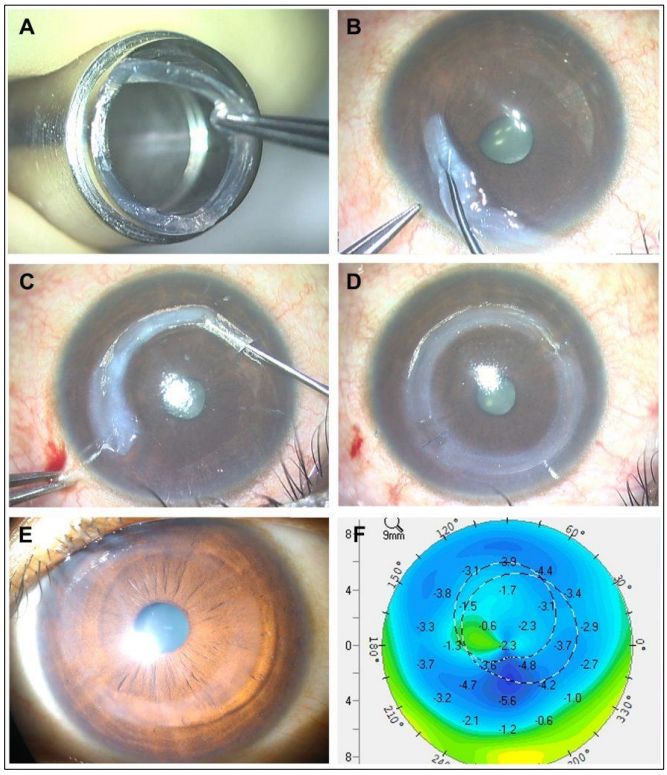


Figure 3. 30:Steps of Intacs Ring insertion

A , B ; Canalisation of the cornea C , D Insertion of the INTACS ring
E: INTACS RING seen from 11o'clock till 6 o'clock flattened cornea.
F:Topogram of the flattened cornea.
Source: https://www.allaboutvision.com/conditions/inserts.htm

3.2 Genes PCR Sanger Sequencing

3.2.1. Ethical Approval and Informed Consent

This study obeys the tenets of the Declaration of Helsinki and was approved by the Institutional Scientific and Research Review Board (IRB) of Universiti Tunku Abdul Rahman, Sungai Long Campus, Selangor.

3.2.2 Study Populations

Participants comprised 42 KC patients who were diagnosed for the first time as KC, 126 unaffected family controls, 97 age-matched normal controls (college students) and 143 young school children (9 -12 yrs old).

3.2.2.1 Inclusion Criteria

i. All patients admitted to the clinical study are as described in chapter 2
ii. Close family members of KC patients who turned up at the clinic for screening after their relatives have been diagnosed as KC
iii. College students and school children who agreed to or had parental permission for KC screening and examination of their eyes

3.2.2.2 Exclusion Criteria

Patients or controls who were unwilling or unable to give informed consent.

3.2.3 Protocol and Methodology

3.2.3.1 Blood Collection

From each participant, finger prick blood was collected onto an FTA card (Whatman, Classic) for transportation to the laboratory at room temperature and

storage at 4^{o}C in the laboratory until required for DNA extraction and analysis (Fig.3.31).

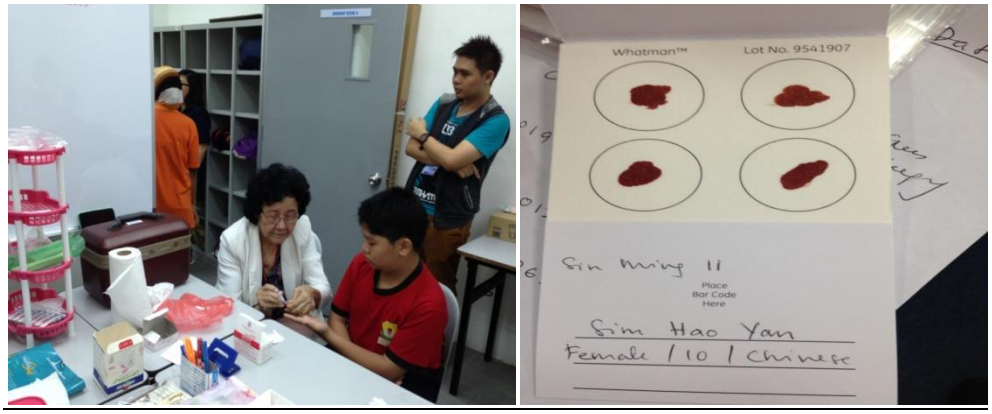


Figure 3. 31: (a) Blood collection and saving on (b) Whatman FTA Cards.

3.2.3.2 DNA Extraction and PCR Amplification

Only 3 candidate genes were selected finally for this study based on other research work. Initially 12 candidate genes were selected, but due to constraint of time, only 3 candidate genes were selected finally. Sanger sequencing of PCR products was done to identify mutations and polymorphism. The candidate genes were namely VSX1Exon3, SOD1Exon2, and COL4A3Exon17. These candidate genes were chosen, based on results from other researchers' findings. (Tanwar et al, 2010; Shetty et al, 2015, Verma et al, 2013; Palliwal et al, 2009; Rabinowitz, 1998 and Udar et al 2006 and Silic Stabuch et al 2015).

3.2.3.3 DNA Extraction by Genomic DNA Purification (Whatman**)**

Genomic DNA purification of dry blood sample, saved on QIA FTA^{TM} Clssic cards.

Materials used: FTA Card (Classic card) Harris Micro punch (3mm). FTA purification Reagent, TE[1] buffer (10mM, Tris-HCL, 0.1mM EDTA, pH 8.0).

PROTOCOL:

i. Collect sample on FTA™ card and dry at room temperature for 1 hour. Cells Are lysed, proteins are denatured and DNA is released, and entangled with the card matrix.

ii. Punch the properly dried card. Cut a 3mm. disc and place in a 1.5ml microcentrifuge PCR tube

iii. Rinse the punch with 500µl of sterile water and vortex 5 times. . Perform 3washes. Cell debris and PCR inhibitors are washed away. Proteins remain bound to the disc.

iv. Remove the water. Centrifuge briefly and remove rinsed water. Use a pipette tip to transfer the washed disc to a clean 0.5 ml microcentrifuge tube.

v. Elute purified DNA. Add 30µl sterile distilled water and incubate in a calibrated heating block or thermal cycler at 95°C for 30mins. After incubating the disc, vortex for 1min. by pulsing the tube 60times to dislodge the DNA from the matrix. During heating DNA is denatured and dissociates from the fibres of the FTA Elute card. Protein and PCR inhibitors remain bound to the matrix.

vi. Centrifuge the tube to recover the condensation from the top of the tube and to pellet the disc. Withdraw the disc from the solution and store eluted DNA.

vii. Perform PCR analysis. Add Master Mix and perform PCR according to the protocol chosen.

3.2.3.4 The Purity and Concentration of Extracted DNA

Samples were determined using a spectrophotometer at 260 nm and 280 nm (PerkinElmer, USA). Stock DNA aliquots were kept at -80°C whereas working aliquots were kept at 4°C for further analysis.

3.2.3.5 PCR Reaction

The Primer Pairs used to amplify each of the 4 coding VSX1 exon variants, are listed below.

PCR reaction (Eppendorf mastercycler, Westbury, NY) was carried out in 20ul reaction mixture set up containing 2ul of 10x PCR buffer with 1.5Mm $MgCl_2$, 200mM dNTP, 0.2mM of each forward (F) and reverse (R) primer, 0.2 U Taq DNA polymerase (Sigma) and 50 ng genomic DNA.

Table 3. 3: Primers used for *VSX1* gene amplification.

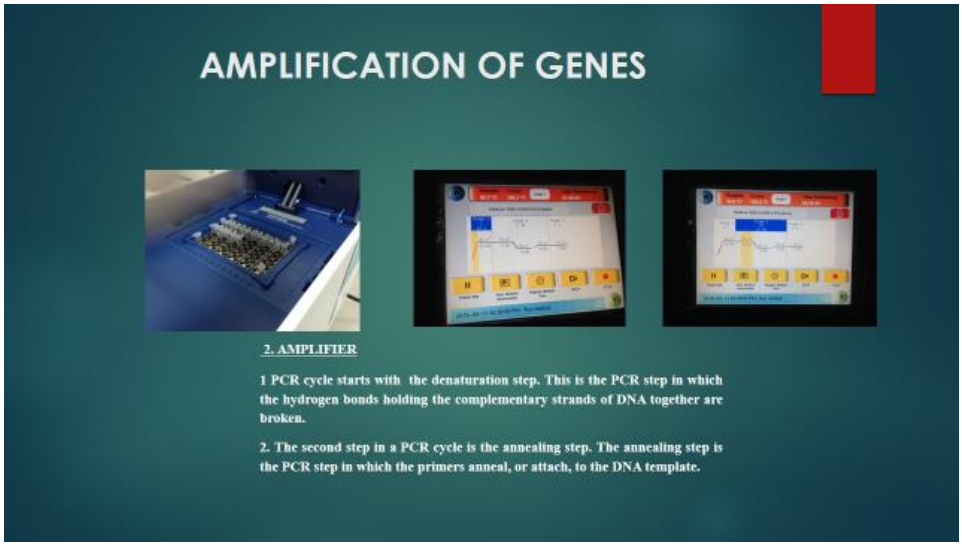
Gene	**Exon**	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**	**Product size (bp)**
VSX1	3	CATTCAGAGGTGGGGTGTT	TCTTGTGGTGCCTTCAGCTA	468
SOD1	2	ACTCCCAAGTCTGGCTGCTGCTT	GGG GTT TTA ACG TTT AGG GGC TA	280
COL4A3	17	ATGAGCGCCCGGACCG	GCCAGGCCCGCGAGTA	110

3.2.3.6 PCR Amplification

Polymerase reaction is a process used in molecular biology to amplify a single copy or piece of DNA, into a few copies, across several orders or magnitude, generating thousands to millions of copies of a DNA sequence.

PCR amplification was carried out in an Eppendorf mastercycler (Westbury, NY) in 20ul reaction mixtures set up containing 2ul of 10x PCR buffer with 1.5Mm MgCl2, 200mM dNTP, 0.2mM of each forward (F) and reverse (R) primer, 0.2 U Taq DNA polymerase (Sigma) and 50 ng genomic DNA.

The genomic DNA underwent initial denaturation for 5mins. at 95'C, followed by 35 cycles, at 94 'C for 1 min, then Exons annealing at 62'C for 1 min, extension at 72'C for 30 seconds and final extension for 5mins. The VSX1 gene coding regions with the Exon3 junction were examined by bidirectional analysis, Forward (F) and Reverse (R).



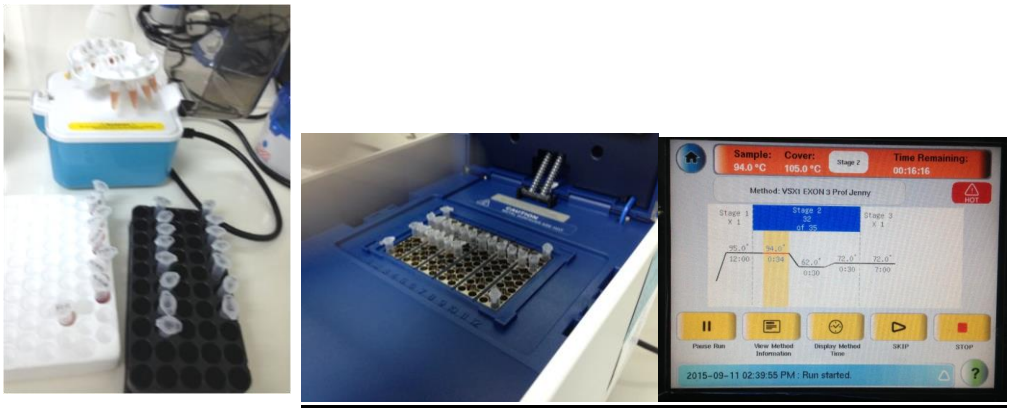


Figure 3. 32: PCR amplification has 4 phases basically. 1. Denaturation 2.Annealing 3. Optimisation 4. Extension

Table 3. 4: PCR amplification details

GENE	Exon	1. Denaturation	2 Denaturation	3. Annealing Optimisation	4. Extension
VSX1	3	95(12)x1	94(1) x35	62 (30)	72(30sec) + 5Mins.
SOD1	2	95(10)x1	94 (15) x35	59 (15)	72(30sec) +5mins
COL4A3	17	95(10)x1	94 (15) x35	49 (15)	72 (30sec) +5mins

Remarks: Temp_.^{0}C (Time_mins) Cycles x1)

The primers were tested against a range of annealing temperatures, before selecting the one that worked for each amplicon.namely 62^{0} C for VSX1, 59^{0} C for SOD1 and 49^{0} C for COL4A3.

3.2.3.7 Gel Electrophoresis

The resulting PCR products (amplicons) were electrophoretically separated on 1.5% w/v pre-stained ethidium bromide agarose gels, at 80V for 30 minutes (or longer depending on the variant and size of the amplicon) in 0.5x tris-borate-EDTA (TBE) buffer. A 1Kb ladder (ThermoFisher) was used to demonstrate amplicon size.

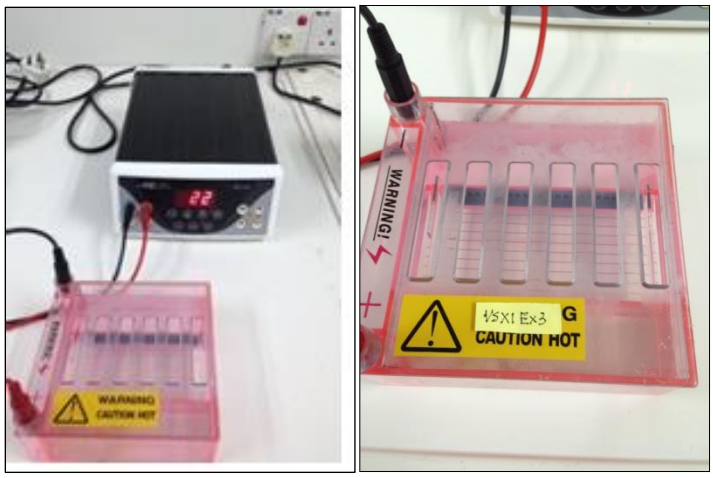


Figure 3. 33: Apparatus for agarose gel electrophoresis

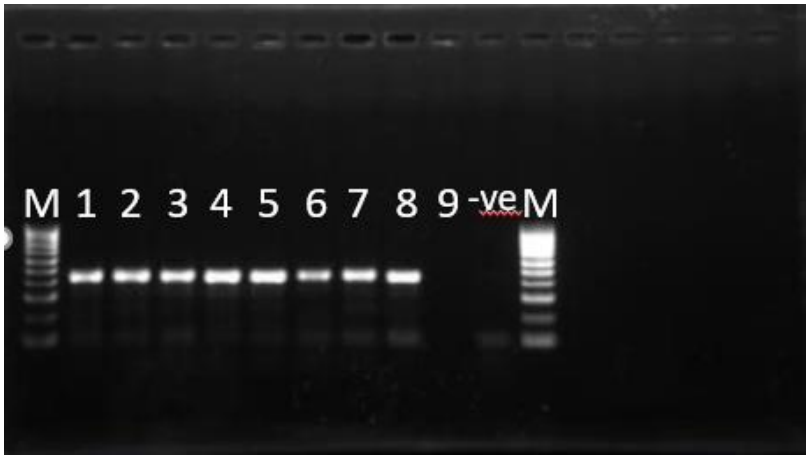


Figure 3. 34: Results of *VSX*1Exon3 gel electrophoresis showing the Ladder(M) and 468bp amplicon band for VSX1. The -ve indicates the control sample (NTC)

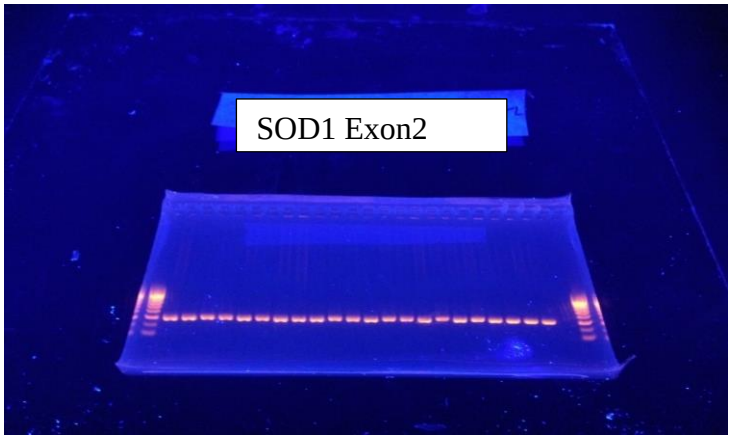


Figure 3. 35:*SOD*1 Exon2 bands at 280bp size . UV lighting

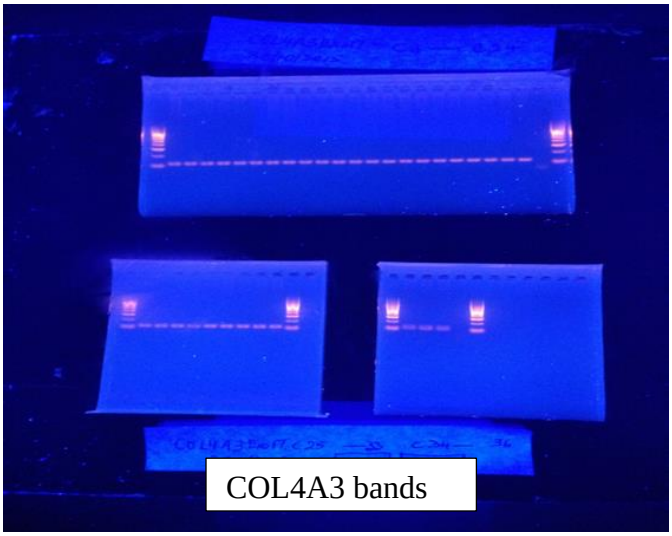


Figure 3. 36:*COL*4A3 bands at 110bp. UV lighting

3.2.3.8 DNA Sequencing

The PCR products were pooled and purified using the gel-elution kit method. The purified PCR products were sent out for commercial Sanger sequencing using Big Dye Terminator ready action mix and analyzed on an ABI-3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequencing data was analyzed using BLAST software and compared against reference sequences for the candidate genes (GenBank: The Gen Bank accession number for VSX1 is NM_014588, SOD1 is NM_0004544, and COL4A3 is NG_011592.1) using an alignment software (Sequencher® ver.5.1.0, Gene Codes Corporation, Ann Arbor, MI, USA). The variations identified were evaluated using Alamut software version 2.1e (Interactive Biosoftware, Rouen, France). The normenclature, location and classification of variations were done based on Alamut output (Apical Science Outsource Company input)

3.3 Exome Sequencing

3.3.1 Ethical Clearance and Informed Consent

Ethical clearance from the Institutional Ethics Review Committee in UTAR was obtained for this study. All samples were outsourced to Bioeasy for analysis and results.

3.3.2 Participant Recruitment

Only 24 participants (5 KC patients and 19 of their family members) were recruited for whole exome sequencing as a pilot study. The KC patients included the proband KC6 who had been tested by PCR-sequencing, together with his family members who were his paternal grandmother, parents and his mother's siblings. KC6 and his family members formed the proband family. Three *VSX1* variants had already been identified in this proband family.

Each participant had to give informed consent and answer a questionnaire. They also underwent comprehensive ocular examinations

3.3.3 Blood collection and Methodology of Exome Sequencing (outsourced)

From each participant, 5 ml of blood was collected by venipuncture for serum extraction and analysis of mutations by whole exome sequencing. The blood samples were collected in EDTA tubes. DNA extraction was done , and at -20^{o} C.

3.3.3.1 Whole Exome Sequencing (WES) Process

All blood samples were processed for DNA extraction, quality and quantity measurement, and library preparation for exome sequencing using the Illumina Mi-seq platform. These procedures were outsourced to a service provider, Bioeasy,

who was also responsible for the assessment of sequence quality, trimming and filtering, alignment, base calling and the identification of sequence variants.

Whole-exome sequencing is a widely used next-generation sequencing (NGS) method that involves sequencing the protein-coding regions of the genome. The human exome represents less than 2% of the genome, but contains ~85% of known disease-related variants,[1] making this method a cost-effective alternative to whole-genome sequencing.

Exome sequencing using exome enrichment can efficiently identify coding variants across a broad range of applications, including population genetics, genetic disease, and cancer studies.

Advantages of Exome Sequencing

Identifies variants across a wide range of applications; achieves comprehensive coverage of coding regions; provides a cost-effective alternative to whole-genome sequencing (4–5 Gb of sequencing per exome compared to ~90 Gb per whole human genome), produces a smaller, more manageable data set for faster, easier data analysis compared to whole-genome approaches.

Exome sequencing is a cost-effective approach when whole-genome sequencing is not practical or necessary. Sequencing only the coding regions of the genome enables researchers to focus their resources on the genes most likely to affect the phenotype, and offers an accessible combination of turnaround time and price. Exome sequencing detects variants in coding exons, with the capability to expand

targeted content to include untranslated regions (UTRs). DNA libraries can be prepared in as little as 1 day and require only 4–5 Gb of sequencing per exome.

Figure below is the exome sequencing workflow. Part1 and part 2.DNA fragmentation, target enrichment & amplification, DNA sequencing

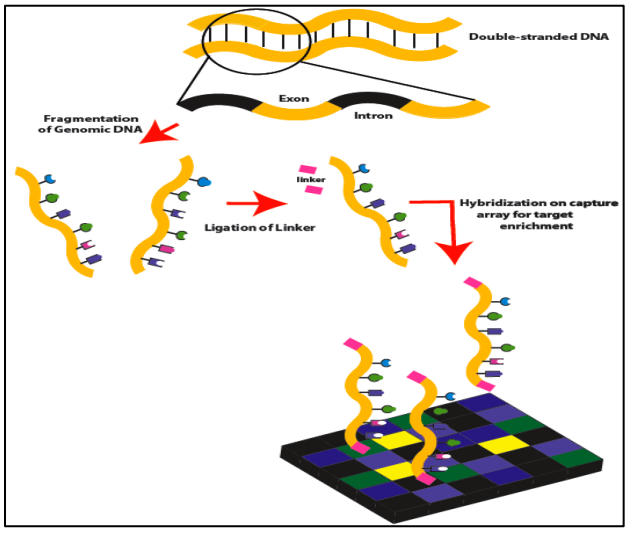


Figure 3. 37: Exome sequencing workflow
Source: https://commons.wikimedia.org/w/index.php?curid=9642877

There are many methods for whole exome sequencing. The most popular platform is provided by Illumina which supplies a DNA Prep with Enrichment sequencing solution that enables fast and flexible library preparation, an integrated DNA extraction protocol for blood samples and optimized performance using bead-based library prep chemistry for reliable and highly reproducible results.

3.3.3.2 Analysis of Whole Exome Sequencing Results

Bioinformatic databases of exome variations and protein prediction software was used to determine the presence and frequency of occurrence of the gene variants in patients and controls.

3.3.3.3 Variant Identification / Variant Calling

The sequencing reads (obtained from the sequencing service provider in FASTQ format) were trimmed and filtered using Fastq-Mcf (version 1.04.636; from ea-utils package; Aronesty. 2011). The reads were trimmed to remove Illumia adapter, trimmed quality score ($Q \leq 20$) and filtered at a minimum length of 70bp. Reads that do not achieve the minimum length were discarded along with its pair. The reads quality was assessed FastQC (version 0.11.2; Andrews. 2010). The trimmed and filtered reads were subjected to alignment via bwa-mem (version 0.7.8-r455; Li & Durbin. 2010) against the hg19 database. Possible PCR and optical duplicates removal were done with Samtools (Li et al. 2009). Next, the GATK (McKenna et al. 2010; DePristo et al. 2011; Auwera et al. 2013) Haplotype caller was used to call variants.

This entire analysis process was streamlined via SeqMule (Guo et al.2015).

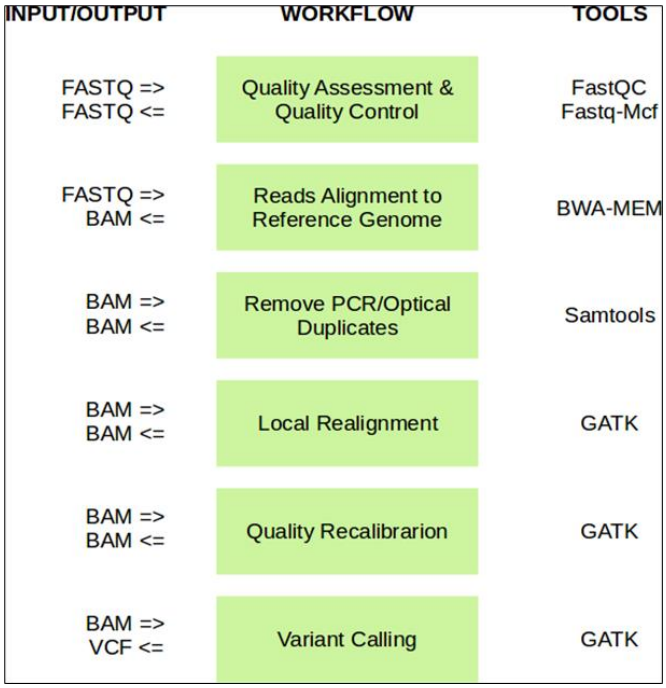


Figure 3. 38: Bioinformatics data analysis process for variant identification using SeqMule

3.3.3.4 Variant Annotation and Counting

The variants called from the analysis of the 24 samples were annotated using SnpEff (version 4.2; Cingolani et al. 2012b). The genomic location of each variant is compared to the Hg19 genes database to see whether the variant was within a known coding region, and the effect of the nucleotide change to the coded protein product. The 24 samples were classified as case or control, and the number of variant genotypes in each group was calculated using SnpSift (version 4.2; Cingolani et al. 2012a). The variant genotype counting was classified by samples with homozygous variant genotype, samples with heterozygous variant genotype and alternative allele.

CHAPTER 4 RESULTS

All statistical analyses were performed using Statistical package for Social Sciences software version 11.5 (SPSS Inc. Chicago, II, USA).

Variables show the overall characteristics or values involved in the thesis. All this data has been compiled and documented in Appendix M.

A variable is a characteristic/ event that has different values. It is measurable. These are linked to the objectives. The areas to be studied in the variables, and can be measured directly or indirectly.However, not all variables are measurable.

4.1 Clinical Keratoconus

4.1.1 Demographic Data

The participants in this study comprised Keratoconus patients (KC), patients' family members (Family) and normal controls who were both adults and school children. The KC study population comprised 42 patients of different ethnic groups (28 Indians, 9 Malays and 5 Chinese). Because the numbers in each group was small, no differentiation by ethnicity was made for statistical analyses. All patients were grouped as KC to be compared with the controls. Nevertheless, it was noted that there were significantly more male (66%) than female KC patients and that most KC patients were adolescents and young adults below 30 years of age (Table 2.3). Although female patients were found to have less severe lesions and were more often affected only in one eye, there was not enough numbers to make this gender difference significant. The proportion of KC was highest in Indians

(66.67%), followed by Malays (21.437%) and Chinese (11.90%). Here again, further studies with larger numbers of patients are needed to assess the significance of these differences.

4.1.2 Risk Factors from the Questionnaire Analysis

The answers from the Questionnaire were analysed to show the difference between KC cases and control groups in the occurrence of risk factors, such as eye rubbing and other allergies that pose an environmental risk to contribute to the multifactorial aetiopathogenesis of KC. The cross-tabulation tables 2.4 and 2.5 show the difference in eye rubbing and severe eye rubbing habits. Corresponding tables for allergy are provided in Tables 2:3- 2.11 for eye itchiness, redness, discharge, eye and nose watering, asthma, food and skin allergy.

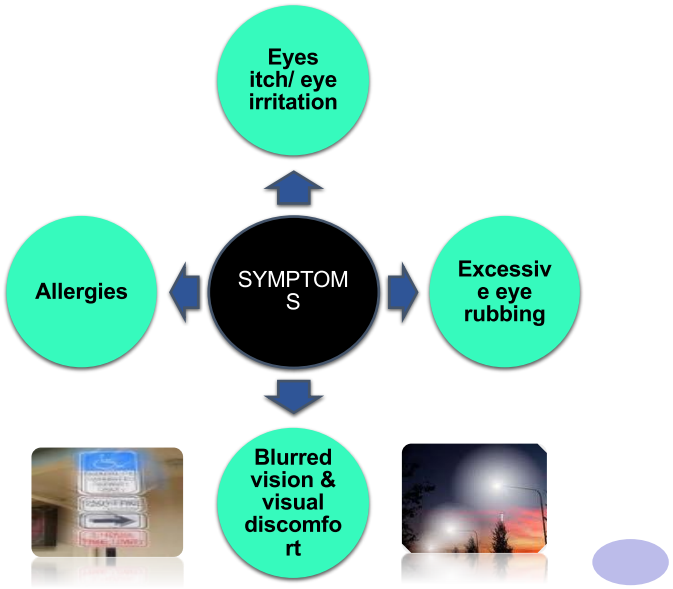


Figure 4. 1: Risk Factors of the Eyes, Eye rubbing, Allergies
Source: www.opt.indiana.edu/lowther/keratoconus.htm

Table 4. 1: Eye rubbing * group

Crosstab

			EYERUB		
			YES	NO	Total
GROUP	KC	Count	33	9	42
		% within GROUP	78.6%	21.4%	100.0%
		% within EYERUB	16.9%	4.1%	10.1%
		% of Total	7.9%	2.2%	10.1%
	FAMILY	Count	63	63	126
		% within GROUP	50.0%	50.0%	100.0%
		% within EYERUB	32.3%	28.5%	30.3%
		% of Total	15.1%	15.1%	30.3%
	NORMAL	Count	56	40	96
		% within GROUP	58.3%	41.7%	100.0%
		% within EYERUB	28.7%	18.1%	23.1%
		% of Total	13.5%	9.6%	23.1%
	SCHOOL CHILDREN	Count	40	104	144
		% within GROUP	27.8%	72.2%	100.0%
		% within EYERUB	20.5%	47.1%	34.6%
		% of Total	9.6%	25.0%	34.6%
Total		Count	195	221	416
		% within GROUP	46.9%	53.1%	100.0%
		% within EYERUB	100.0%	100.0%	100.0%
		% of Total	46.9%	53.1%	100.0%

Tables 4.3 and 4.4 show that in all KC Eye rubbing and Severe Rubbing were more significantly present (78.6%) as a habit than the control Family (50%) and Normal (58.3%) groups. school children (27.8%) have less of these habits.

Table 4. 2: Severe eye rubbing*Group

Crosstab

			ESEVERERUB		
			YES	NO	Total
GROUP	KC	Count	33	9	42
		% within GROUP	78.6%	21.4%	100.0%
		% within ESEVERERUB	28.4%	3.0%	10.1%
		% of Total	7.9%	2.2%	10.1%
	FAMILY	Count	41	85	126
		% within GROUP	32.5%	67.5%	100.0%
		% within ESEVERERUB	35.3%	28.3%	30.3%
		% of Total	9.9%	20.4%	30.3%
	NORMAL	Count	15	81	96
		% within GROUP	15.6%	84.4%	100.0%
		% within ESEVERERUB	12.9%	27.0%	23.1%
		% of Total	3.6%	19.5%	23.1%
	SCHOOL CHILDREN	Count	24	120	144
		% within GROUP	16.7%	83.3%	100.0%
		% within ESEVERERUB	20.7%	40.0%	34.6%
		% of Total	5.8%	28.8%	34.6%
Total		Count	116	300	416
		% within GROUP	27.9%	72.1%	100.0%
		% within ESEVERERUB	100.0%	100.0%	100.0%
		% of Total	27.9%	72.1%	100.0%

From Table 4.4, there seems to be significance in severity of eye rubbing (78.6%) within the KC group, remained high, in comparison with other control groups

Both Family (32.%) and Normal (15.6%) controls had lower proportion within their groups . Only 16.7% of school children had severe rubbing.

Table 4. 3: Eye itchiness * GROUP

Crosstab

			EITCH		
			YES	NO	Total
GROUP	KC	Count	15	27	42
		% within GROUP	35.7%	64.3%	100.0%
		% within EITCH	12.5%	9.1%	10.1%
		% of Total	3.6%	6.5%	10.1%
	FAMILY	Count	53	73	126
		% within GROUP	42.1%	57.9%	100.0%
		% within EITCH	44.2%	24.7%	30.3%
		% of Total	12.7%	17.5%	30.3%
	NORMAL	Count	31	65	96
		% within GROUP	32.3%	67.7%	100.0%
		% within EITCH	25.8%	22.0%	23.1%
		% of Total	7.5%	15.6%	23.1%
	SCHOOL CHILDREN	Count	19	125	144
		% within GROUP	13.2%	86.8%	100.0%
		% within EITCH	15.8%	42.2%	34.6%
		% of Total	4.6%	30.0%	34.6%
Total		Count	120	296	416
		% within GROUP	28.8%	71.2%	100.0%
		% within EITCH	100.0%	100.0%	100.0%
		% of Total	28.8%	71.2%	100.0%

In Table 4.5 The proportion is slightly lower regarding Eye Itchiness for KC (35.7%), Family (42.1%), Normal (32.3%) and in school children (13.3%).

There does not seem to be any relationship or association between eye rubbing or severe eye rubbing and eye itchiness.

Table 4. 4: Group * Skin allergy

Crosstab

			SKINALLERGY		
			YES	NO	Total
GROUP	KC	Count	28	14	42
		% within GROUP	66.7%	33.3%	100.0%
		% within SKINALLERGY	31.5%	4.3%	10.1%
		% of Total	6.7%	3.4%	10.1%
	FAMILY	Count	20	106	126
		% within GROUP	15.9%	84.1%	100.0%
		% within SKINALLERGY	22.5%	32.4%	30.3%
		% of Total	4.8%	25.5%	30.3%
	NORMAL	Count	23	73	96
		% within GROUP	24.0%	76.0%	100.0%
		% within SKINALLERGY	25.8%	22.3%	23.1%
		% of Total	5.5%	17.5%	23.1%
	SCHOOL CHILDREN	Count	18	126	144
		% within GROUP	12.5%	87.5%	100.0%
		% within SKINALLERGY	20.2%	38.5%	34.6%
		% of Total	4.3%	30.3%	34.6%
Total		Count	89	327	416
		% within GROUP	21.4%	78.6%	100.0%
		% within SKINALLERGY	100.0%	100.0%	100.0%
		% of Total	21.4%	78.6%	100.0%

Table 4.6 shows how Skin allergy (Atopy) has a higher risk factor (66.7%) in KC cases in comparison with other controls.15.5% of Family controls, 24.0% of Normal controls and 12.5% of in school children within their groups.

Table 4. 5: Group * Asthma

Crosstab

			ASTHMA		
			YES	NO	Total
GROUP	KC	Count	16	26	42
		% within GROUP	38.1%	61.9%	100.0%
		% within ASTHMA	22.5%	7.5%	10.1%
		% of Total	3.8%	6.3%	10.1%
	FAMILY	Count	24	102	126
		% within GROUP	19.0%	81.0%	100.0%
		% within ASTHMA	33.8%	29.6%	30.3%
		% of Total	5.8%	24.5%	30.3%
	NORMAL	Count	9	87	96
		% within GROUP	9.4%	90.6%	100.0%
		% within ASTHMA	12.7%	25.2%	23.1%
		% of Total	2.2%	20.9%	23.1%
	SCHOOL CHILDREN	Count	21	123	144
		% within GROUP	14.6%	85.4%	100.0%
		% within ASTHMA	29.6%	35.7%	34.6%
		% of Total	5.0%	29.6%	34.6%
Total		Count	71	345	416
		% within GROUP	17.1%	82.9%	100.0%
		% within ASTHMA	100.0%	100.0%	100.0%
		% of Total	17.1%	82.9%	100.0%

Similarly Asthma (38.1%) too, is significantly linked as a risk factor for the KC group, compared with other controls which are lower, Family controls (19.0%), Normal controls (9.4%) and school children 14.6% only..

Tables for Eye Redness, Eye Discharge, Eye Watering, ,Noseblock and Food Allergy (Tables 4.9 – 4.13) are to be found in Appendix.

Table 4. 6:Summary of ocular risk factors in KC and family and Normal controls.

Ocular variable	KC	FAMILY	NORMAL	SCHOOL CHILDREN	P VALUE
EYERUBBING, n (%)	33 (78.6%)	63 (50.0%)	56 (58.3%)	40(27.8%)	0.000
SEVERERUBBING, n (%)	33 (78.6%)	41 (32.5%)	15 (15.6%)	24 (16.7%)	0.000
ITCHINESS, n (%)	15 (35.7%)	53 (42.1%)	31 (32.3%)	19 (13.2%)	0.000
EYEREDNESS, n (%)	11 (26.2%)	25 (19.8%)	12 (12.5%)	25 (17.4%)	0.355
EYEDISCHARGE, n (%)	21 (50%)	13 (10.3%)	4 (4.2%)	33 (22.9%)	0.000
EYEWATERING, n (%)	7 (16.7%)	40 (31.70%)	22 (22.9%)	8 (5.6%)	0.004
NOSEBLOCK, n (%)	8 (19%)	28 (22.2%)	16 (16.7%)	13 (9%)	0.054
SKINALLERGY, n (%)	28 (66.7%)	20 (15.9%)	23 (24%)	18 (12.5%)	0.000
ASTHMA, n (%)	16 (38.1%)	24 (19%)	9 (9.4%)	21(14.6%)	0.001
FOOD ALLERGY, n (%)	15 (35.7%)	13 (10.3%)	17 (17.7%)	0 (0%)	0.000

The Chi square test on results in Table 4.6 shows a significant association between KC and eye rubbing, severity of eye rubbing, eye discharge, skin allergy, asthma, and food allergy. The association with eye rubbing (78.6 %) as a risk factor, in aetiopathogenesis of KC, is not surprising as eye rubbing causes the thinning of keratocytes. This finding is concordant with that reported by many other researchers. Assiri et al., (2005) , found 44.8% of KC cases had the habit of eye rubbing. Rabinowitz et al.,(2003) , also found 83% of his KC patients had this habit, compared to only 58% in his normal controls. Gordon- Shaag et al., (2013) observed that the degree of KC also depended on the period and force (severity) of eye rubbing. More recently, Rabinowitzet al (2019) in a new study, reaffirmed that "mechanical trauma due to rubbing of eyes is significant in keratoconus aetiopathogenesis. "

In this study, eye rubbing seems to be 4.85 times increased risk compared to normal controls. Eye itchiness seems to be 4.39 times more a risk in KC. Perhaps the highly increased risk in these 2 groups, show that Eye Itchiness produces the need for rubbing, and culturally another factor, is Asians tend to rub the eyes very intensely and also with knuckles.

4.1.3 Clinical Examination Results

All KC patients and control groups were examined for clinical features of keratoconus. Only KC patients had the phenotype. All other control groups had normal clear cornea without any of these KC clinical features.

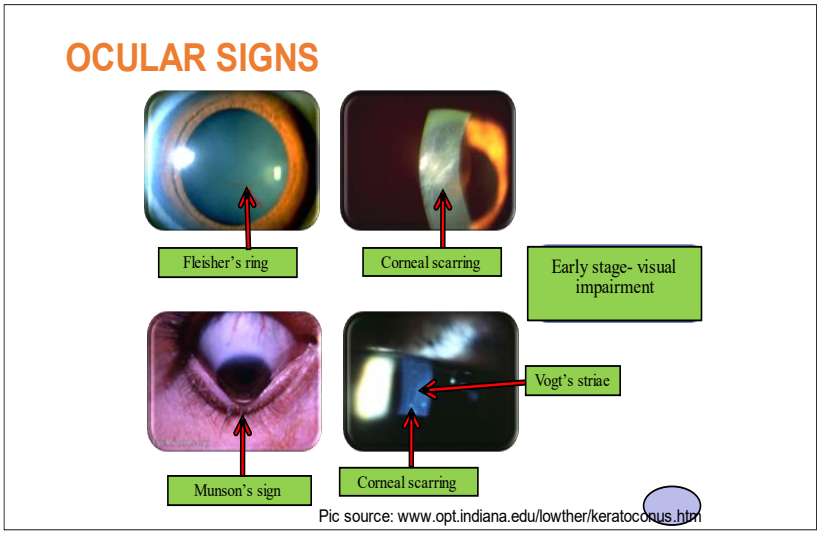


Figure 4. 2: Ocular Signs

Table 4. 7: Slit Lamp Findings of 42 KC Cases

				Munsen sign		Vogt's striae		Hydrops		Scarring	
Pt ID	Race	Age	Sex	OD	OS	OD	OS	OD	OS	OD	OS
KC1	C	32	F	/	/	x	**x**	**x**	**x**	**x**	**x**
KC2	M	28	M	/	/	x	**x**	**x**	**x**	**x**	**x**
KC3	M	17	M	/	/	/	**x**	/	/	/	**x**
KC4	I	32	M	/	/	/	**x**	**x**	**x**	/	**x**
KC5	M	19	F	/	/	**x**	/	**x**	**x**	**x**	**x**
KC6	I	21	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC7	M	16	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC8	I	32	F	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC9	I	29	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC10	M	17	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC11	I	19	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC12	I	22	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC13	I	30	F	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC14	M	26	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**
KC15	I	24	M	/	/	**x**	/	**x**	**x**	**x**	**x**
KC16	M	19	M	/	/	/	/	**x**	**x**	**x**	**x**
KC17	M	23	M	/	/	**x**	**x**	**x**	**x**	**x**	**x**

KC18	C	24	F	/	/	x	x	x	x	x	x
KC19	C	18	M	/	/	x	x	x	x	x	x
KC20	I	18	M	/	/	x	x	x	x	x	x
KC21	M	33	M	/	/	/	/	x	x	x	x
KC22	M	22	F	/	/	/	/	x	x	x	x
KC23	C	20	M	/	/	/	/	x	x	x	x
KC24	C	31	F	/	/	x	x	x	x	x	x
KC25	C	29	M	/	/	x	x	x	x	x	x
KC26	I	28	M	/	/	x	x	/	x	/	x
KC27	I	25	M	/	/	/	/	x	x	/	x
KC28	M	30	F	/	/	/	x	x	x	x	x
KC29	I	33	F	/	/	/	x	x	x	x	x
KC30	I	27	M	/	/	/	x	x	x	x	x
KC31	I	32	F	/	/	/	x	x	x	x	x
KC32	I	28	M	/	/	/	/	x	x	x	x
KC33	I	35	F	/	/	/	x	x	x	x	x
KC34	I	22	F	/	/	x	/	x	x	x	x
KC35	I	18	M	/	/	x	x	x	x	x	x
KC36	I	14	M	/	/	x	x	x	x	x	x
KC37	I	19	M	/	/	x	x	x	x	x	/
KC38	I	24	M	/	/	x	x	x	x	x	x
KC39	M	19	F	/	/	x	x	x	x	x	/
KC40	I	20	M	/	/	x	x	x	x	x	x
KC41	M	33	F	/	/	x	/	x	x	/	x
KC42	I	25	M	/	/	x	x	x	x	/	x

Table 4.9 shows the keratoconus patients main clinical features. Munsen's sign is present in 100% of patients, as the steepness of the corneas (> 45 dioptres) produce the V-shaped lower lid appearance when they look downwards. Vogts lines are present in 20 eyes (4.71%) , which are breaks in Bowman's membrane. Only 4 (7.14 %) eyes had Hydrops. These eyes cleared after CXL treatment .. Seven (8.33%) eyes had scarring , which was not central and did not need Penetrating Keratoplasty. Calculations are based on 42 cases (84 eyes)

4.1.3.1 Classification of KC

The table below shows the grading of keratoconus by K-max values according to Krumeich Classification for Keratoconus.

Table 4. 8: Krumeich Classification for Keratoconus

Characteristics	Krumeich Classification
K-max	
<45	Mild
45-50	Moderate
51-60	Advanced
61-70	Severe

In the RE groups, the group with K_{MAX} 61-70 dioptres were the steepest group, those who presented late or presented with advanced keratoconus with much thinner corneas, and high irregular astigmatism.

In both RE and LE groups (Table 4.15), most patients had a K_{MAX} of 45-60D (80.96 % in the RE and 85.72 % in the LE). Thus, this indicates that according to Krumeich classification, Mild, Moderate, Advanced Keratoconus all clinical types were seen in this study.

4.1.3.2 Video-Topograph-Morphology of KC Corneal Shapes

The normal cornea shape is an aspheric surface, is more curved in the central area, less in the paracentral area, with a shape called “prolate”.

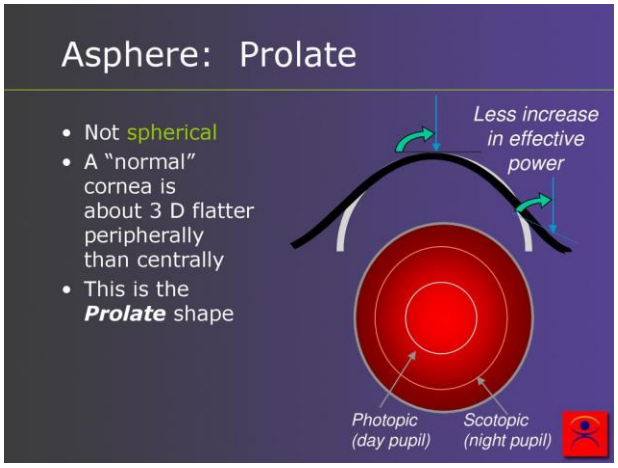


Figure 4. 3:the normal cornea is much flatter at the periphery than at the centre.
Source: https://slideplayer.com/slide/15098852/

The normal cornea has a very low astigmatism, due to the upper lids resting on the cornea vertically, resulting in "With the rule" astigmatism.

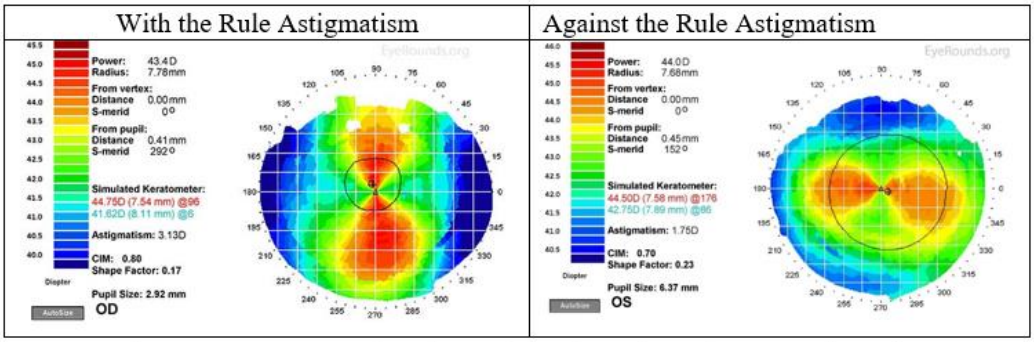


Figure 4. 4: Topography of Normal Regular Astigmatism, RE astigmatism at 90 degrees and LE astigmatism at 180 degrees.
Source: JPD

Table 4. 9: K-max values in KC patients graded with Krumeich Classification

Characteristics	Frequency, n	Percentage (%)	Krumeich Classification
	42	100	
K-max (RE)			
<45	4	9.52	Mild
45-50	18	42.86	Moderate
51-60	16	38.10	Advanced
61-70	4	9.52	Severe
K-max (LE)			
<45	4	9.52	Mild
45-50	20	47.62	Moderate
51-60	16	38.10	Advanced
61-70	2	4.76	Severe

In the RE groups, the group with K_{MAX} 61-70 dioptres were those who presented late or presented with advanced keratoconus with much thinner corneas, and high irregular astigmatism. In this study, it was common to see inequality in advancement and progress of keratoconus between the eyes.

In both RE and LE groups, most patients had a K_{MAX} of 45-60D . 34 in the RE (80.96%) and 36 in the LE (85.72%). Thus, according to Krumeich classification, all grading types, Mild, Moderate, Advanced and Severe Keratoconus clinical types were seen in this study.

4.1.3.3 Comparison and Summary Table with Kmax for All Study Groups

Table 4. 10: Family, Normal, and School Children

		KC	Family	Normal Control	School Children
Kmax Right	Mean	54.46	44.05	44.32	44.75
	Standard Deviation	8.04	1.86	4.76	1.43
Kmax Left	Mean	52.87	44.95	45.30	44.38
	Standard Deviation	6.73	2.75	3.71	1.45
CCT (both eyes)	Mean	475.85	530.40	517.00	562.5
	Standard Deviation	4.898	5.140	5.29	4.89

*All calculations for Mean and Standard Deviation (STD) were averaged with both eye readings taken and calculated collectively

Unlike the steeper readings of the KC cases, the above table shows flatter KMax readings for all control groups as expected. Similarly the corneal thickness was much thinner in KC groups but thicker in all control groups.

4.1.3.4 Ocular Response Analyser (ORA, Reichert)

The KC patients and normal controls provided contrasting spikes and signals in the ORA graph.

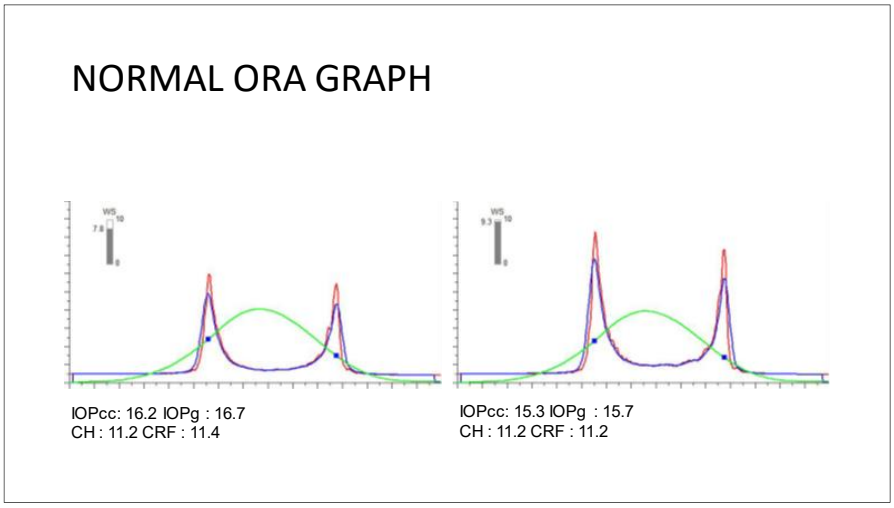


Figure 4. 5: Typical bilateral strong spikes in the normal ORA graph.
Source: JPD

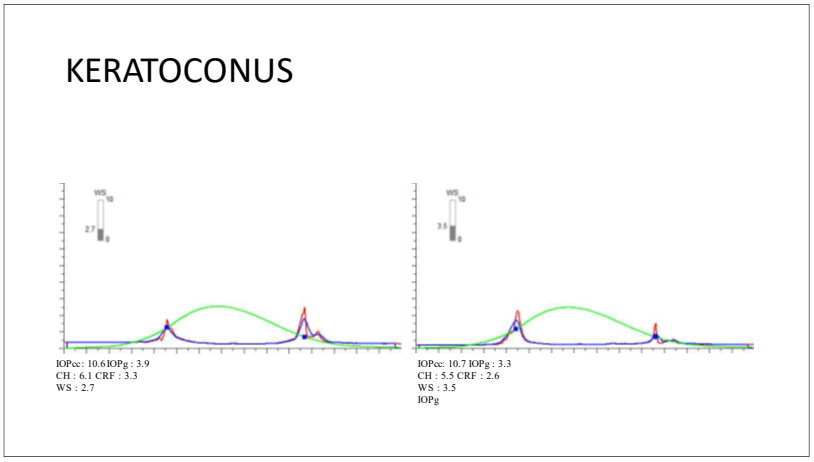


Figure 4. 6: Typical Bilateral diminished spikes in the ORA graph of a KC patient. Normal Curves
Source: JPD

The following tables and figures summarize the ORA readings for the 42 KC patients and their family members who had no KC (Please refer to Appendix for other groups). Each eye had the Mean and STD calculated separately.

Table 4. 11: RE KC Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	42	42	42	42
	Missing	0	0	0	0
Mean		12.32	14.55	8.44	9.20
Median		12.35	14.80	8.90	9.40
Std. Deviation		1.84	1.533	1.41	1.249
Variance		3.39	2.35	1.99	1.56
Range		6.00	5.50	5.90	6.00
Minimum		9.20	11.30	5.20	6.20
Maximum		15.20	16.80	11.10	12.20

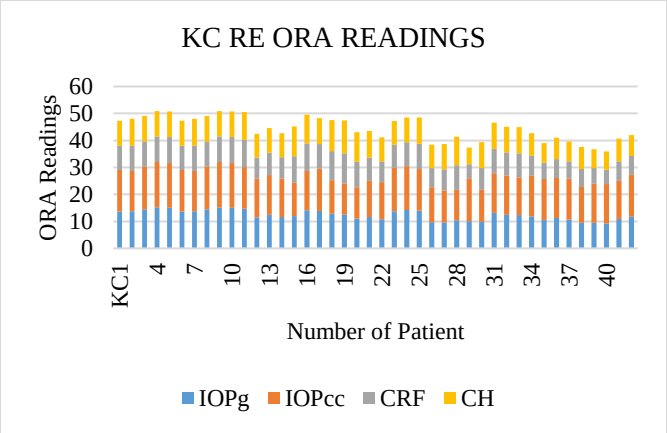


Table 4. 12: LE KC Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	42	42	42	42
	Missing	0	0	0	0
Mean		6.91	10.30	6.49	8.82
Median		6.50	10.05	6.60	9.00
Std. Deviation		1.78	2.366	.93	1.19
Variance		3.15	5.60	.87	1.41
Range		7.40	8.60	4.00	4.40
Minimum		4.10	6.80	4.00	6.50
Maximum		11.50	15.40	8.00	10.90

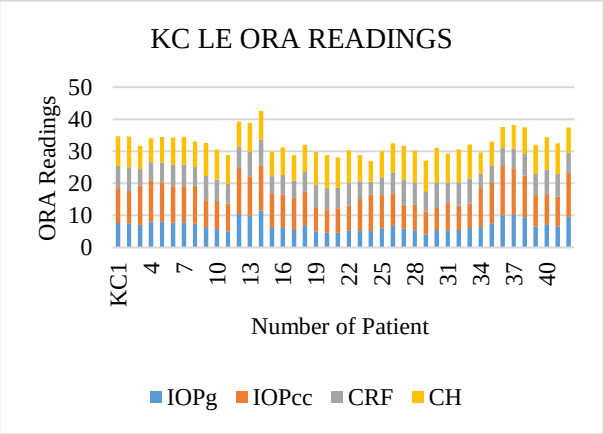


Table 4. 13: RE Family Frequency

	IOPg	IOPcc	CRF	CH
N Valid	126	126	126	126
Missing	0	0	0	0
Mean	14.51	14.22	10.66	11.45
Median	14.45	14.15	10.60	10.30
Std. Deviation	2.89	3.19	1.58	11.09
Variance	8.33	10.19	2.50	123.01
Range	15.30	18.50	8.80	128.60
Minimum	8.30	4.20	6.30	4.40
Maximum	23.60	22.70	15.10	133.00

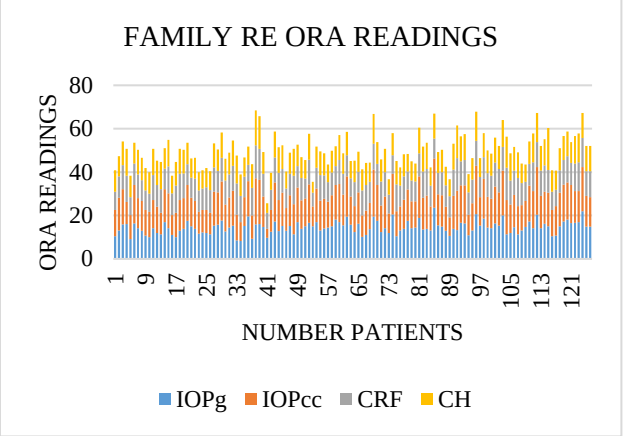


Table 4. 14: LE Family Frequency

	IOPg	IOPcc	CRF	CH
N Valid	126	126	126	126
Missing	0	0	0	0
Mean	14.64	14.68	11.14	10.88
Median	14.50	14.60	11.10	10.70
Std. Deviation	2.72	3.29	1.89	2.20
Variance	7.42	10.79	3.58	4.82
Range	14.60	19.00	11.30	13.90
Minimum	8.70	4.30	6.80	4.20
Maximum	23.30	23.30	18.10	18.10

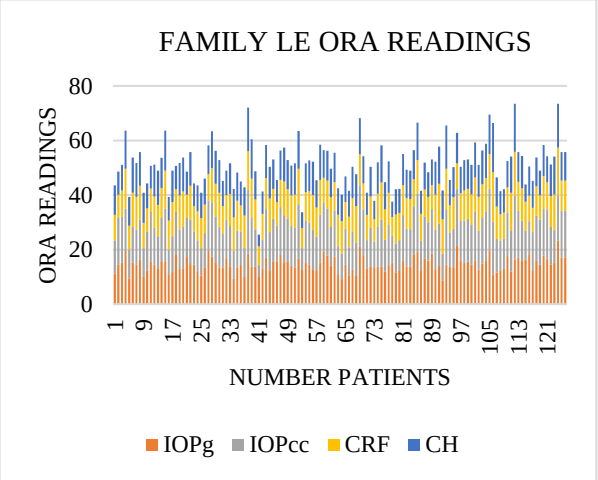


Table 4. 15: RE Normal Control Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	96	96	96	96
	Missing	0	0	0	0
Mean		15.04	15.03	10.78	10.96
Median		14.80	14.65	10.90	11.00
Std. Deviation		3.60	3.05	2.16	1.77
Variance		12.94	9.32	4.66	3.12
Range		18.50	16.90	12.40	10.90
Minimum		4.20	8.30	4.40	5.30
Maximum		22.70	25.20	16.80	16.20

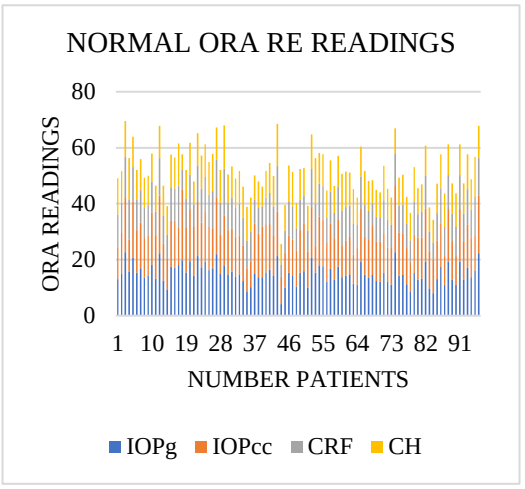


Table 4. 16: LE Normal Control Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	96	96	96	96
	Missing	0	0	0	0
Mean		15.32	14.86	11.15	11.25
Median		15.00	14.90	11.05	11.10
Std. Deviation		3.44	2.94	2.25	1.92
Variance		11.86	8.65	5.08	3.67
Range		19.00	15.70	13.90	12.60
Minimum		4.30	8.30	4.20	5.50
Maximum		23.30	24.00	18.10	18.10

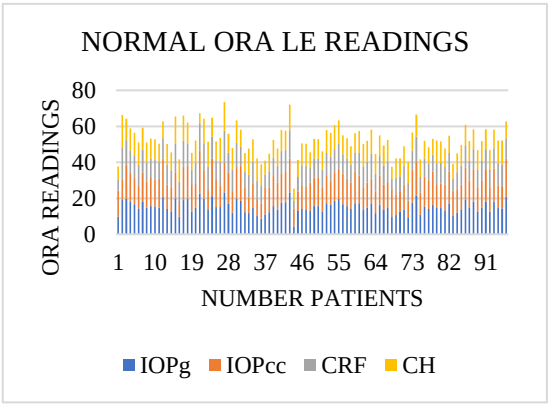


Table 4. 17: RE School Children Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	141	141	141	141
	Missing	0	0	0	0
Mean		17.27	16.61	11.85	11.93
Median		17.30	16.40	11.70	12.0
Std. Deviation		4.898	4.898	4.898	4.898
Minimum		12.40	12.60	9.8	10.0
Maximum		19.10	18.50	13.0	12.20

Table 4. 18: LE School Children Frequency

		IOPg	IOPcc	CRF	CH
N	Valid	141	141	141	141
	Missing	0	0	0	0
Mean		16.70	16.31	12.10	11.92
Median		16.45	16.50	12.40	12.40
Std. Deviation		4.898	4.898	4.898	4.898
Minimum		14.40	13.40	8.90	9.0
Maximum		19.90	18.00	13.50	13.10

The tables above of school children ORA frequencies illustrate and confirm the findings of other research, that Biomechanics of the cornea reduce with age E.Spoerle's (2005).

From the ORA results, the mean, median and interquartile ranges of CH, CRF and CCT were compared for KC patients, and all other controls. Figures below summarizes these differences in box-and whisker plots.

The Box and Whisker plots, in Figure 2.18 to 2.22 illustrate that KC group has the overall lowest reading for biomechanical strength. Figure 2.18 shows the difference between CH and CRF is highest., indicating that the KC cornea is biomechanically weak and therefore has the lowest reading in all the measurements.

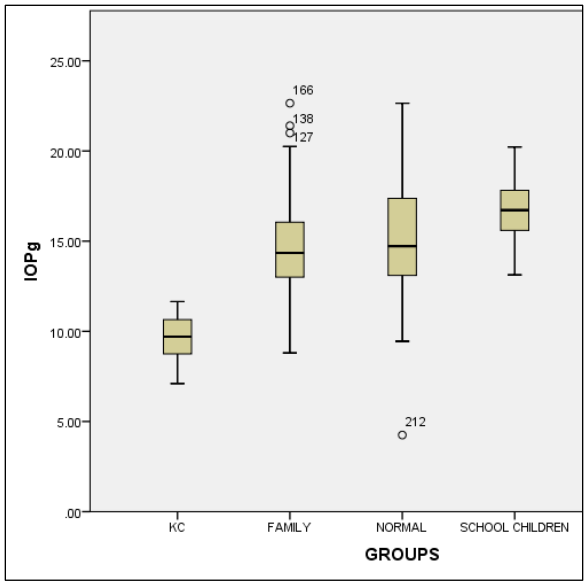


Figure 4. 7: Box-and Whisker plots of IOPg readings in different groups.

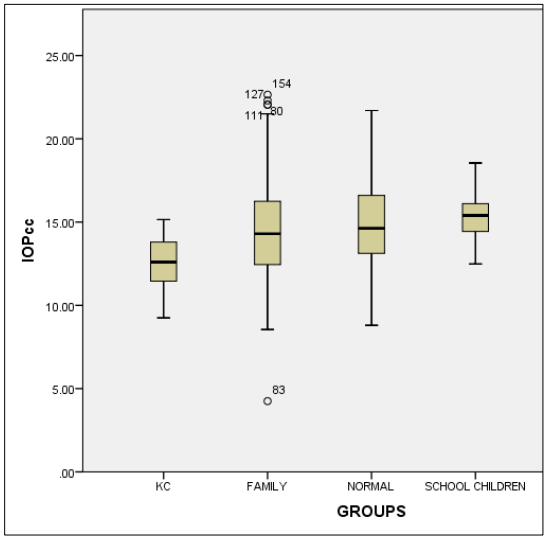


Figure 4. 8: Box-and Whisker plots of IOPcc readings in different groups.

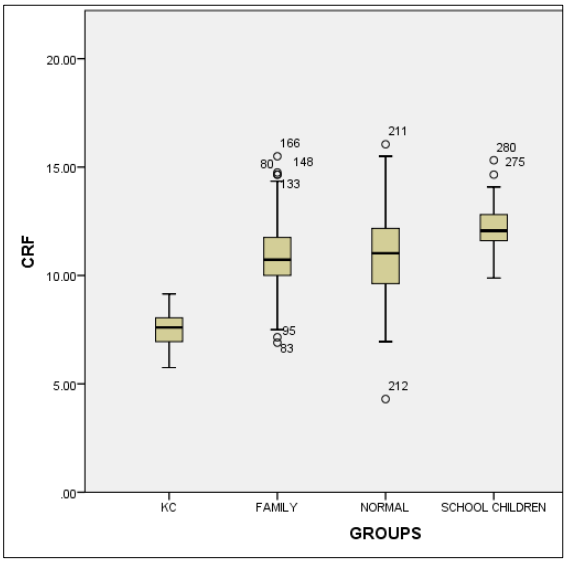


Figure 4. 9: Box-and Whisker plots of CRF readings in different groups.

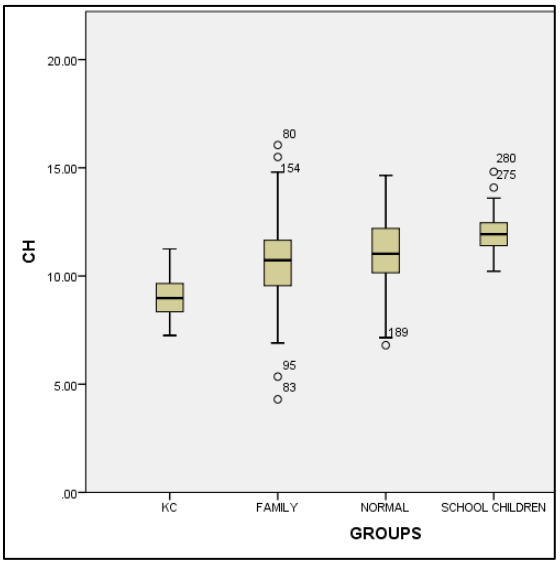


Figure 4. 10: Box-and Whisker plots of CH readings in different groups.

In summary, the analysis of the mean difference between CH and CRF showed that mean CH was higher than mean CRF in the keratoconus group ($p< 00.1$)

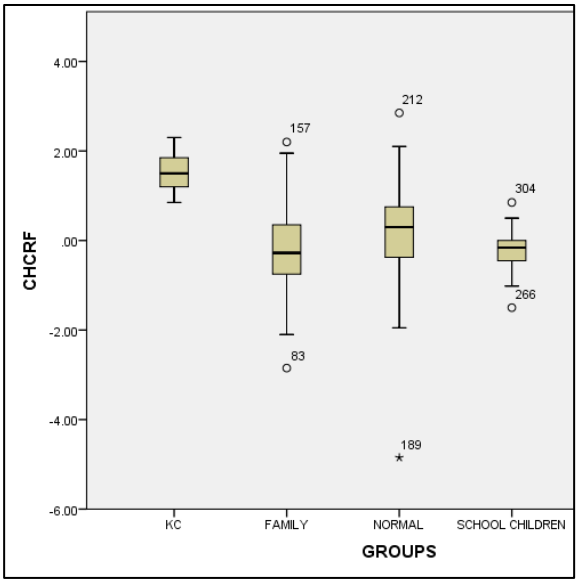


Figure 4. 11: Box-and Whisker plots of CH-CRF readings in different groups

The highest difference between CH and CRF was in the keratoconus group. At present, there is no specific or accepted criteria for categorizing an eye as overtly KC suspect (Schlegel, *et al*., 2008). However, from this study, it was seen that CH and CRF were lower in KC than normal eyes. The difference between CH and CRF, described by Touboul *et al* (2008) was more positive in the KC suspect group than in the normal ($p = 0.006$). In this study, the Mean Central Corneal Thickness (CCT) between the groups was different between the 3 groups. The CH and CRF difference was more positive in the KC cases compared with other control groups. This finding suggests that the natural history of KC (evolution from a normal shape to KC shape) is associated with progressive modifications of the corneal biomechanics reflected in the decrease in CH and CRF and increase in CH-CRF difference (Saad, *et al.*, 2018).

4.1.3.5 Comparison of CH and CRF ORA Readings in KC and Family Controls

Table 4. 19: Comparison of CH and CRF values in KC patients and family controls.

CH	No. (eyes)	Mean mm Hg	Range mmHg	STD mm Hg
KC	84	9.01	6.20 - 12.20	1.219
FAMILY	250	11.3	4.40- 13.30	11.09
CRF				
KC	84	7.47	4.0- 11.1	1.17
FAMILY	250	10.6	6.80-18.0	1.89

CH= corneal hysteresis; CRF= Corneal Resistance Factor; STD= Standard Deviation

Table 4.26 shows that, on the average, KC eyes with corneal thinning yield significantly lower CH ($p=0.002$) and CRF ($p<0.0001$) readings than their family members with no KC. This finding is consistent with that reported by Wollensak and Spoerle (2005) and many other studies listed in Table 2.21. In addition, the difference between CH and CRF was more positive in the KC group ($p = 0.006$), indicating possible improved biomechanical strength after CXL, as reported by Touboul *et al*. (2008).

Table 4. 20: Summary table from similar research for Comparison of ORA.

	Normal				Keratoconus		
	n	CH	CRF		n	CH	CRF
Luce (2005)	339	9.6	NA		60	8.1	NA
Shah et al (2007)	207	10.7± 2.0	NA		93	9.6± 2.2	NA
Ortiz et al (2007)	165	10.8± 1.5	11.0± 1.6		21	7.5± 1.2	6.2± 1.9
Mollan et al (2008)	118	10.6± 2.2	10.0± 2.5		76	8.7± 2.2	6.9± 2.4
Touboul et al (2008)	122	10.3±	11.0		88	8.3	7.6
Hajmi et al	252	10.6± 1.4	10.6± 1.6		172	8.1± 1.4	7.1± 1.6
Present study (JPD)	192	10.96± 1.77	10.87± 2.16		84	9.2± 1.24	8.4± 1.4

Data expressed as mean mm Hg ± SD

4.1.4 Treatment Results

The basic original research done by Wollensak *et al*., (2005) clearly showed that CXL is a chemical photosensitive process. The photochemical process yields immediate stiffening of the collagen fibrils and the stromal tissue. As a result, there is corneal remodelling, regularisation of the corneal surface, and the migration of the apex of the cone towards the central cornea (Spoerle, 2005). Refractive error is also improved by 1-2 diopters. The cornea became thinner and vision was only improved slightly. One long-term study mentioned mild increase of CCT by 5.86u in the third year (Raiskup *et al*., 2012).

4.1.4.1 CXL Followed By RGPs

Rigid Gas Permeable (RGP) Contact lenses were used to maximize visual acuity before as well as after UV Crosslinking. The anterior spherical surface of the RGPs served to correct all astigmatism, whether regular or irregular. The secondary function of the RGPs was also to act as a "splint in a fractured arm", to hold and flatten the conical cornea (molding) for long-term and longer lasting biomechanical support.

In all KC patients, the post-CXL KC corneal abnormalities were stabilized and the progression of KC was halted. Visual acuity was improved by at least 40-60% and was maximized with the wearing of RGPs to 6/12- 6/5. The LogMar equivalents made it to an almost perfect vision of LogMar 0 (6/6). Even with a highly irregular astigmatic cornea of 10 or more dioptres of cylindrical error, satisfactory reduction of astigmatism could be achieved. This stresses the importance and efficacy of fitting the post-CXL eye with RGPs early to give and

maintain a superior quality of vision. It may thus be inferred that “remolding” of the irregular corneal surface results. The data in the following tables and figures describe the visual outcomes before and after CXL and CXL plus RGPs fitting (Tables 2.28 and Figures 2.22).

Table 4. 21: Comparison Of Visual Acuity Before CXL (PreCXL) And After CXL(PostCXL) and After Wearing Special KC RGPs .

	Keratometry (in diopters)		Ultrasonic pachymetry (in µm)		Visual acuity in LogMar (PreCXL)		Visual acuity in LogMar PostCXL		Visual acuity in LogMar PostRGP	
Pt ID	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS
KC1	47.33	55.2	542	489	0.5	1	0.3	0.6	0.1	0.5
KC2	55.59	56.39	435	509	1.5	1.2	1	1	0.2	0.2
KC3	44.47	45.2	449	362	1.8	0.6	pk0.5	0.3	0.3	0.1
KC4	65.6	48	466	488	1	1	1	0.8	0.3	0
KC5	45.32	45.31	430	500	0.6	0.2	0.4	0	0.2	0
KC6	47.23	59.71	555	608	0.9	0.5	0.2	0.4	0.1	0.1
KC7	48.7	55.14	507	476	0.5	1	0.3	0.5	0.2	0.2
KC8	52.2	48.25	482	534	0.9	0.5	0.7	0.2	0	0
KC9	58.81	51.76	523	519	1.5	0.3	1	0.1	0.1	0
KC10	48.61	50.58	328	329	1	1	0.6	0.4	0.1	0.1
KC11	50.1	47.9	365	415	0.8	0.3	0.5	0.2	0.2	0.2
KC12	48.2	44.61	444	481	0.6	0.2	0.4	0.1	0.1	0.1
KC13	47.78	54.24	583	512	0.8	0.9	0.3	0.3	0.1	0.1
KC14	54.37	52.6	456	462	1	1	0.6	0.6	0	0
KC15	50.1	49	472	470	1	1	0.3	0.4	0	0
KC16	50.1	51.2	531	493	1	1	0.6	0.4	0.3	0.3
KC17	54.3	58.2	456	442	0.9	1.5	0.6	0.9	0.2	0.2
KC18	52.9	55.2	521	487	0.9	1	0.6	0.6	0.2	0.2
KC19	56.5	54.4	512	488	1	1	0.4	0.5	0.2	0.2
KC20	47.25	43.25	529	514	0	0.3	0	0.3	0	0.2
KC21	61.5	60.3	390	440	0.5	0.7	0.4	0.5	0.3	0.5
KC22	58.7	56.8	502	411	1	1	0.6	0.6	0.2	0.3
KC23	53.8	50.1	489	529	1.5	0.3	0.7	0.2	0.4	0.2
KC24	47.8	58	496	307	0.6	1	0.4	0.6	0.2	0.4
KC25	55.3	58.31	425	418	0.5	1.5	0.3	0.9	0.2	0.4
KC26	78.5	65	412	372	1.5	0.5	0.9	0.4	0.5	0.4
KC27	49.43	43.6	485	450	1	0.3	0.6	0.1	0	0
KC28	54.75	54.75	515	519	0.6	0.9	0.3	0.5	0.2	0.2
KC29	65.78	46.59	393	536	1.5	1	0.9	0.5	0.3	0.1

KC30	46.51	51.18	515	455	0	0.6	0	0.4	0.2	0.2
KC31	47.15	50	548	565	1	0.4	0.6	0.2	0.3	0.1
KC32	52.1	45.8	432	465	1	1	0.4	0.4	0.2	0.2
KC33	45	45.25	581	549	0.2	1.5	0.2	0.9	0.1	0.3
KC34	48.2	51.5	410	416	0.9	0.6	0.4	0.4	0	0.2
KC35	55.7	49.4	449	549	0.9	0.3	0.6	0.2	0.1	0
KC36	48.4	44.9	502	573	0.8	0.2	0.5	0	0	0
KC37	45.8	50.7	574	522	0.2	0.7	0.2	0.2	0	0.1
KC38	45.98	45.19	489	471	1	0.7	0.4	0.3	0.1	0.1
KC39	45.6	44.8	508	518	0.6	0	0.3	0	0	0
KC40	44.1	43.5	494	489	0.8	0.8	0.3	0.3	0	0
KC41	48.73	63.07	453	414	0.8	1.5	0.6	1	0.1	0.4
KC42	48.7	59.6	486	327	0.3	1	0.2	0.8	0	0.4

Table 4. 22: Kmax descriptive statistics before and after CXL

		Before CXL	After CXL	Validation (Wilcoxon P.)
Kmax Right	Mean	54.6103	51.2022	
	Median	53.5	49.465	
	Standard Deviation	7.3226	6.2797	0.000
Kmax Left	Mean	53.1087	51.3325	
	Median	51.8	50.64	
	Standard Deviation	6.5294	5.3047	0.000
ASTIG Right	Mean	6.0747	4.3297	
	Median	5.365	3.72	
	Standard Deviation	3.1613	3.2708	0.000
ASTIG Left	Mean	5.5815	3.9352	
	Median	5.4	3.375	
	Standard Deviation	2.9853	2.7796	0.000
CCT Right	Mean	479.5254	485.0508	
	Median	489	489	
	Standard Deviation	57.1614	62.3219	0.328
CCT Left	Mean	471.5085	469.339	
	Median	481	477	
	Standard Deviation	68.3269	55.9198	0.748

Spherical Equivalent Right	Mean	-6.9417
	Median	-6
	Standard Deviation	4.9643
	Mean	-6.7292
Spherical Equivalent Left	Median	-6
	Standard Deviation	4.895

POSTCXL FOLLOWUP SCHEDULE:

However, though patients were followed up on a PostCXL schedule, of the First day, 1 week, 2 Weeks, 1month, 3months, 6 months, and one year., the patients did not always keep their postCXL review schedule, once they felt they could see better, and their contact lenses already maximised their vision.

Average followup, was perhaps annually, once their vision was maximised.

They personally returned for review whenever, they had any complications like eye irritation, or RGPs broke, or had to be cleaned. Thus, all recorded data was based on Maximum corrected visual acuity, at their last visit, as is seen in the Figures: 4.11-4. 12

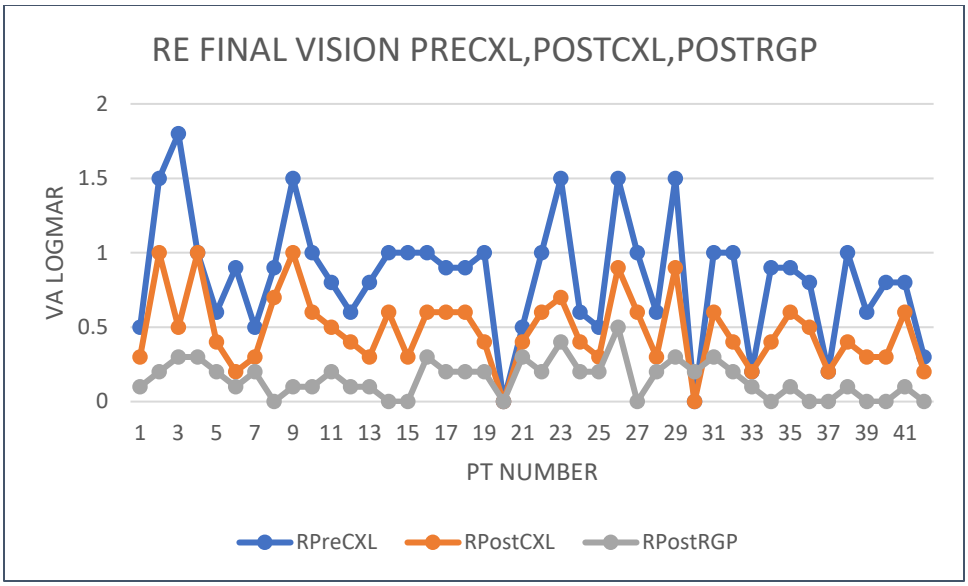


Figure 4. 12: Improvement in RE visual acuity after CXL and RGP fitting.

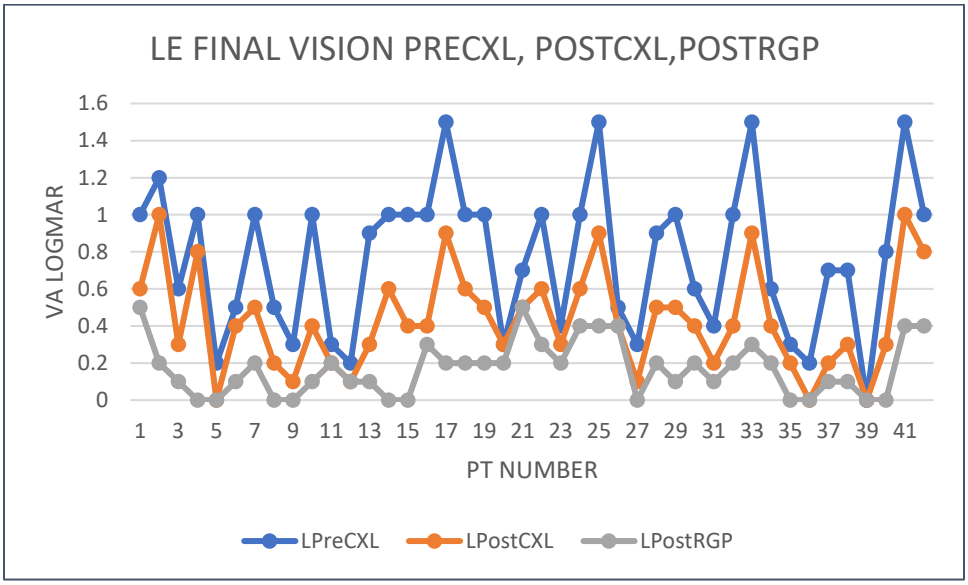


Figure 4. 13 LE visual acuity after CXL and RGP fitting

Figures 4.12 and 4.13 illustrate the improvement, by Crosslinking in treatment of Keratoconus. It is very clear that this is due to reduction in steepness of the cornea, directly reducing the Myopic Astigmatism. The average reduction of Myopia ranged from 1.00-5.00 dioptres, With the reduction in these refractive modalities,

there is improved visual acuity. The two figures (4.12-4.13) above, show the blue line, the initial PreCXL vision. This is further improved postCXL as the brown line indicates.Finally all this vision is maximized and optimized, as the Grey line shows,

An example would be the timeline of the PostCXL measurements,as patients can come initially , regularly PostCXL. An average regiment schedule would be: 1st Day, 1 week, 1 month, 3 months, 6 months upto one year. Then reschedule as needs. Annual checkup to assess vision and correction with RGPs. In this study, KC patients have had their best visual acuity last taken and recorded.

All the Visual Acuity , PreCXL, PostCXL and PostRGP thus have been compiled, and converted to a Graphic presentation Fig. 4.12 and 4.13. to illustrate the improvement in Visual Acuity.

4.1.5 Case Studies of KC

The following four case studies illustrate mild to advanced clinical keratoconus features. These cases also illustrate the variability in amount of biomechanical strength gained in response to CXL treatment in each case. Also the outcome in Vision gained is also variable, but improved and eventually maximised with RGPs.

4.1.5.1 Case Study1: Unilateral KC In 15 Yr-Old Male

Case C1, a 15-year-old boy presented with a history of blurred vision in both eyes, more in the LE. On examination, he had the readings below:

RVA 6/18	LE CF 3/60
45.0 DS	58 DS
ASTIG. -2.0DS	-6.0DS
Vision 6/9 with RGP	Vision 6/12 with RGP

He had CXL done on both eyes, as it is usual for the other eye to progress. Post-CXL he had to only wear RGP contact lens in the LE. His visual acuity was RE 6/9 and LE 6/12 with RGP. However, he was non-complaint and gave up RGP wearing, using his RE vision to enable him to work binocularly.

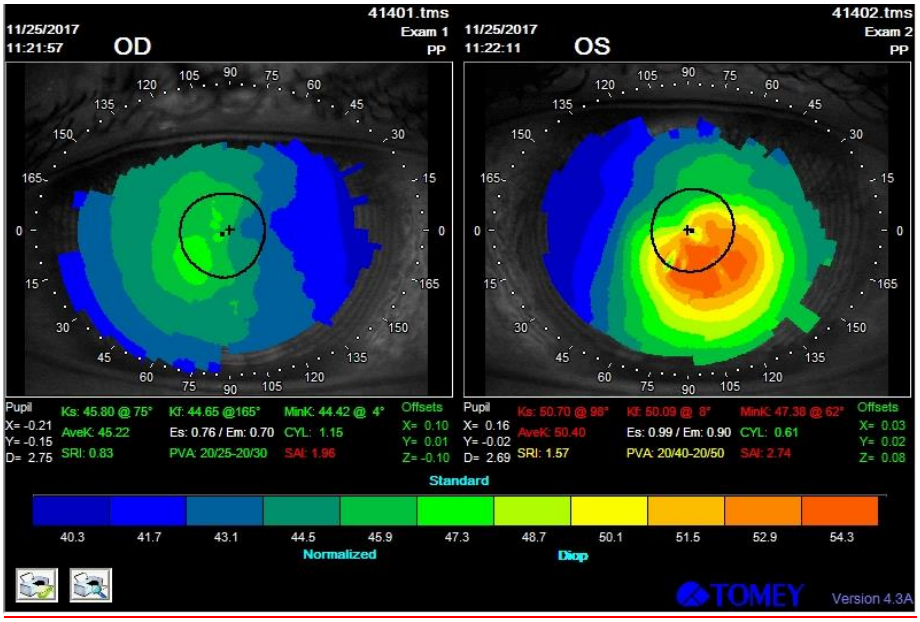


Figure 4. 14: Topography (RE) Post CXL Cornea and (LE) Post CXL with remnant astigmatism.

Source: JPD

PostCXL History (7years)

It is already since he crosslinked his eyes. His Cornea has been maintained from progression with the wearing of the RGP on the RE. His LE cornea has increased in steepness.

4.1.5.2 Case Study 2: CXL And Post-CXL RGP Fitting In Early KC

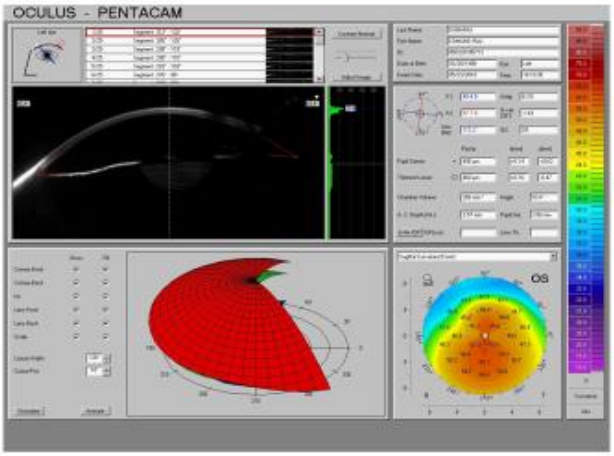


Figure 4. 15: Advanced Keratoconus
source: JPD

Case C2 presented at the age of 20 yrs with poor visual acuity of RE6/24 and LECF3/60. His best spectacle corrected vision was RE6/15 and LE 6/24. With RGP correction, his vision improved to RE 6/7.5 and LE 6/12. After CXL treatment the high astigmatism in both eyes were reduced and the best vision with RGPs became RE6/6 and LE 6/9. The corneal thickness has remained the same.

PostCXL History: (12 years) After 10 years of wearing RGPs, Case C2 changed to Semisclerals which allowed him to have clearer and sharper vision, and longer (12-16) hours of comfortable wearing of Semisclerals.

4.1.5.3 Case Study 3: Moderate KC. Phakic IOL implantation post-CXL after stabilization of KC progression

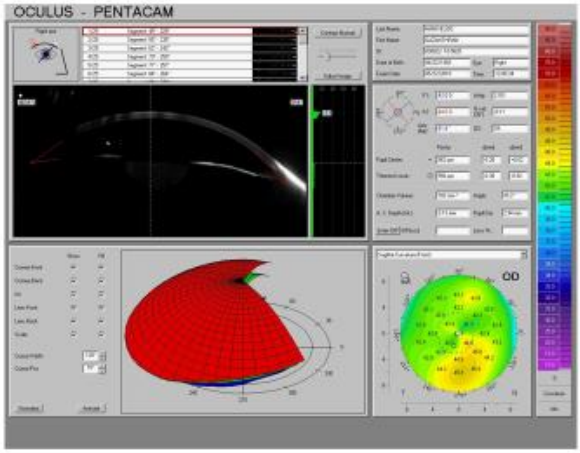


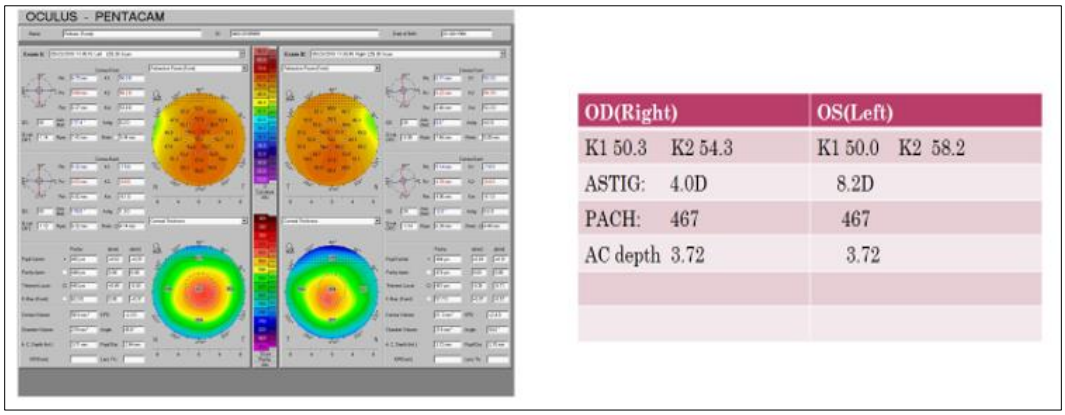
Figure 4. 16: Moderate Keratoconus.
Source: JPD

Case C3 presented at the age of 25 years with poor visual acuity of RE6/24 and LE CF3/60. His best spectacle- corrected vision was RE6/24 and LE CF 2/60. After RGP correction his vision became RE6/9 and LE 6/18. After CXL treatment the high astigmatism in both eyes were reduced and the best vision with RGPs was RE6/6 and LE 6/9

After 5years of wearing RGPs, Case C3 still has the same vision and the same K readings, as he wears his RGPs regularly. He, however, has slight discomfort in his LE due to the steep fit of his contact lenses. After CXL, astigmatism was reduced in both his eyes, and with RGPs he was able to see 6/6 binocularly, although the vision in his LE could be best corrected to 6/9 only, due to the residual higher myopic astigmatism. Two years Post-CXL, C3 decided to have Phakic IOL implantation in both eyes, as he had no more KC progression (the

refractive power remained at -4.00DS and no further corneal thinning occurred). Since then, he has maintained good vision of 6/6 in both eyes with the Phakic IOLs

4.1.5.4 Case Study 4: Intolerance of RGPs after CXL



OD(Right)	OS(Left)
K1 50.3 K2 54.3	K1 50.0 K2 58.2
ASTIG: 4.0D	8.2D
PACH: 467	467
AC depth 3.72	3.72

Figure 4. 17: Unequal progression of topographic picture resulting in high difference inastigmatism. Source: JPD

Case 4 was a 21-year-old male who presented with poor vision. He was however, correctable with RGPs to RE6/6 and LE 6/12. Binocularly he achieved 6/6 due to his better RE. However, he could not adapt to the RGPs, and eventually wore them only part time, alternating with using his spectacles. This however, gave him only 80% vision whenever, he wore RGPs. However, the intermittent wearing of RGP did not contribute to any further KC progression.

4.1.5.5 Case Study 5: Unusual case of KC recurrence 15 years after penetrating keratoplasty

(This Case was excluded from statistical analysis, by exclusion criteria)

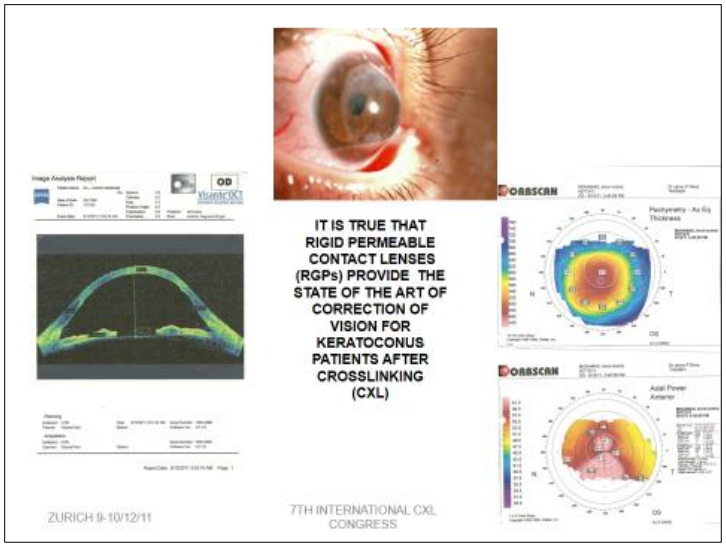
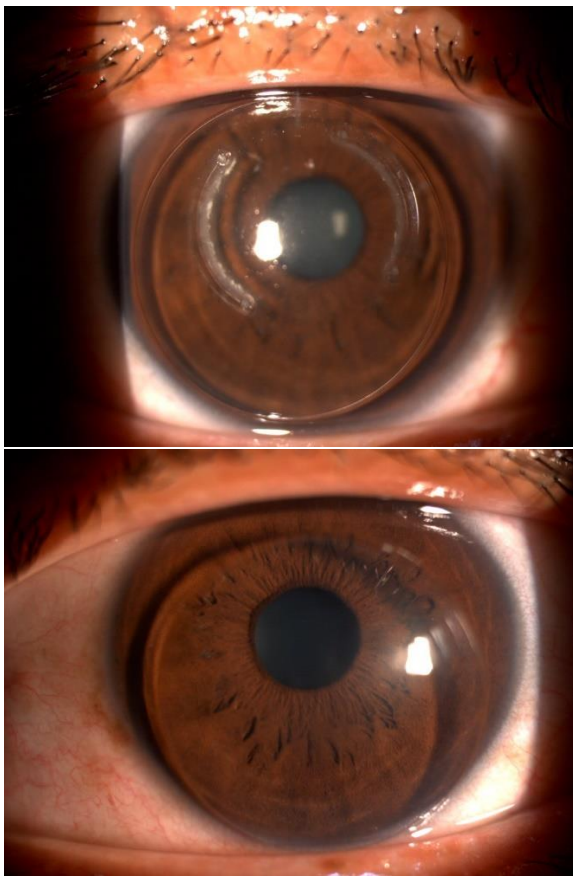


Figure 4. 18:Slit lamp view of protruding cornea due to KC recurrence, and similar documentation in the OCT and topographic mapping.
Source: JPD

Case C5 was a 50- year-old Malay male presented with very blurred vision in both eyes for about a year. He had, had KC and was treated with Penetrating Keratoplasty in Australia 15 years ago. After the surgery, he could see much better with the help of spectacles but did not have perfect vision. However, over the years, his vision deteriorated and became very bad in the past two years, particularly in his right eye. Together with presbyopia, it became impossible for him to read as well.

On examination, it was clear he had a recurrence of KC. Although his corneal thickness was still >500u, he was advised to have CXL to stabilize and stop any further progression of KC. After CXL, his vision was improved especially after he started to wear RGPs. He had very steep corneas with 72 dioptres steeper axis in the right eye and 56 dioptres in the left eye. His RE RGP contact lens fitted well and he could see 6/15 on Snellen's chart. His RE posed a problem because of excessive steepness, and though he could see 6/12p when the RGP was in more central cornea, the RGP could not stay in position, because of the very high astigmatism. Reading and near visibility was solved by just wearing reading spectacles. With CXL, his RE vision improved as the astigmatism was reduced tremendously. The left eye, however, could not tolerate the small RGP lens, and was eventually fitted with a semi scleral lens.

4.1.5.6: Case Study 6: Intacs In RE and RGP In LE



RE: INTACS inserted into corneal stroma to correct steeper axis 65^{o} with high astigmatism.. CXL was done to maximise VA.

LE: RGP FITTED ON CORNEA to correct POSTCXL remnant astigmatism 1.00 Dioptre

Figure 4. 19: Intacs Patient Details
Source: JPD

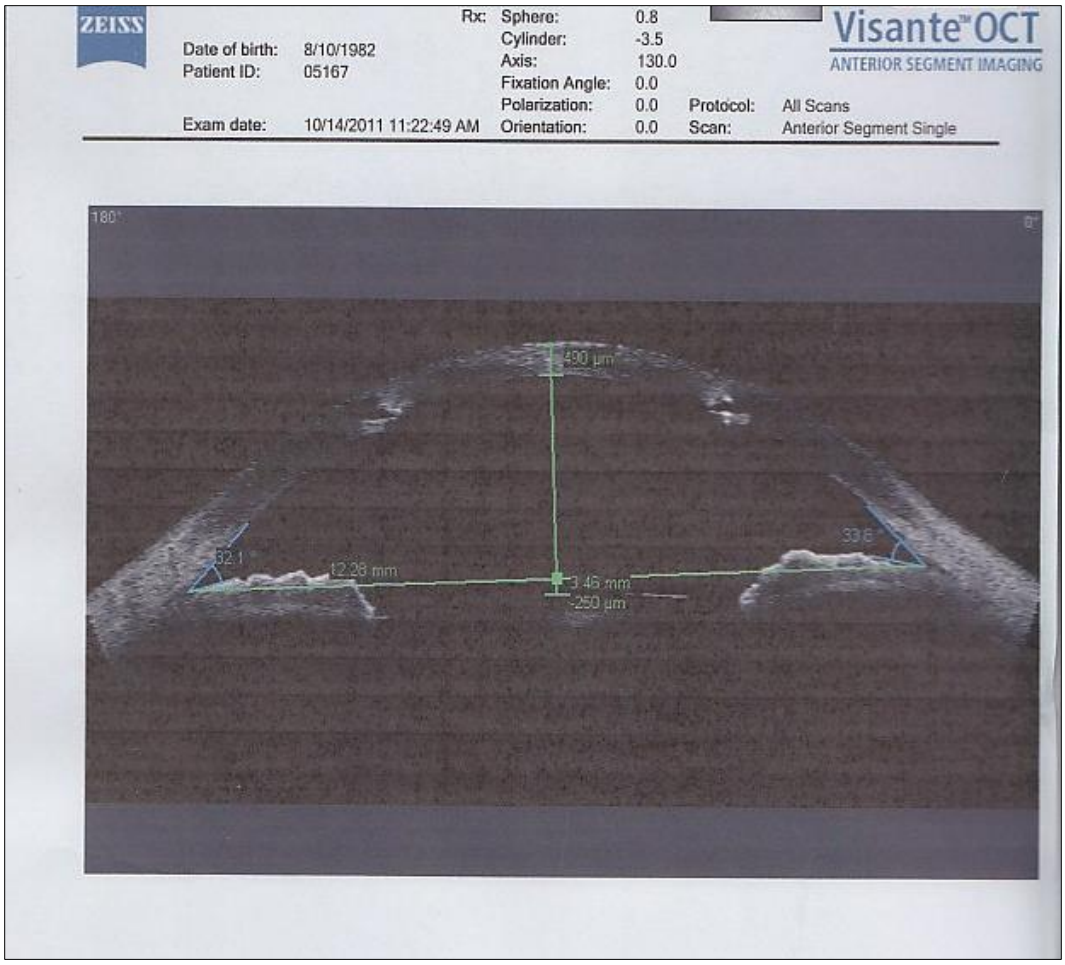


Figure 4. 20: Ocular Coherent Tomography (OCT) of the RE. Cross-section of the INTACS is seen in the corneal stroma of the patient.
Source: JPD

Case C6 was a 34-year-old keratoconus patient. He had poor vision RE 6/60 even with RGP contact lens. The LE was better, being correctable to 6/9p with RGP. He wanted further enhancement of his RE vision, as he worked as an administrator, and always had paper work. He complained the inequality of vision, especially the RE caused him very bad headaches. He was a good candidate for INTACs as his cornea was still >450u, the limit for insertion. After implantation of INTACS and CXL treatment, his vision improved to 6/9p. After the treatment, the

RE vision balanced his LE and , Case C6 had good binocular vision, for both far and near. He also had no more headaches.

4.1.5.7 Summary and Conclusion of Six Case Studies

The above six clinical cases illustrate the variations in KC presentation as well as response to treatment. Clearly, both early or late detection can affect the outcome of treatment and early detection is essential for a better prognosis in KC patients.

4.2 Results Genes- PCR Sequencing

4.2.1 Sequence variants observed in *VSX*1Exon3

Four variants were observed in *VSX*1Exon3 and are described in Table 4.30

Table 4. 23: Sequence variants observed in *VSX*1

SNP ID	VSX transcript ID	c.DNA change	VSX1 protein ID	Amino acid change
rs12480307	NM_014588	c.546A>G	NP_055403	pA182A
rs6138482	NM_014588	c.627+23G>A	NP_055403	p.R217H
rs56157240	NM_014588	c.627+84T>A	NP_055403	p.P237P
(IVS3-24C)	NM_014588	c.504-24C>T	NP_055403	

A gene variant is a permanent change in the DNA sequence that makes up a gene. This type of genetic change used to be known as a gene mutation, but because changes in DNA do not always cause disease, it is thought that gene variant is a more accurate term. Variants can affect one or more DNA building blocks (nucleotides) in a gene.

4.2.2 Chromatogram Identification for Each Vsx1 Variant

All the other groups, 126 family controls, 97 normal controls and 143 school children were similarly analysed and screened for these same four reported single nucleotide polymorphisms (SNP) are shown in Figure 4.22.

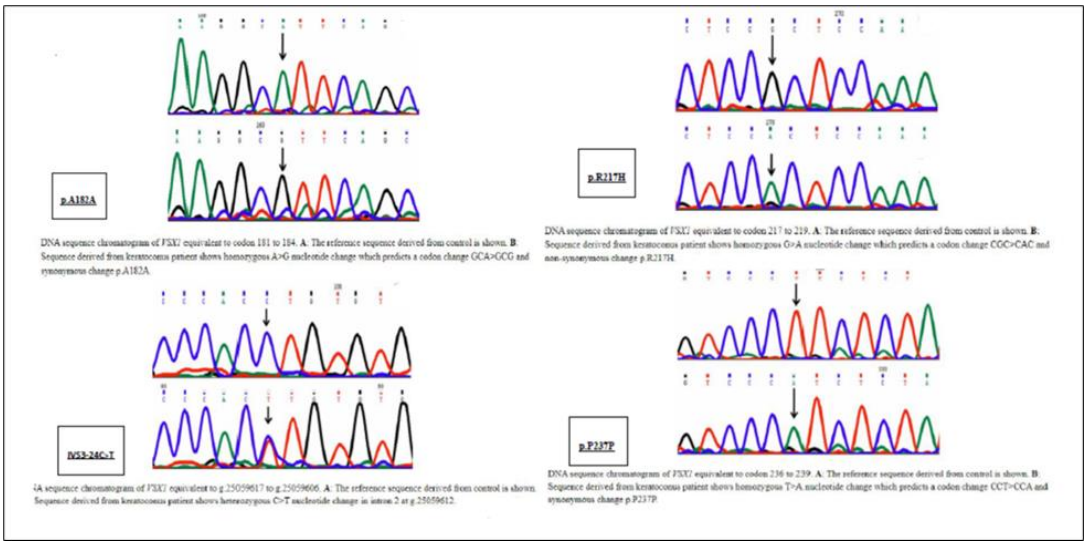


Figure 4. 21: Chromatograms for VSX1 variants, A182A; P237P; R217H and c.64-24C>T

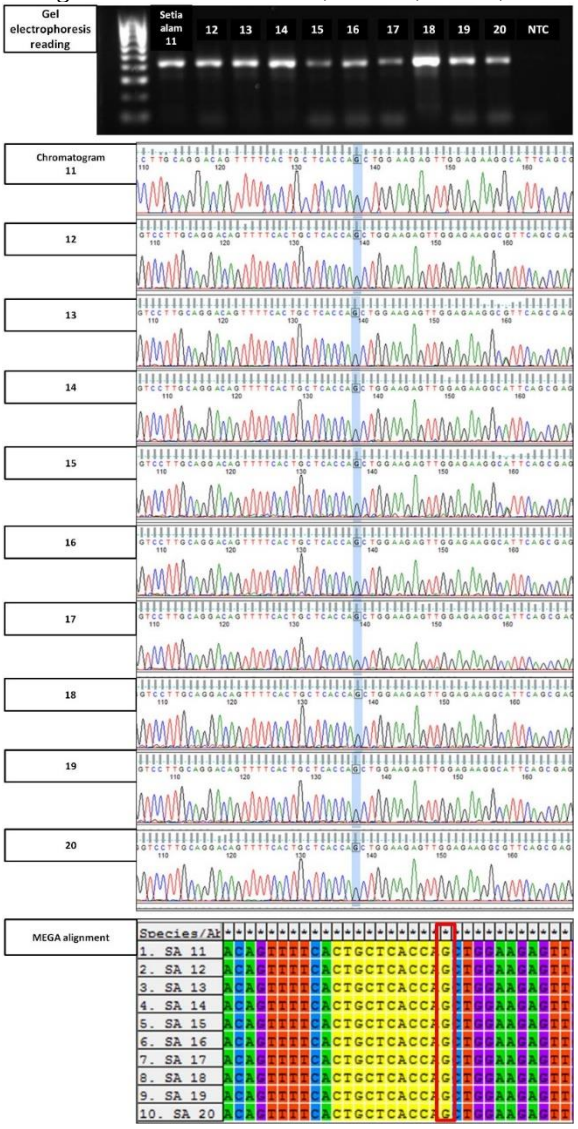


Figure 4. 22: Example of a chromatogram and MEGA alignment for *VSX*1Exon3 sequences
Source: JPD

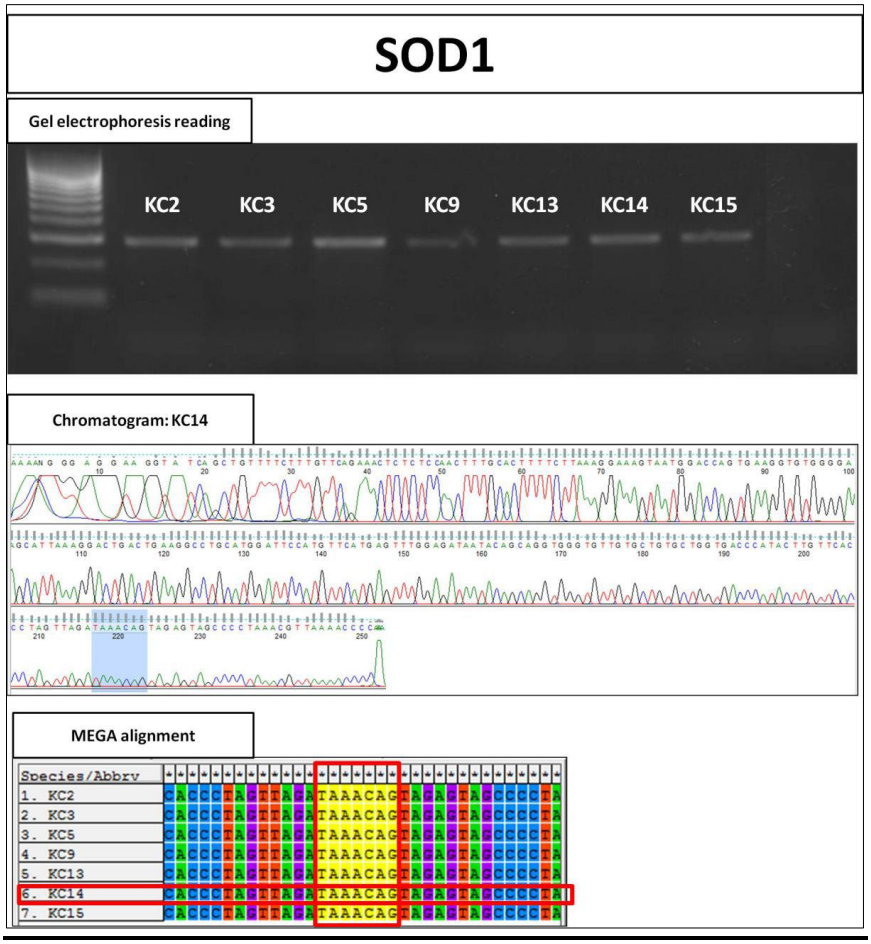


Figure 4. 23: Example of a chromatogram and MEGA alignment for *SOD1Exon2*
The SOD1Exon2 was only detected in patient KC6; KC15 and KC18
Source:JPD

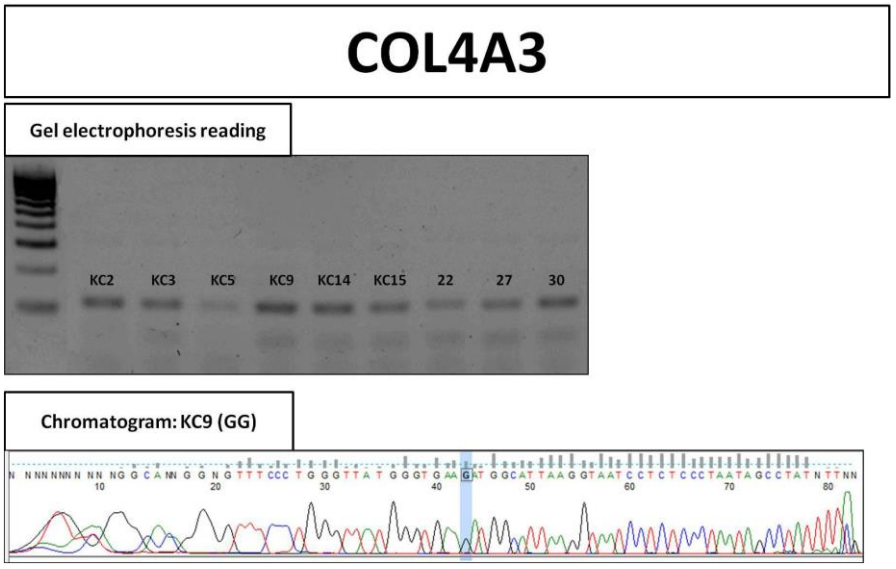


Figure 4. 24: Example of a chromatogram and MEGA alignment for *COL4A3Exon17*
The COL4A3Exon17 was not detected in any KC patients
Source: JPD

4.2.3 Results of PCR Sequencing

All KC cases and different control groups were analyzed for coding and flanking exonic regions of the three candidate genes. Table 3.5 shows the presence of gene variants for the 42 KC cases. The results for control groups are provided in Appendix.

Table 4. 24: Gene variants of *VSX*1Exon3, *SOD*1Exon2 and *COL*4A3Exon17 in 42 KC cases

R/N	***VSX1* Exon 3**	***SOD1* Exon 2**	***COL4A3* Exon 17**
KC1	c.627+23G>A (het)		None
KC2	p.A182A; c.50424C>T;c.627+84T>A (all het)	None	
KC3	p.A182A; c.627+84T>A (all het)		
KC4	None		
KC5	None		
KC6	None	g.9339G>A	
KC7	None		
KC8	p.A182A;c.504-24C>T; c.627+84A (all het)		
KC9	c.627+23G>A (het)		
KC10	p.A182A; c.627+84 T>A (all het)		
KC11	c.627+23G>A (het)	None	
KC12	c.627+23G>A (homo)		
KC13	c.627+23G>A (homo)		
KC14	c.627+23G>A (homo)		
KC15	c.627+38C>A(novel), c.627+23G>A (all het)	g.9339G>A	
KC16	p.A182A; c.627+84 T>A (all het)		
KC17	None		
KC18	p.A182A; c.627+84T>A (all het)	g.9339G>A	
KC19	c.627+23G>A (homo)		
KC20	c.627+23G>A (het)	None	
KC21	c.627+23G>A (homo)		
KC22	c.627+23G>A (het)	None	
KC23	None		
KC24	None		
KC25	c.627+23G>A (homo)		
KC26	c.627+23G>A (het)		
KC27	None		
KC28	None		

KC29	c.627+23G>A		
KC30	c.627+23G>A (het)		
KC31	p.A182A; c.627+84T>A; c.627+23G>A (all het)		
KC32	c.627+23G>A		
KC33	p.A182A; c.627+84T>A		
KC34	None		
KC35	p.A182A; c.627+84T>A; c.627+23G>A; c.504-24C>T (all het)		
KC36	p.A182A; c.627+84T>A (all het)		
KC37	p.A182A;c.627+84T>A (all het)		
KC38	p.A182A; c.627+84T>A (all het)		
KC39	None		
KC40	None		
KC41	p.A182A; c.627+84T>A; c.627+23G>A		
KC42	p.A182A; c.627+84T>A (all het)		

*het: heterozygous; homo: homozygous

All empty cells indicate, no results. There were no SOD1 and COL4A3 variants

Table 4. 25: The Frequencies of different VSX1 gene variants

VSX1 VARIANT (N, %)	A182A (N, %)	P237P (N, %)	R217H (N, %)	c.504-24C>T (N, %)
KC (42:100)	14, 33.3	14, 33.3	18, 42.83	3, 7.14
Family Control (126:100)	20, 15.74	20, 15.74	13, 10.24	1, 0.78
Normal Control (97:100)	12, 12.37	12, 12.37	48, 49.48	6, 6.18
School Children (143:100)	20, 17.54	20, 17.54	54, 61.56	4, 4.56

Detail tables of Frequencies of Family, Normal controls and school children are presented in Appendix

This table shows the KC group to be having 33.3% of both A182A and P237P. Risk of developing keratoconus, when the person carries these 2 variants, can possibly be inferred that, those having these two are likely to be keratoconus phenotypes. In the Odds Ratio calculation this is seen more clearly as a 3-4 times likely to be a risk of getting keratoconus (OR 3.14 {*pvalue*0.*026* CI 95, (1.14 - 8.60). However, the reverse is true for variant R217H which is carried by 49.48% of the Normal controls. These are unlikely to develop KC, and possibly carry a protective role against the development of KC. (OR 0.082[*pvalue* <0.0001, CI 95 (0.0313 – 0.237). Full tables in appendix stanstats section G1 to G7. In the school children the frequency also was high, but when compared, there is no special association between the two groups, as OR is 1.

In the *VSX*1 gene screening, no pathogenic (associated with disease) mutations were identified as the variants in KC cases were also seen in the controls. However, four previously reported SNPs (Tanwar *et al*, 2010; Shetty *et al* ,2015; and Abu-Amero, 2011) were present in different KC patients.

Table 3. 5: Frequency of *VSX*1Exon3 gene variants (allele changes) observed in sporadic KC cases and Family controls

No	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
1	KC44	1-KC	C	F	32	A/A	T/T	A/A	C/C
2	KC48	1-KC	M	M	28	G/A	T/T	A/A	C/C
3	KC1	1-KC	M	M	17	G/A	T/T	A/A	C/C
4	KC10	1-KC	I	M	32	G/G	T/A	A/G	C/T
5	KC11	1-KC	M	F	19	G/A	T/T	A/A	C/C
6	KC13	1-KC	I	M	21	G/G	T/A	A/G	C/C
7	KC14	1-KC	M	M	16	G/A	T/T	A/A	C/C
8	KC15	1-KC	I	F	32	A/A	T/T	A/A	C/C
9	KC16	1-KC	I	M	29	A/A	T/T	A/A	C/C
10	KC18	1-KC	M	M	17	A/A	T/T	A/A	C/C
11	KC19	1-KC	I	M	19	G/A	T/T	A/A	C/C
12	KC2	1-KC	I	M	22	G/G	T/A	A/G	C/T
13	KC21	1-KC	I	F	30	G/G	T/A	A/G	C/C
14	KC22	1-KC	M	M	26	G/G	T/T	A/A	C/C
15	KC24	1-KC	I	M	24	G/G	T/A	A/G	C/C
16	KC25	1-KC	M	M	19	A/A	T/T	A/A	C/C
17	KC26	1-KC	M	M	23	G/A	T/T	A/A	C/C
18	KC27	1-KC	C	F	24	A/A	T/T	A/A	C/C
19	KC28	1-KC	C	M	18	G/A	T/T	A/A	C/C
20	KC3	1-KC	I	M	18	G/A	T/A	A/A	C/C
21	KC33	1-KC	M	M	33	G/G	T/T	A/A	C/C
22	KC35	1-KC	M	F	22	G/G	T/T	A/A	C/C
23	KC36	1-KC	C	M	20	A/A	T/T	A/A	C/C
24	KC37	1-KC	C	F	31	G/A	T/T	A/A	C/C
25	KC38	1-KC	C	M	29	G/G	T/T	A/A	C/C

26	KC4	1-KC	I	M	28	G/G	T/T	A/A	C/C
27	KC40	1-KC	I	M	25	G/G	T/T	A/A	C/C
28	KC45	1-KC	M	F	30	G/A	T/T	A/A	C/C
29	KC47	1-KC	I	F	33	G/A	T/A	A/G	C/C
30	KC49	1-KC	I	M	27	G/G	T/A	A/G	C/C
31	KC5	1-KC	I	F	32	G/G	T/T	A/A	C/C
32	KC50	1-KC	I	M	28	G/G	T/T	A/A	C/C
33	KC54	1-KC	I	F	35	G/A	T/A	A/G	C/T
34	KC56	1-KC	I	F	22	G/G	T/A	A/G	C/C
35	KC6	1-KC	I	M	18	G/G	T/T	A/A	C/C
36	KC62	1-KC	I	M	14	G/G	T/A	A/G	C/C
37	KC63	1-KC	I	M	19	G/G	T/A	A/G	C/C
38	KC64	1-KC	I	M	24	G/G	T/T	A/A	C/C
39	KC66	1-KC	M	F	19	G/G	T/T	A/A	C/C
40	KC67	1-KC	I	M	20	G/A	T/A	A/G	C/C
41	KC68	1-KC	M	F	33	G/G	T/A	A/G	C/C
42	KC8	1-KC	I	M	25	G/G	T/T	A/A	C/C

G, glutamine; A, adenine; T, thymine; C, cytosine.

A comparative statistical analysis of genotype and allele frequency was done using chi-square or Fisher's exact test, to assess differences in the distribution of *VSX*1 polymorphisms between cases and controls. The allelic p-value and odds-ratio were calculated for each of the identified *VSX*1 gene variants, using SNPSTATS for SNP analysis.

4.2.4 Genetic Association Studies

Genetic association studies for relationship between genetic variation and keratoconus status for VSX1 gene variant, A182A; P237P; R217H, c.504-24C>T were performed. All the SNPs conformed to Hardy-Weinberg Equilibrium.

The following tables show the outcomes of analysis and calculations of Odds Ratio for Gene Variant Results of PCR sequencing, for each individual group versus the 42 Keratoconus patients.

Table 4. 26: Outcomes of analysis and calculations of Odds Ratio for Gene Variant Results of PCR sequencing

SNP ID	VSX transcript ID	c.DNA change	VSX1 protein ID	Amino acid change
rs12480307	NM_014588	c.546A>G	NP_055403	pA182A
rs6138482	NM_014588	c.627+23G>A	NP_055403	p.R217H
rs56157240	NM_014588	c.627+84T>A	NP_055403	p.P237P
(IVS3-24C)	NM_014588	c.504-24C>T	NP_055403	

4.2.4.1 Association/Interpretation Studies

Three of the four SNPs - A182A; P237P; R217H, were significantly associated with keratoconus. The odds of patients caring the mutant allele of A182A; P237P were 3.14 s to 4 times higher than family and normal control. The details of results in this study are documented in the following tables. The results of student children did not show a significant association to keratoconus. All tables

related to school children can be found in the Appendix.

Table 4. 27: KC cases with 126 Family controls. Allelic and genotypic frequencies of VSX1 p.A182A (rs12480307)

p.A182A (rs12480307)	Frequency, *n* (%)		Pearson x^2 or Fisher's test (p value)	OR (95% CI; OR p value)	
	Keratoconus N = 42	FAMILY Control N = 126			
Genotype					
A/A	29 (69.0)	105(83.3)		-	-
A/G	13 (30.9)	17 (13.4)			
G/G	1(1.02)	4 (3.3)			
Allele					
A	71 (85)	227 (91)			
G	13(15)	23 (9)			
Carrier					
AA and AG versus GG(R)	42(100)	123(96.8)	0.15	1.0	
	0 (0)	3(2.4)		0.00 (0.00-NA)	
AA versus AG and GG(D)	29 (69)	106(84.1)	0.026	1.0	
	13(30.1)	20(16)		3.14(1.14-8.60)	

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 28:KC cases with 126 Family controls Allelic and genotypic frequencies of VSX1p.P237P (rs5615740)

p.P237P (rs5615740)	Frequency, *n* (%)		Pearson x^2 or Fisher's test (p value)	OR (95% CI; OR p value)	
	Keratoconus N = 42	FAMILY Control N = 125			
Genotype					
AA	0 (0)	3(2.38)		-	-
TA	14 (33.3)	17 (13.49)			
TT	28(67)	106 (84.1)			
Allele					
T	70 (83)	227 (91)			
A	14(15)	23 (9)			
Carrier					
AA and AG versus AA(R)	42(100) 0 (0)	123(97.6) 3(2.4)	0.15	0.00(0.0-NA)	
TT versus TA and AA(D)	28 (66.7) 14(33.3)	106(84.1) 20(15.8)	0.012	3.61(1.13-9.94.)	

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells

Table 4. 29: KC cases with 126 Family controls Allelic and genotypic frequencies of VSX1p.R217H (rs6138482)

p.R217H rs6138482	Frequency, n (%)		Pearson x^2 or Fisher's test (p value)	OR (95% CI; OR p value)	
	Keratoconus N = 42	FAMILY Control N = 126			
Genotype					
AA	7 (17)	3(2)		-	-
GA	13 (31)	17 (14)			
GG	22(52)	105 (84)			
Allele					
G	57 (68)	232 (93)			
A	27(32)	18 (7)			
Carrier					
GG and GA versus AA(R)	35(83.3) 7 (16.7)	121(96.8) 4 (3.2)	0.004	0.124 (0.175-0.549)	
GG versus GA and AA(D)	22(52.4) 20(47.6)	111 (88.8) 14 (11.2)	<0.0001	0.086 (0.0313 -0.237)	

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 30: KC cases with 126 family controls Allelic and genotypic frequencies of VSX1 c.504-24C>T (rs201363715)

c.504-24C>T (rs201363715)	Frequency, *n* (%)		Pearson x^2 or Fisher's test (p value)	OR (95% CI; OR p value)	
	Keratoconus N = 42	FAMILY Control N = 126			
Genotype					
CC	39 (93)	124(98.4)		-	-
CT	3 (7)	2(1.58)			
Allele					
C	81	249			
T	3	1			
Carrier					
CC	39(92.9)	124(98.4)	0.15	1.0	
CT	3 (7.1)	2(1.58)	0.27	1.0	

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

4.2.4.2 Summary report and interpretation: (Tables 4.28 to 4.32 family controls) All the SNPs in this analysis conformed to Hardy Weinberg Equilibrium (HWE*) p >0.05*. Two of the SNPs namely, A182A and P237P were associated to KC significantly. The OR for A182A was 3.14(CI 95%. 1.14 _ 8.60, *p value> 0.026*) and OR for P237P, was 3.61(CI 95%. 1.31 _ 9.94, *pvalue <0.012*).

Thus, the odds of the KC Patients carrying the mutant allele for A182A and P237P was approximately 3 – 4 times higher than that of family controls. This confirms that Risk of development of keratoconus, is increased to 3-4times by presence of these two variants.

However, SNP R217H is also associated with KC, but inversely related, as this variant is much more frequent in family controls rather than KC patients. OR is instead low, 0.086 (CI 95%, 0.0313 – 0.237) pvalue <0.0001. This perhaps then can be interpreted to have a protective role.

Table 4. 31: KC cases with Normal Controls. Allelic and genotypic frequencies of VSX1 p.A182A (rs12480307)

p.A182A (rs12480307)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	NORMAL Control		
	N = 42	N = 125		
Genotype				
A/A	29 (69)	84(88)		-
A/G	13 (31)	18 (12)		
G/G				
Allele				
A	71 (85)	180 (94)		
G	13(15)	12 (6)		
Carrier				
AA and AG versus GG(R)	29 (69)	84(87.5)	0.15	1.0
AA versus AG and GG(D)	13(30.1)	12(12.5)	0.029	3.78(1.11 – 2.79)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 32: KC cases with Normal Controls. Allelic and genotypic frequencies of VSX1p.P237P (rs5615740)

p.P237P (rs5615740)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	NORMAL Control		
	N = 42	N = 96		
Genotype				
AA	0 (0)	0 (0)		-
TA	14 (33)	12(12)		
TT	28(67)	84 (88)		
Allele				
T	70 (83)	180 (94)		
A	14(17)	12 (6)		
Carrier				
TT	28(66.7)	84(87.5)		1.00
TA	14(33.3)	12(12.5)	0.02	4.05 (1.21-13.56)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 33: KC cases with Normal Controls. Allelic and genotypic frequencies of VSX1p.R217H (rs6138482)

p.R217H rs6138482	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus N = 42	Normal Control N = 96		
Genotype				
AA	7 (16.)	17(18)		-
GA	13 (31)	30 (31)		
GG	22(52.)	49 (51)		
Allele				
G	57 (68)	128 (67)		
A	27(32)	64 (33)		
Carrier				
GG and GA	35(83.3)	79(82.3)	0.51	1.0
versus AA(R)	7 (16.7)	17(17.7)		1.49 (0.46 -4.88)
GG versus GA	22(52.4)	49 (51)		1.0
and AA(D)	20(47.6)	47(49)	0.33	1.59(0.62 -4.07)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 34: KC cases with Normal Controls. Allelic and genotypic frequencies of VSX1 c.504-24C>T (rs201363715)

c.504-24C>T (rs201363715)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus N = 42	NORMAL Control N = 96		
Genotype				
CC	39 (93)	88(92)		-
CT	3 (7)	8(8)		
Allele				
C	81(96)	184(96)		
T	3(3)	8(4)		
Carrier				
CC	39(92.9)	88(91.7)		1.0
CT	3 (7.1)	8(8.3)	0.98	1.02(0.18-5.75)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

4.2.4.3 Summary report and interpretation for Normal Controls

All the SNPs in this analysis conformed to Hardy Weinberg Equilibrium (HWE*) p >0.05*.

Two of the SNPs namely, A182A and P237P were associated to KC significantly. The OR for A182A was 3.78 (CI 95%. 1.11 _12.79, *p value> 0.029*) and OR for P237P, was 4.05(CI 95%. 1.21 _ 13.55, *pvalue <0.02*). Table 4.33 and 4.36.

Thus, the odds of the KC Patients carrying the mutant allele for A182A and P237P was approximately 4 times higher than that of Normal controls.

Again, with Variant R217H, OR is low, 1.49 and 1.59, Perhaps also having a protective role, as in the Family controls.

Table 4. 35: KC cases with School Children. Allelic and genotypic frequencies of VSX1 p.A182A (rs12480307)

p.A182A (rs12480307)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	SCHOOL CHILDREN		
	N = 42	N = 114		
Genotype				
A/A	29 (69)	95(83)		-
A/G	13 (31)	16 (14)		
G/G	0(0)	3(3)		
Allele				
A	71 (85)	206 (90)		
G	13(15)	22 (10)		
Carrier				
AA and AG	42 (100)	111(97.4)	1	1.0
versus GG(R)	0	3(2.6)		
AA versus AG	29(69.0)	95(83.3)	1	1.0
and GG(D)	13(30.9)	19 (16.7)		NA(0.00-NA)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 36: KC cases with School Children. Allelic and genotypic frequencies of VSX1p.P237P (rs5615740)

p.P237P (rs5615740)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	SCHOOL CHILDREN		
	N = 42	N = 114		
Genotype				
AA	0 (0)	4 (4)		-
TA	14 (33)	15(13)		
TT	28(67)	95 (83)		
Allele				
T	70 (83)	205 (90)		
A	14(17)	23 (10)		
Carrier				
TT	28(66.7)	95(83.3)	1	1.00
T/A-A/A (D)	14 (33.3)	19 (16.67)		NA(0.00-NA)
T/T-T/A	42(100)	110(96.5)	1	1.00
A/A(R)	0(0)	4(3.5)		NA(0.00-NA)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 37: KC cases with School Children. Allelic and genotypic frequencies of VSX1p.R217H (rs6138482)

p.R217H rs6138482	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	SCHOOL CHILDRENl		
	N = 42	N = 114		
Genotype				
AA	7 (17)	11(10)		-
GA	13 (31)	32 (28)		
GG	22(52.)	71 (62)		
Allele				
G	57 (68)	174 (76)		
A	27(32)	54 (24)		
Carrier				
GG and GA	35(83.3)	103(90.3)	1	1.0
versus AA(R)	7 (16.7)	11(9.7)		0.00(0.00-NA)
GG versus GA	22(52.4)	71 (63.3)		1.0
and AA(D)	20(47.6)	43(37.7)	1	0.00(0.00-NA)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

Table 4. 38: KC cases with School Children. Allelic and genotypic frequencies of VSX1p.P237P (rs5615740)

c.504-24C>T (rs201363715)	Frequency, *n* (%)		(p value)	OR (95% CI; OR p value)
	Keratoconus	SCHOOL CHILDREN		
	N = 42	N = 114		
Genotype				
CC	39 (93)	108(95)		-
CT	3 (7)	6(5)		
Allele				
C	81(96)	222(97)		
T	3(4)	6(3)		
Carrier				
CC	39(92.9)	108(94.7)	1	1.0
CT	3 (7.1)	6(5.3)	1	0.03(0.00-NA)

OR: Mantel-Haenzel odds ratio; CI: confidence interval; *Minimum expected count in 2x3 table = 3.07, except 2 cells; †Minimum expected count in 2x2 table = 22.79 in all cells; ‡Fisher's test was used, minimum expected count in 2x2 table = 3.07, except 2 cells; §Minimum expected count in 2x2 table = 19.72 in all cells.

4.2.4.4 Summary report and interpretation for School Children

All SNPS were insignificant in the children's group. 29 children were excluded from analysis due to bad quality of DNA extraction and absence of sequencing VSX1 data. Only 114 children blood samples were tested.

Odds Ratio calculations, for school children shows, there is no significant association as OR is 1.0.

4.3 Haplotype Block Association (details are combination of family, normal and school children data)

Haplotype block association is to show whether there is association of two visual system homeobox 1(VSX1) SNPS p. A182A (SNP2) and p.P237P [c.627+84T>A (SNP3)].

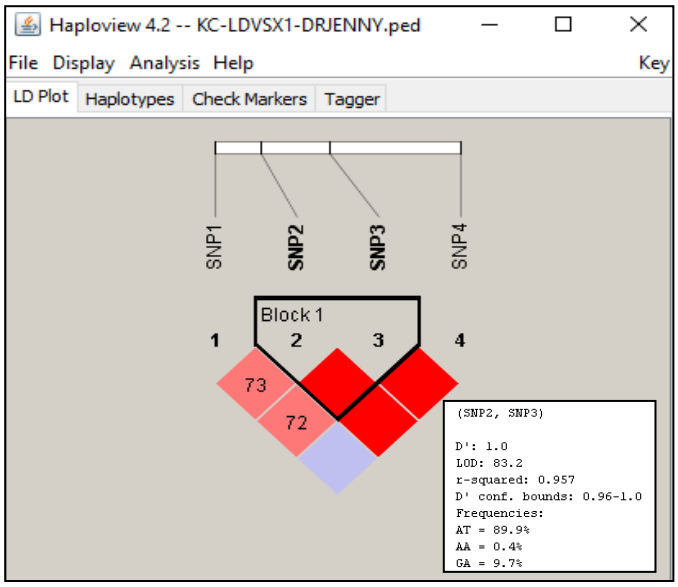


Figure 4. 25: Haplotype block (Block 1)

Figure 4.26 shows a haplotype block (Block 1) was generated using Haploview softwarewhich showed association of two visual system homeobox 1 SNPs p.A182A (SNP2) and c.627+84T>A (SNP3). With the LOD score of >2.0, r^2 of 0.957, minimum allele frequency value of 0.05 and D' value falls within confidence interval boundary between 0.96 – 1.00, both SNPs were in linkage disequilibrium

Table 4. 39: Legends for each SNP mentioned in the analysis:

t		
SNP1	IVS2+479C>T (IVS3-24C>T)	rs201363715
SNP2	p.A182A	rs12480307
SNP3	IVS3+84T>A	rs56157240
SNP4	IVS3+23G>A	rs6138482

4.3.1 Results from Haploview Analysis

A haplotype association test was used to assess whether a haplotype of polymorphisms was associated with keratoconus. The LOD score, confidence internalof D' values and minor allele frequencies (MAF) were calculated.

For the association test on VSX1, four VSX1 SNPs were included in the analysis. A haplotype block labelled as Block 1 which included SNPs p.A182A, and c.627+84T>A was generated (Figure 1). These SNPs were labelled as SNP2 and SNP3 respectively. The SNPs that were grouped in Block 1 had sufficient MAF valueof at least 0.05 with LOD score ≥ 2.0 and D' = 1.0. SNP1 (c.504-24C>T) did not haveMAF values of at least 0.05 and were thus excluded from Block 1. The Haploview analysis revealed three haplotype matchups based on the alleles from p.A182A and c.627+84T>A. The three matchups were AT (89.9%), GA (9.7%) and AA (0.4%). For the AA and GA haplotypes, there was no difference between patients and controls ($p > 0.05$). The AA haplotype is not included as part of Block 1 haplotype distribution. However, the AT haplotype showed a difference in distribution between patients and controls ($p = 0.0354$). Moreover, the result from $\chi 2$ and allelic analysis for all VSX1 SNPs using Haploview showed similar results for the $\chi 2$ analysis from SPSS.

This result indicates that the association was closely linked between SNP2 and SNP3 , namely A182 A and P237P respectively. The linkage association was less with R217H comparatively. These associations were described in a single haplotype block. This finding is similar to a Korean study (Mok JW et al 2008), in which the authors reported association of the

three SNPs in a haplotype. There was however a fourth SNP, c.627+22C>T found, which was not found in this study.

Linkage association studies of VSX1 gene is rare in literature, so finding this was useful in finding the genetic pattern. Is is important to recognize that associating VSX1 in a given population does not necessarily, imply the SNPs are pathogenic in all populations.

Literature on molecular genetics of keratoconus indicates that changes that occurred in VSX1 genetic sequence, were suggestive of pathogenic mutations in certain populations (Dehkordi FA et al 2013) whereas other studies suggested the same variations were merely non-pathogenic polymorphisms in other populations (Dash DP et al , 2010; Abu-Amero KK et al ,2011;). With those studies suggesting pathogenic mutations, when a particular SNP allele is associated with keratoconus, the alleles could be directly contributing to the disease phenotype, or is in linkage disequilibrium with the actual allele that contributes to keratoconus.

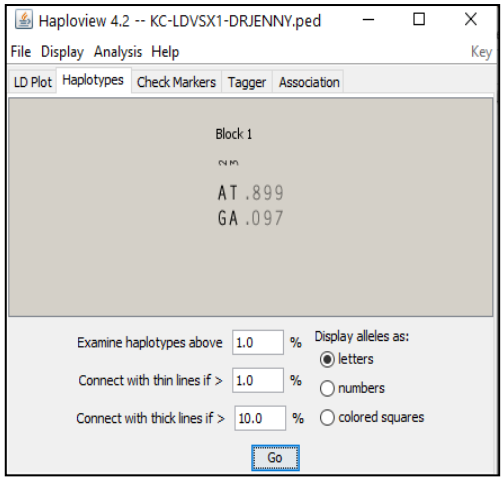


Figure 4. 26 The haplotype alleles found in Block 1. The AT haplotype is the wild type alleles that are grouped from two SNPs: p.A182A (rs12480307)and IVS3+84T>A (rs56157240).

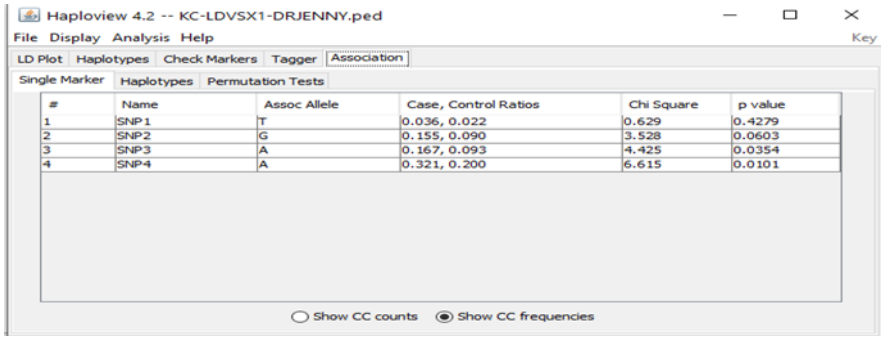


Figure 4. 27: The associated allele frequencies for each SNP, in terms of case-control ratios (with p-value calculated)

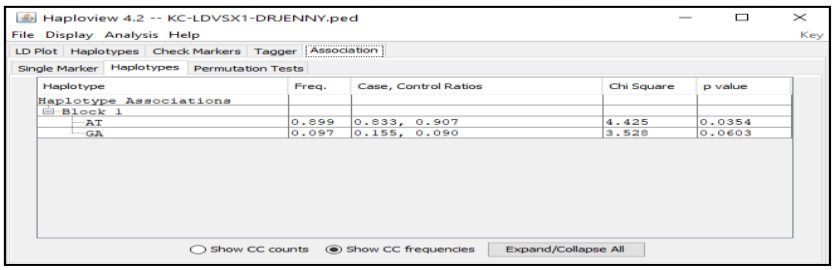


Figure 4. 28: The haplotype associated allele frequencies for haplotype block, interms of case-control

4.3.2 Haploview Analysis for KC Patients Versus Family Controls

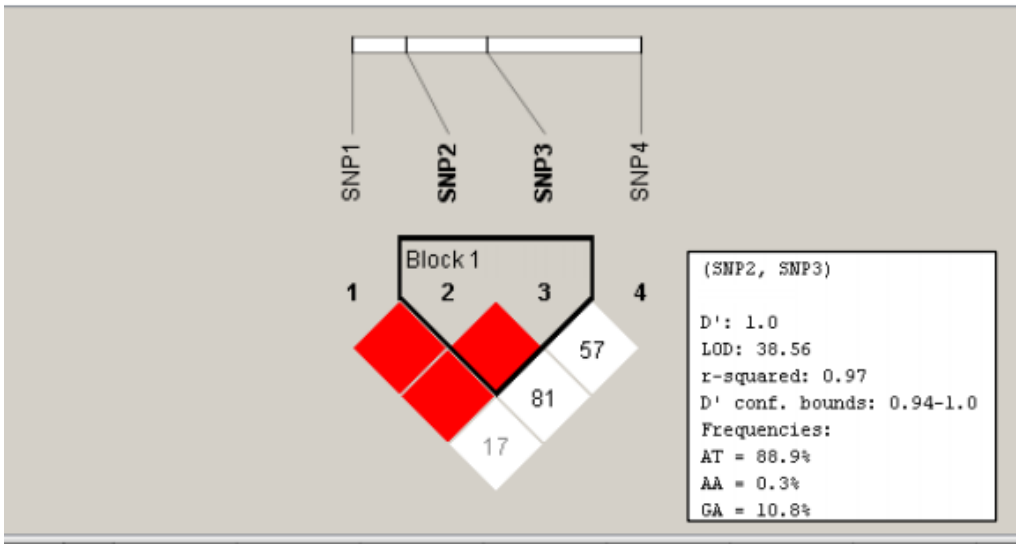


Figure 4. 29: Haploview Analysis for KC Patients Versus Family Controls

For patients versus family controls, a haplotype block (labelled as Block 1) was generated using Haploview software which showed association of two visual system homeobox 1 SNPs p.A182A (SNP2) and c.627+84T>A (SNP3). With the LOD score of > 2.0, r2 of 0.970, minimum allele frequency value of 0.05 and D' value falls within confidence interval boundary between 0.94 – 1.00, both SNPs were in linkage disequilibrium

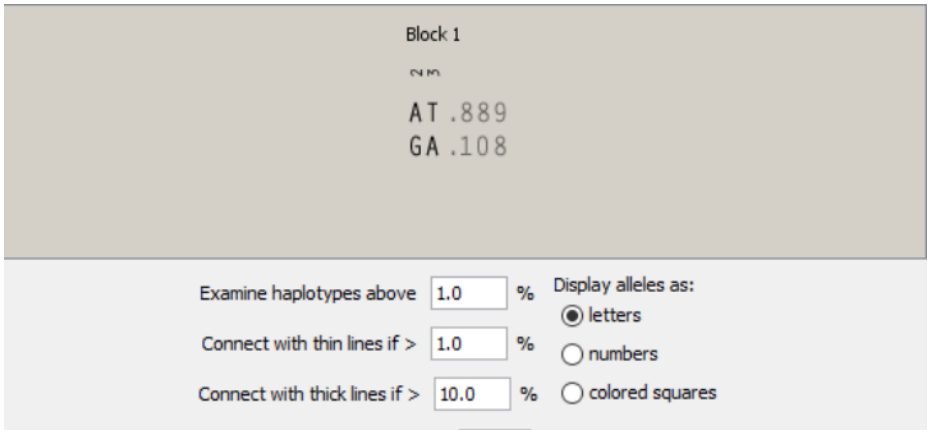


Figure 4. 30: The haplotype alleles found in Block 1.

The haplotype alleles for patients versus family controls that was found in Block 1. The AT haplotype is the wild type alleles that are grouped from two SNPs: p.A182A (rs12480307) and IVS3+84T>A (rs56157240).

Single Marker | Haplotypes | Permutation Tests

#	Name	Assoc Allele	Case, Control Ratios	Chi Square	p value
1	SNP1	T	0.036, 0.004	5.344	0.0208
2	SNP2	G	0.155, 0.092	2.575	0.1085
3	SNP3	A	0.167, 0.092	3.558	0.0592
4	SNP4	A	0.321, 0.072	33.554	6.9303E-9

Figure 4. 31: The associated allele frequencies between patients and family controls for each SNP, in terms of case-control ratios (with p-value calculated)

Single Marker | Haplotypes | Permutation Tests

Haplotype	Freq.	Case, Control Ratios	Chi Square	p value
Haplotype Associations				
⊟ Block 1				
AT	0.889	0.833, 0.908	3.558	0.0592
GA	0.108	0.155, 0.092	2.575	0.1085

Figure 4. 32:The haplotype associated allele frequencies between patients and family controls for haplotype block, in terms of case-control ratios (with p-value calculated)

Similar allele and similar haplotype, as p value for haplotype AT are at 0.0592 and GA 0.1085. As these are Family controls, it is expected, that, Haplotypes would be similar.

4.3.2.1 Results from Haploview Analysis for Patients Versus Family Controls

A haplotype association test was used to assess whether a haplotype of polymorphisms was associated with keratoconus. The LOD score, confidence internal of D' values and minor allele frequencies (MAF) were calculated. For the association test on VSX1, four VSX1 SNPs were included in the analysis. A haplotype block labelled as Block 1 which included SNPs p.A182A, and c.627+84T>A was generated (Figure 5). These SNPs were labelled as SNP2 and SNP3 respectively. The SNPs that were grouped in Block 1 had sufficient

MAF value of at least 0.05 with LOD score ≥ 2.0 and D' = 1.0. The Haploview analysis revealed three haplotype matchups based on the alleles from p.A182A and c.627+84T>A. The three matchups were AT (88.9%), GA (10.8%) and AA (0.3%). Although the AA haplotype is not included as part of Block 1 haplotype distribution, there was no difference between patients and family controls for all three haplotypes ($p > 0.05$). The result from $\chi 2$ and allelic analysis for all VSX1 SNPs using Haploview showed similar results for the $\chi 2$ analysis from SPSS

Fig 4.29 shows AT Haplotype as 83.3% in KC cases , and 90.8% in Family controls. P-value is 0.592, and 0.1085, confirming the distribution findings are similar and not significant.

4.3.3 Haploview Analysis for KC Patients Versus Normal Controls

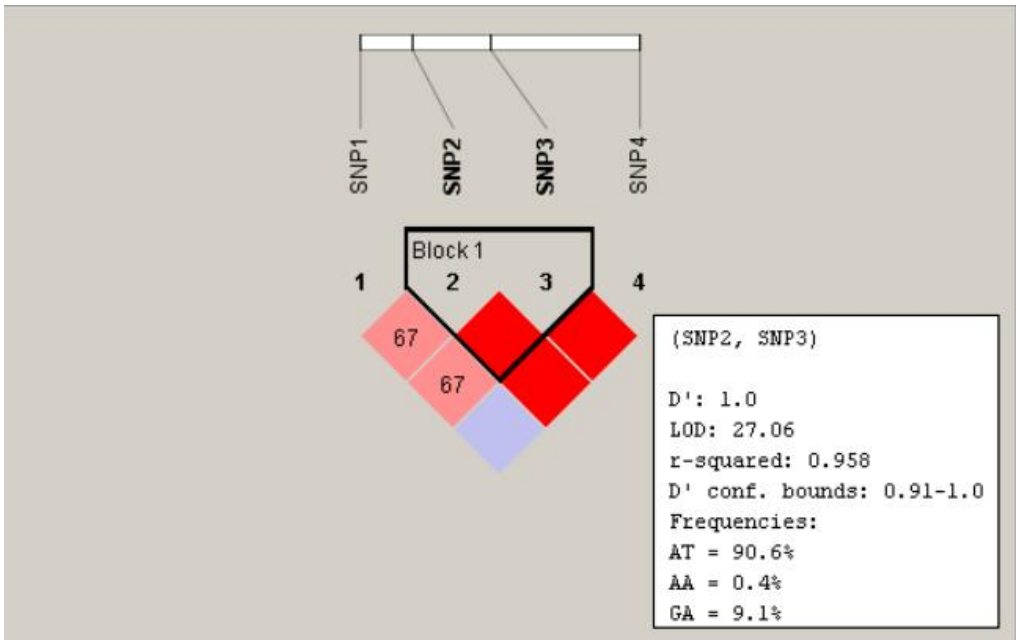


Figure 4. 33:Haploview analysis for KC patients versus normal controls

For patients versus normal controls, another haplotype block (labelled as Block 1) was generated using Haploview software which showed association of two visual system homeobox 1 SNPs p.A182A (SNP2) and c.627+84T>A (SNP3). With the LOD score of > 2.0, r2 of 0.958, minimum allele frequency value of 0.05 and D' value falls within confidence interval boundary between 0.91 – 1.00, both SNPs were in linkage disequilibrium

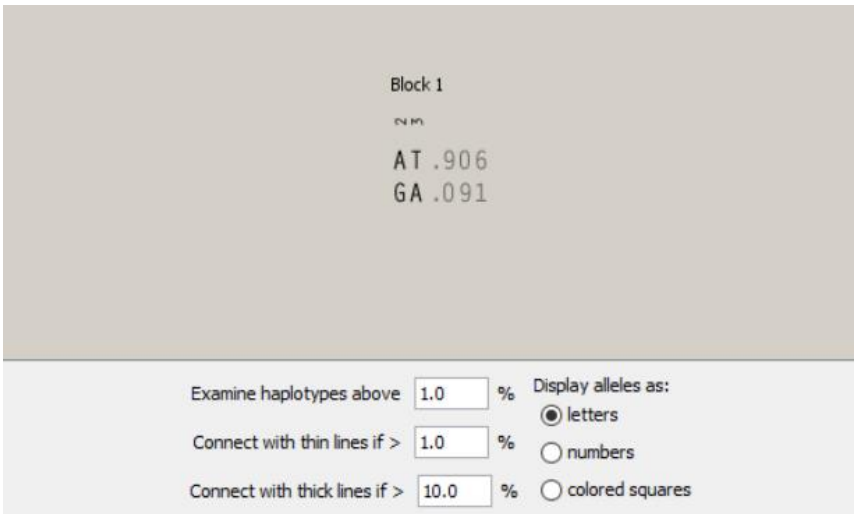


Figure 4. 34: The haplotype alleles for patients versus normal controls found in Block 1.

The AT haplotype is the wild type alleles that are grouped from two SNPs: p.A182A (rs12480307) and IVS3+84T>A (rs56157240)

Single Marker | Haplotypes | Permutation Tests

#	Name	Assoc Allele	Case, Control Ratios	Chi Square	p value
1	SNP1	C	81:3, 184:8	0.054	0.8161
2	SNP2	G	13:71, 12:180	6.038	0.014
3	SNP3	A	14:70, 12:180	7.431	0.0064
4	SNP4	G	57:27, 128:64	0.037	0.8465

Figure 4. 35: The associated allele frequencies between patients versus normal controls for each SNP, in terms of case-control ratios (with p-value calculated)

Single Marker | Haplotypes | Permutation Tests

Haplotype	Freq.	Case, Control Ratios	Chi Square	p value
Haplotype Associations				
Block 1				
AT	0.906	70.0 : 14.0, 180.0 : 12.0	7.431	0.0064
GA	0.091	13.0 : 71.0, 12.0 : 180.0	6.038	0.014

Figure 4. 36: The haplotype associated allele frequencies between patients versus normal controls for the haplotype block, in terms of case-control ratios (with p-value calculated)

Here the Haplotype pvalue are significan in both cases and Normal controls, as pvalue is AT 0.0064 and GA 0.014. The cases do not carry similar haplotype as Normal controls.

4.3.3.1 Results from Haploview Analysis For Patients Versus Normal Controls

A haplotype association test was used to assess whether a haplotype of polymorphisms was associated with keratoconus. The LOD score, confidence internal of D' values and minor allele frequencies (MAF) were calculated. For the association test on VSX1, four VSX1 SNPs were included in the analysis. A haplotype block labelled as Block 1 which included SNPs p.A182A, and c.627+84T>A was generated (Figure 9). These SNPs were labelled as SNP2 and SNP3 respectively. The SNPs that were grouped in Block 1 had sufficient MAF value of at least 0.05 with LOD score ≥ 2.0 and D' = 1.0. The Haploview analysis revealed three haplotype matchups based on the alleles from p.A182A

and c.627+84T>A. The three matchups were AT (90.6%), GA (9.1%) and AA (0.4%). For the AA haplotype, there is no difference between patients and unrelated, normal controls ($p > 0.05$), and the AA haplotype is not included as part of Block 1 haplotype distribution. However, the AT and GA haplotypes showed a difference in distribution between patients and controls ($p < 0.05$). Moreover, the result from $\chi 2$ and allelic analysis for all VSX1 SNPs using Haploview showed similar results for the $\chi 2$ analysis from SPSS

This difference is to be expected as there is no Family relationship.

4.4 Results for Exome Sequencing

4.4.1 Variant Identification from Exome Sequencing Data

The sequence mapping data for 24 exome sequencing samples were re-analysed according to various hypotheses to attain novel insight. The analysis methods and results are summarized below

To compare the samples side-by-side, the quality-processed sequence mapping files were re-analysed together to identify variants. The table below summarises the total number of variants that was identified, and the number of different genotypes called for each sample.

Whole Genome Sequencing (WGS) is sequencing of the entire genetic code of a person. Whole Exome Sequencing (WES) is only sequencing the part of the genome that contains protein-coding regions of genes in a genome. This part is called the "Exome". The exome is only 1% of the whole genome. It is the part of the gene which gives instructions to make all the proteins in the body. Sometimes the fastest way to find what may cause a disease, is just looking at this exome region.

4.4.2 Variants in Genes of Interest

Table 4. 40: Number of variants and sample genotypes

Sample ID	Total no. of variants	No. of genotypes called (./.)	Homozygous Reference (R/R)	Heterozygous (R/A)	Homozygous Alternate (A/A)
KC14	149,704	5,273	88,579	33,182	22,670
KC24	149,704	4,334	87,535	36,304	21,531
KC19	149,704	4,542	86,445	36,777	21,940
KC28	149,704	5,095	89,100	32,312	23,197
KC6	149,704	9,783	86,795	32,649	20,477
KC14A	149,704	9,243	86,916	33,187	20,358

KC14B	149,704	5,023	88,058	34,615	22,008
KC14C	149,704	5,197	88,253	33,741	22,513
KC24B	149,704	7,724	86,632	34,563	20,785
KC19A	149,704	5,041	88,270	34,088	22,305
KC28A	149,704	5,596	86,808	35,715	21,585
KC28B	149,704	4,808	88,749	32,657	23,490
KC6A	149,704	9,011	86,990	33,070	20,633

Several genes have previously been reported to be associated with KC, such as *VSX1*, *SOD1*, *CDH11*, *NUB1*, *HGF*, *COL4A3*, *COL7A1*, *COL6A1*, *COL8A1*, *MMP9*, *MMP2*, *IL1A*, and *IL1B*. The identified variants were annotated to predict the effect or function of an individual SNP using SNP annotation tools. One of the fundamental levels of variant annotation includes categorizing each variant based on its relationship to coding sequences in the genome and how it may change the coding sequence and affect the gene product. The variant can be categorized into four different effect classes

Table 4. 41: Number of variants in genes of interest

Gene NameD2:I18	Total Variants	NONE	SILENT	MISSENSE	NONSENSE
VSX1	4	0	2	2	0
SOD1	1	1	0	0	0
CDH11	6	2	1	3	0
NUB1	9	6	2	1	0
HGF	7	6	1	0	0
COL7A1	6	0	3	3	0
COL6A1	13	5	4	4	0
COL8A1	1	1	0	0	0
MMP9	7	2	2	3	0
MMP2	10	5	5	0	0
IL1A	5	3	0	2	0
IL1B	2	1	1	0	0
COL4A3	23	11	4	8	0
SOD3	6	3	1	2	0
Other Collagen Genes	642	238	213	188	3

NONE: Variants which do not have a direct effect on the protein product (typically variants in non-coding regions).
S I LENT: Variants which do not alter the amino acid in the protein product (mutated codon codes for the same amino acid).
MI S S ENSE: Variants which alter the amino acid in the protein product (mutated codon codes for a different amino acid).
NONSENSE: Variants which introduces a premature stop codon to the protein product (mutated codon codes for a stop/nonsense codon).

4.4.3 Comparison of Variants from This Study with Those in Other Reports

The variants within *VSX1*, *SOD1*, *COL4A3* and *COL4A4* detected in this study are compared to those previously reported by Tanwar *et al.* (2010), Shetty *et al.* (2015), Mok *et al.* (2008), Al-Muammar *et al.* (2015) and Štabuc-Šili *et al.* (2010).

4.4.3.1 The *VSX1* gene

Various publications have reported the association of variants within *VSX1* gene with Keratoconus. Tanwar et al. (2010) reported four nucleotide changes, three of which are also found in the present study. The two

variants reported by Shetty et al. (2015) and the two variants reported by Mok et al. (2008) were not found in this study. The five variants reported by Štabuc-Šilih et al. (2010) are also not found (three in coding region not identified and two in non-coding regions not covered). Table 4.44 lists the single nucleotide variants (SNVs) that are identified from the exome sequencing data of the 24 individuals within the *VSX1* gene in this study.

Table 4. 42: Variants identified within *VSX1* gene in this study

Gene	VSX1_Exon3			
CHROMOSOME	20	20	20	20
SNPEFF_EXON_ID	3	3	3	3
SNPEFF_AMINO_ACID_CHANGE	P237	T222K	R217H	A182
POSITION	25059381	25059427	25059442	25059546
rsID	rs56157240	.	rs6138482	rs12480307
REF NUCLEOTIDE	A	G	C	T
ALT NUCLEOTIDE	T	T	T	C
SNPEFF_IMPACT	LOW	MODERATE	MODERATE	LOW
SNPEFF_FUNCTIONAL_CLASS	SILENT	MISSENSE	MISSENSE	SILENT
SNPEFF_TRANSCRIPT_ID	NM_199425.2	NM_199425.2	NM_199425.2	NM_014588.5
SNPEFF_CODON_CHANGE	ccT/ccA	aCa/aAa	cGc/cAc	gcA/gcG
KC-HIR018	x	x	/	x
KC-HIR023	/	x	x	/
KC-HIR025	x	/	x	x
KC-HIR027	x	x	/	x
KC-HIR036	x	x	x	x
NORM-HIR010	x	x	x	x
NORM-HIR011	/	x	x	/
NORM-HIR019	x	x	x	x
NORM-HIR020	x	x	/	x
NORM-HIR021	x	x	x	x
NORM-HIR022	/	x	x	/
NORM-HIR024	x	x	x	x
NORM-HIR026	/	/	x	/
NORM-HIR028	x	x	/	x
NORM-HIR029	x	x	x	x
NORM-HIR030	x	x	x	x

NORM-HIR031	x	x	x	x
NORM-HIR032	/	x	x	/
NORM-HIR041	/	x	x	/
NORM-HIR045	/	x	x	/
NORM-HIR009	x	x	x	x
NORM-HIR012	/	x	x	/
NORM-HIR013	/	x	x	/
NORM-HIR035	x	x	x	x
x : the sample is homozygous reference				
/: the sample is heterozygous, carrying 1 copy of each of the REF and ALT alleles				
/*: the sample is homozygous alternate				
./. : No genomic data detected				

To facilitate comparison of the variants found in the current study with PCR and Sanger sequencing results, Table 4.45 lists the variants within *VSX1* gene exon 3 for each sample. Column 4 lists one variant per line, and uses up to four different nomenclature to represent the variant, including describing the variant on genomic level, dbSNP ID transcript level, and protein level. Below is the list of variant found in the patients.

Table 4. 43: Variants identified within VSX1 gene exon 3 for each sample comparing NGS and PCR results**.**

Variant	20:25059442C>T NM_199425.2:c.650G>A p.Arg217His (R217H)		20:25059381A>T NM_199425.2:c.711T>A p.Pro237Pro rs56157240 (P237)		20:25059546T>C NM_014588.5:c.546A>G p.Ala182Ala rs12480307 (A182)		20:25059427G>T NM_199425.2:c.665C>A p.Thr222Lys (T222K)	
Family	NGS	PCR	NGS	PCR	NGS	PCR	NGS	PCR
KC28	/	/	x	x	x	x	x	x
KC28A	/	/	x	x	x	x	x	x
KC28B	x	x	x	x	x	x	x	x
KC24	x	x	/	/	x	/	x	x
KC24B	x	x	x	x	x	x	x	x
KC14	/	/	x	x	x	x	x	x
KC14A	x	x	x	x	x	x	x	x

KC14 B	/	/	x	x	x	x	x	x
KC14 C	x	X	x	x	x	x	x	x
KC19	x	/	x	x	/	x	x	x
KC19 A	x	x	/	x	/	x	/	x
HIR01 3	x	x	/	/	/	/	x	x
HIR00 9	x	x	x	x	x	x	x	x
HIR01 0	x	x	x	x	x	x	x	x
HIR01 1	x	/	/	/	/	/	/	/
HIR02 2	x	/	/	/	/	/	x	x
HIR01 2	x	X	/	/	/	/	x	x
HIR03 0	x	X	x	x	x	x	x	x
HIR03 1	x	x	x	x	x	x	x	x
HIR03 2	x	x	x	/	x	/	x	x
HIR03 5	x	x	x	x	x	x	x	x
KC6	x	x	x	x	x	x	x	x
KC6A	x	/	/	/	/	x	x	x
HIR04 5	x	x	/	/	/	/	x	x

KC28, KC24, KC14, KC19, KC6 represent the proband. The letters A, B, C, D represent father, mother, siblings, respectively. All the variants found below is heterozygous. (/) indicates the variant is present, (x) indicates no variant found.

Various publications have reported the association of variants within the *VSX1* gene with KC. Tanwar *et al*. (2010) reported four nucleotide changes, three of which were also found in the present study. The two variants reported by Shetty *et al*. (2015) and the two variants reported by Mok *et al*. (2008) were not found in this study. The five variants reported by Štabuc-Šilih *et al*. (2010) were also not found (three in the coding region not identified and two in non-coding regions not covered). Table 4.3. lists the single nucleotide variants (SNVs) that were identified from the exome sequencing data of the 24 individuals in this study.

Table 4. 44: Variants identified within the VSX1 gene in this study

Nucleotide Change	dbSNP ID	Transcript ID	Protein alteration	Exon	KC* (n=5)	Normal* (n=15)
20:25059381A>T	rs56157240	NM_199425.2	P237	3	0,1	0,6
20:25059427G>T		NM_199425.2	T222K	3	0,1	0,1
20:25059442C>T	rs6138482	NM_199425.2	R217H	3	0,2	0,2
20:25059546T>C	rs12480307	NM_014588.5	A182	3	0,1	0,6

*The two integers (separated by comma) represent the number of samples with homozygous variant (A/A) genotype and the number of samples with heterozygous (R/A) genotype.

To facilitate comparison of the variants found in the current study with PCR-Sanger sequencing results, Table 4.6 lists the variants within the *VSX1* gene exon 3 for each sample. Column 2-5 list one variant per line and uses up to four different nomenclature to represent the variant, including describing the variant on genomic level, dbSNP ID transcript level, and protein level.

Table 4. 45: Variants identified within the VSX1 gene exon 3 for each sample comparing WES and PCR results.

Variant	20:25059442C>T NM_199425.2:c.650G>A p.Arg217His (R217H)		20:25059381A>T NM_199425.2:c.711T>A p.Pro237Pro rs56157240 (P237)		20:25059546T>C NM_014588.5:c.546A>G p.Ala182Ala rs12480307 (A182)		20:25059427G>T NM_199425.2:c.665C>A p.Thr222Lys (T222K)	
Family	WES	PCR	WES	PCR	WES	PCR	WES	PCR
KC28	/	/	x	x	x	x	x	x
KC28A	/	/	x	x	x	x	x	x
KC28B	x	x	x	x	x	x	x	x
KC24	x	x	/	/	x	/	x	x
KC24B	x	x	x	x	x	x	x	x
KC14	/	/	x	x	x	x	x	x
KC14A	x	x	x	x	x	x	x	x
KC14B	/	/	x	x	x	x	x	x
KC14C	x	x	x	x	x	x	x	x
KC19	x	/	x	x	/	x	x	x
KC19A	x	x	/	x	/	x	/	x
KC6	x	x	x	x	x	x	x	x
KC6A	x	/	/	/	/	x	x	x

KC28, KC24, KC14, KC19, KC6 represent the proband. The letters A,B, C, D represent father, mother, siblings, respectively.
All the variants found above are heterozygous. (/) indicates the variant is present, (x) indicates no variant found.

4.4.3.2 The *SOD1* gene

Al-Muammar *et al*. (2015) reported four nucleotide changes in the *SOD1* gene. All these four nucleotide changes are, however, non-coding, and are not targeted by exome sequencing. Table 4.51 lists the single nucleotide variants (SNVs) that are identified from the exome sequencing data of the 24 individuals within the *SOD1* gene in the present study.

Table 4. 46 Only one variant was identified within the *SOD1* gene in the present study (g.9339G>A)

Nucleotide Change	dbSNP ID	Transcript ID	Exon	KC* (n=5)	Normal* (n=15)
21:33041230C>T	rs17880487	NM_000454.4	5	0,1	0,1

*The two integers (separated by comma) represent the number of samples with homozygous variant (A/A) genotype, the number of samples with heterozygous (R/A) genotype.

To facilitate comparison of the variants found in the current study with those detected by PCR-Sanger sequencing, Table above lists the variants within *SOD1* gene for each sample. Column 4 lists one variant per line and uses up to four different nomenclature to represent the variant, including describing the variant on genomic level, dbSNP ID transcript level, and protein level.

The variant was only identified in KC28 and KC28B (son and mother). It was not found in any other of the 24 samples. Al-Muammar et al. (2015) reported four nucleotide changes in the *SOD1* genes. All these four nucleotide changes are, however, non-coding, and are not targeted by exome sequencing. Table 4.49 lists the single nucleotide variants (SNVs) that are identified from the exome sequencing data of the 24 individuals within the *SOD1* gene in the present study.

Table 4. 47: Variants identified within *SOD1* gene in the present study

Gene	SOD1
CHROMOSOME	21

SNPEFF_EXON_ID	5
SNPEFF_AMINO_ACID_CHANGE	.
POSITION	33041230
rsID	rs17880487
REF NUCLEOTIDE	C
ALT NUCLEOTIDE	T
SNPEFF_IMPACT	MODIFIER
SNPEFF_FUNCTIONAL_CLASS	NONE
SNPEFF_TRANSCRIPT_ID	NM_000454.4
SNPEFF_CODON_CHANGE	339
KC-HIR018	x
KC-HIR023	x
KC-HIR025	x
KC-HIR027	/
KC-HIR036	x
NORM-HIR010	x
NORM-HIR011	x
NORM-HIR019	x
NORM-HIR020	x
NORM-HIR021	x
NORM-HIR022	x
NORM-HIR024	x
NORM-HIR026	x
NORM-HIR028	x
NORM-HIR029	/
NORM-HIR030	x
NORM-HIR031	x
NORM-HIR032	x
NORM-HIR041	x
NORM-HIR045	x
NORM-HIR009	x
NORM-HIR012	x
NORM-HIR013	x
NORM-HIR035	x

x : the sample is homozygous reference

/: the sample is heterozygous, carrying 1 copy of each of the REF and ALT alleles

/*: the sample is homozygous alternate

./. : No genomic data detected

*The two integers (separated by comma) represent the number of samples with homozygous variant (A/A) genotype, the number of samples with heterozygous (R/A) genotype.

To facilitate comparison of the variants found in the current study with PCR and Sanger sequencing results, Table 4.50 lists the variants within *SOD1* gene for each

sample. Column 4 lists one variant per line, and uses up to four different nomenclature to represent the variant, including describing the variant on genomic level, dbSNP ID transcript level, and protein level.

The variant is only identified on KC28 and KC28B (from the same family). It is not found in any other 24 samples.

Table 4. 48: Variants identified within *SOD1* gene for each sample.

Variant	21:33041230C>T NM_000454.4:c.*339C>T rs17880487	
Family	NGS	PCR
KC28	/	x
KC28A	x	x
KC28B	/	x

KC28 represent the proband. The letters A, B and C represent father, mother, siblings, respectively. All the variants found below is heterozygous. (/) indicates the variant is present, (x) indicates no variant found.

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4.4.3.3 The COL4A3 GENE

Six of the eight variants in the *COL4A3* gene reported by Štabuc-Šilih *et al.* (2010) were identified in this study. Table 4.51 lists the single nucleotide variants (SNVs) that were identified from the exome sequencing data of the 24 participants in this study.

Table 4. 49:Variants identified within the *COL4A3* gene

Gene	**COL4A3**					
CHROM	2	2	2	2	2	2
SNPEFF_EXON_ID	2	7	9	17	21	21
SNPEFF_AMINO_ACID_CHANGE	G43R	L141P	E162G	D326Y	L399	R408H
POS	228102723	228111435	228113175	228121101	228128540	228128568
ID	rs13424243	rs10178458	rs6436669	rs55703767	rs10205042	rs34505188
REF	G	T	A	G	C	G
ALT	C	C	G	T	T	A
SNPEFF_IMPACT	MODERATE	MODERATE	MODERATE	MODERATE	LOW	MODERATE
SNPEFF_FUNCTIONAL_CLASS	MISSENSE	MISSENSE	MISSENSE	MISSENSE	SILENT	MISSENSE
SNPEFF_TRANSCRIPT_ID	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4
SNPEFF_CODON_CHANGE	Ggg/Cgg	cTg/cCg	gAa/gGa	Gat/Tat	Ctg/Ttg	cGc/cAc
KC-HIR018	x	/*	/*	x	/*	x
KC-HIR023	x	/*	/*	x	/*	x
KC-HIR025	x	/	/	/	/*	x
KC-HIR027	x	/*	/*	x	/*	x
KC-HIR036	x	/	/	x	/	x
NORM-HIR010	x	/*	/*	x	/*	/
NORM-HIR011	x	/*	/*	x	/	x
NORM-HIR019	x	/*	/*	x	/*	x
NORM-HIR020	x	/*	/*	x	/*	x
NORM-HIR021	x	/*	/*	x	/*	x
NORM-HIR022	/	/	/	x	/*	x
NORM-HIR024	x	/*	/*	x	/*	x
NORM-HIR026	/	/*	/*	x	/*	x
NORM-HIR028	x	/*	/*	/	/	x

NORM-HIR029	x	/	/	x	/*	x
NORM-HIR030	/	/*	/*	/	/	x
NORM-HIR031	x	/*	/*	/	x	x
NORM-HIR032	x	/*	/*	/	x	x
NORM-HIR041	x	/	/	/	x	x
NORM-HIR045	x	/	/	x	/	x
NORM-HIR009	x	/*	/*	x	/	x
NORM-HIR012	x	/*	/*	x	/*	x
NORM-HIR013	x	x	x	/*	x	x
NORM-HIR035	x	/	/	/	/	x

Gene	COL4A3					
CHROM	2	2	2	2	2	2
SNPEFF_EXON_ID	22	22	23	25	26	42
SNPEFF_AMINO_ACID_CHANGE	H451R	H451	G484	P574L	Q621	M1209I
POS	228131169	228131170	228131752	228135631	228137769	228162451
ID	rs11677877	rs189364374	rs34019152	rs28381984	.	rs200562865
REF	A	C	G	C	A	G
ALT	G	T	A	T	G	A
SNPEFF_IMPACT	MODERATE	LOW	LOW	MODERATE	LOW	MODERATE
SNPEFF_FUNCTIONAL_CLASS	MISSENSE	SILENT	SILENT	MISSENSE	SILENT	MISSENSE
SNPEFF_TRANSCRIPT_ID	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4
SNPEFF_CODON_CHANGE	cAc/cGc	caC/caT	ggG/ggA	cCg/cTg	caA/caG	atG/atA
KC-HIR018	x	x	x	x	x	x
KC-HIR023	x	x	x	/	x	x
KC-HIR025	x	x	x	/*	x	x
KC-HIR027	x	/	x	/*	x	x
KC-HIR036	x	x	x	/	x	x

NORM-HIR010	/	x	/	/	x	x
NORM-HIR011	x	x	x	/	x	x
NORM-HIR019	x	x	x	x	x	x
NORM-HIR020	x	x	x	/	x	x
NORM-HIR021	x	x	x	x	x	x
NORM-HIR022	x	x	x	/*	x	x
NORM-HIR024	x	x	x	/*	x	x
NORM-HIR026	x	x	x	/	/	x
NORM-HIR028	x	/	x	/	x	/
NORM-HIR029	x	x	x	/	x	x
NORM-HIR030	x	x	x	/	x	x
NORM-HIR031	x	x	x	x	x	x
NORM-HIR032	x	x	x	x	x	x
NORM-HIR041	x	x	x	x	x	x
NORM-HIR045	x	x	x	/	x	x
NORM-HIR009	x	x	x	/	x	x
NORM-HIR012	x	x	x	/*	x	x
NORM-HIR013	x	x	x	/	x	x
NORM-HIR035	x	x	x	/	x	x

Gene	COL4A3					
CHROM	2	2	2	2	2	2
SNPEFF_EXON_ID	52	52	52	52	52	52
SNPEFF_AMINO_ACID_CHANGE	.	.	.	.	.	.
POS	228176872	228176901	228177479	228177567	228177751	228177825
ID	rs6436677	rs2070735	rs7587228	rs1134745	rs28554165	rs10188531
REF	C	A	C	C	G	C
ALT	T	C	T	T	A	G
SNPEFF_IMPACT	MODIFIER	MODIFIER	MODIFIER	MODIFIER	MODIFIER	MODIFIER

SNPEFF_FUNCTIONAL_CLASS	NONE	NONE	NONE	NONE	NONE	NONE
SNPEFF_TRANSCRIPT_ID	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4
SNPEFF_CODON_CHANGE	286	315	893	981	1165	1239
KC-HIR018	x	/*	/*	/*	x	./.
KC-HIR023	/	/	/	/	x	/*
KC-HIR025	x	/*	/*	x	x	./.
KC-HIR027	x	/*	/*	x	/*	/*
KC-HIR036	/	/	/	x	./.	./.
NORM-HIR010	x	/	/	x	/	./.
NORM-HIR011	/	/	x	x	x	./.
NORM-HIR019	x	/*	/*	/*	x	/*
NORM-HIR020	x	/*	/*	/	x	./.
NORM-HIR021	x	/*	/*	/*	x	./.
NORM-HIR022	x	/*	/*	x	/*	./.
NORM-HIR024	/	/	/	x	./.	./.
NORM-HIR026	x	/*	/*	x	x	./.
NORM-HIR028	x	/*	/*	x	/	/*
NORM-HIR029	x	/*	/*	/	/	/*
NORM-HIR030	x	/*	/*	x	/*	./.
NORM-HIR031	/	/	/	x	x	./.
NORM-HIR032	/	/	/	x	x	/*
NORM-HIR041	/	/	/	x	x	./.
NORM-HIR045	/	/	/	x	/	./.
NORM-HIR009	/	/	/	x	/*	./.
NORM-HIR012	x	/*	/*	x	./.	./.
NORM-HIR013	x	/*	/*	x	x	./.
NORM-HIR035	x	/*	/*	x	x	./.

Gene	**COL4A3**				
CHROM	2	2	2	2	2
SNPEFF_EXON_ID	52	52	52	52	52
SNPEFF_AMINO_ACID_CHANGE	.	.	.	.	.
POS	228178645	228178780	228179038	228179238	228179328
ID	rs4290648	rs7567291	rs4470338	rs57817160	rs59257065
REF	C	A	T	G	C
ALT	G	C	C	A	T
SNPEFF_IMPACT	MODIFIER	MODIFIER	MODIFIER	MODIFIER	MODIFIER
SNPEFF_FUNCTIONAL_CLASS	NONE	NONE	NONE	NONE	NONE
SNPEFF_TRANSCRIPT_ID	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4	NM_000091.4
SNPEFF_CODON_CHANGE	2059	2194	2452	2652	2742
KC-HIR018	./.	/*	/*	x	x
KC-HIR023	./.	/*	/	x	x
KC-HIR025	/*	/*	x	/*	/*
KC-HIR027	./.	/*	x	x	x
KC-HIR036	/*	/*	x	x	x
NORM-HIR010	./.	/*	x	x	x
NORM-HIR011	./.	./.	x	x	x
NORM-HIR019	./.	./.	/*	x	x
NORM-HIR020	./.	/*	/	/	/
NORM-HIR021	./.	/*	/*	x	x
NORM-HIR022	./.	./.	x	x	x
NORM-HIR024	./.	/*	x	/	/
NORM-HIR026	./.	/*	x	/*	/*
NORM-HIR028	./.	/*	x	/	/
NORM-HIR029	./.	/*	/	x	x
NORM-HIR030	./.	/*	x	/	./.

NORM-HIR031	./.	./.	x	/	./.
NORM-HIR032	/*	/*	x	/	/*
NORM-HIR041	./.	./.	x	/	/
NORM-HIR045	./.	/*	x	x	x
NORM-HIR009	./.	/*	x	x	x
NORM-HIR012	./.	/*	x	x	./.
NORM-HIR013	./.	/*	x	/	x
NORM-HIR035	./.	./.	x	/*	/*

Legend

x : the sample is homozygous reference

/: the sample is heterozygous, carrying 1 copy of each of the REF and ALT alleles

/*: the sample is homozygous alternate

./. : No genomic data detected

Table 4. 50:Variants identified within the *COL4A3* gene

Nucleotide Change	dbSNP ID	Transcript ID	Protein alteration	Exon	KC* (n=5)	Normal* (n=15)
2:228102723G>C	rs13424243	NM_000091.4	G43R	2	0,0,0	0,3,3
2:228111435T>C	rs10178458	NM_000091.4	L141P	7	3,2,8	11,4,26
2:228113175A>G	rs6436669	NM_000091.4	E162G	9	3,2,8	11,4,26
2:228121101G>T	rs55703767	NM_000091.4	D326Y	17	0,1,1	0,5,5
2:228128540C>T	rs10205042	NM_000091.4	L399	21	4,1,9	8,4,20
2:228128568G>A	rs34505188	NM_000091.4	R408H	21	0,0,0	0,1,1
2:228131169A>G	rs11677877	NM_000091.4	H451R	22	0,0,0	0,1,1
2:228131170C>T	rs189364374	NM_000091.4	H451	22	0,1,1	0,1,1
2:228131752G>A	rs34019152	NM_000091.4	G484	23	0,0,0	0,1,1
2:228135631C>T	rs28381984	NM_000091.4	P574L	25	2,2,6	2,8,12
2:228137769A>G		NM_000091.4	Q621	26	0,0,0	0,1,1
2:228162451G>A	rs200562865	NM_000091.4	M1209I	42	0,0,0	0,1,1
2:228176872C>T	rs6436677	NM_000091.4		52	0,2,2	0,6,6
2:228176901A>C	rs2070735	NM_000091.4		52	3,2,8	8,7,23
2:228177479C>T	rs7587228	NM_000091.4		52	3,2,8	8,6,22
2:228177567C>T	rs1134745	NM_000091.4		52	1,1,3	2,2,6
2:228177751G>A	rs28554165	NM_000091.4		52	1,0,2	2,4,8
2:228177825C>G	rs10188531	NM_000091.4		52	2,0,4	4,0,8
2:228178645C>G	rs4290648	NM_000091.4		52	2,0,4	1,0,2
2:228178780A>C	rs7567291	NM_000091.4		52	5,0,10	10,0,20
2:228179038T>C	rs4470338	NM_000091.4		52	1,1,3	2,2,6
2:228179238G>A	rs57817160	NM_000091.4		52	1,0,2	1,7,9
2:228179328C>T	rs59257065	NM_000091.4		52	1,0,2	2,4,8

*The three integers (separated by comma) represent the number of samples with homozygous variant (A/A) genotype, the number of samples with heterozygous (R/A) genotype, and the number of alternative alleles (each diploid sample has two alleles, homozygous genotypes count as 2 while heterozygous genotypes count as 1)

4.4.4 Comparison of Current Study With PCR-Sanger Sequencing

Table 4.53 lists the variants within *COL4A3* gene exon 17 and exon 25 respectively for each sample.

Table 4. 51: Variants identified within *COL4A3* gene exon 25 for each sample

Variant	2:228135631C>T rs28381984 NM_000091.4(COL4A3):c.1721C>T (p.Pros574Leu) P574	
Family	NGS	PCR (Not Done)
KC28	/	
KC28A	/	
KC28B	/	
KC24	/*	
KC24B	/*	
KC14	x	
KC14A	x	
KC14B	/	
KC14C	x	
KC19	/*	
KC19A	/	
KC6	/	
KC6A	×	

KC28, KC24, KC14, KC19, KC6 represent the proband. The letters A, B, C, D represent father, mother, siblings, respectively. All the variants found above are heterozygous except when indicated as (*) which denotes a homozyous variant, (/) indicates the variant is present, (x) indicates no variant found,

It is that though COL4A3 Exon 17 was chosen for PCR sequencing, none of the 24 KC and family members had the variant. Unlike in WES, the COL4A3 Exon25 yielded more results in the same group. Unfortunately, this was only discovered after PCR sequencing was done. Perhaps, in future PCR work, this exon can be further explored.

Table 4. 52: Variants identified within *COL4A3* gene exon 17 for each sample

Variant	2:228121101G>T rs55703767 NM_000091.4:c.976G>T p.Asp326Tyr	
Family	NGS	PCR
KC28	x	x
KC28A	/	x
KC28B	x	x
KC24	x	x
KC24B	x	x
KC14	x	x
KC14A	x	x
KC14B	x	x
KC14C	x	x
KC19	/	x
KC19A	x	x
HIR013	/*	x
HIR009	x	x
HIR010	x	x
HIR011	x	x
HIR022	x	x
HIR012	x	x
HIR030	/	x
HIR031	/	x
HIR032	x	x
HIR035	/	x
KC6	x	x
KC6A	/	x
HIR045A	x	x

KC28, KC24, KC14, KC19, KC6 represent the proband. The letters A, B, C, D represent father, mother, siblings, respectively. All the variants found below is heterozygous. (/) indicates the variant is present, (x) indicates no variant found, (*) indicates the variant found is homozygous.

4.5 Summary and Discussion: Comparison of Results (Tables 4.53-4.54)

The concordance between VSX1 between PCR and WES is >75% with the data obtained from Exome sequencing. The SNPs location is based on the study and reported publication from Tanwar et al 2010.

Various publications have reported the association of variants within the *VSX1* gene with KC. Tanwar *et al*. (2010) reported four nucleotide changes, three of which were also found in the present study. The two variants reported by Shetty *et al*. (2015) and the two variants reported by Mok *et al*. (2008) were not found in this study. The five variants reported by Štabuc-Šilih *et al*. (2010) were also not found (three in the coding region not identified and two in non-coding regions not covered). (Table 4.44).

From this study, the other reported mutations in SOD1 and COL4A3 genes (Stabuc-Silih et al 2010), that is ,COL4A3exon9 (E162G) was present in all except HIR013.

From the whole exome sequencing results, the mutations appear to be random within the group. Table 4.51 lists the single nucleotide variants (SNVs) g.9339G>A, that was identified from the exome sequencing data of the 24 individuals within the *SOD1* gene in the present study. (table 4.48)

Mutation found in COL4A3 Exon25 (p.P574) (rs28381984) however, is interesting. It was present in 4 members of the family of the proband.K6 (4/4:100%) . 4KC (4/4:100%) but only 60%(10/15) of Family controls.

The SNPs locations in VSX1 are similar to those in the PCR-sequencing results: Three VSX1variants , A182A (rs 1248030307) , p.P237P(rs 56157240) and R217H (rs6138482). Were identified.

In this study, KC cases and different non-KC control groups were examined by targeted gene PCR-sequencing and whole exome sequencing. Selection was based on gene mutations previously reported , associated with the KC phenotype. The overall results showed some association and linkiage of KC with some of these mutations.

There is however, no association of KC with rare or novel mutations, not reported before in medical literature.

Owing to the formidable cost of large volume PCR-sequencing, only 3 targeted genes, VSX1, SOD1 and COL4A3 with specific exons, were also screened in 5KC patients and 19 Family controls by Exome Sequencing as a pilot study.

With whole exome sequencing, it was possible to look at a larger number of genes for previously identified gene variants. However, this pilot study identified specific variants linked to KC and their family members. COL4A3 Exon 25 and Exon52

The variants found in KC patients appeared not to be dominant alleles conforming to Mendelian inheritance. A possible explanation for this observation is that the KC phenotype is linked not to the presence of the gene but to its expression.

In heterozygous individuals, the alleles are different in each copy of the gene. Assuming that both gene copies are equally expressed at any given time, a heterozygous parent may express the gene from the non-variant copy, while the

heterozygous child expresses the gene from the variant copy (or vice versa), resulting in the child developing KC while the parent may appear to be normal. If this was the case, future further investigations with RNA sequencing for gene expression might lead to a clearer understanding of the genetic determinants of KC.

In addition , it is interesting to note that KC6 (proband) had clinical phenotype features of keratoconus yet did not have the 2 gene variant (A182) and (P9237) found in family studies . It is important to consider that the variant COL4A3 exon 25 may have a complementary role in expression of keratoconus phenotypes in KC6.

The variants in the VSX1 exon 3 obtained by PCR-sequencing and whole exome sequencing showed a concordance of >75%. The 2 SNPs, A182A *(pvalue 0.026*.OR3.14(CI 95%, 1.14- 7.60), P237P (*pvalue0.0.12* OR 3.61(CI 95%, 1.31-9.94) shows higher risk for KC in Family controls having these 2 VSX1 variants.

With third variant R217H (*p value0.0.004*,Inverse Ratio OR=11.58 is 0.086,(CI 95%, ,4.21-31.88) suggests low risk ,and perhaps “protective role” of this gene variant against KC in Family controls.

In this study, KC patients and different groups of non-KC controls were examined by targeted gene PCR-sequencing and whole exome sequencing for gene mutations previously reported to be associated with the clinical manifestation of KC. The overall results showed some linking of KC with some of these mutations.

There is however, no association of KC with rare mutations not reported before in medical literature. Owing to the formidable cost of large volume PCR-sequencing, only 3 targeted genes, VSX1, SOD1 and COL4A3 with specific exons, were screened for their occurrence in 5KC patients and 19 Familycontrols by Exome Sequencing as a pilot study.

With whole exome sequencing, it was possible to look at a larger number of genes for previously identified gene variants. However, this exploration also failed to identify specific variants linked to KC and their family members. The variants found in KC patients appeared not to be dominant alleles conforming to Mendelian inheritance. A possible explanation for this observation is that the KC phenotype is linked not to the presence of a gene but to its expression.

In heterozygous individuals, the alleles are different in each copy of the gene. Assuming that both gene copies are equally expressed at any given time, a heterozygous parent may express the gene from the non-variant copy, while the heterozygous child expresses the gene from the variant copy (or vice versa), resulting in the child developing KC while the parent appears to be normal. If this was the case, future further investigations with RNA sequencing for gene expression might lead to a clearer understanding of the genetic determinants of KC.

In addition , it is interesting to note that KC6 (proband) had clinical phenotype features of keratoconus yet did not have the 2 gene variant (A182) and (P9237) found in family studies (chapter 2). It is important to consider that the variant COL4A3 exon 25 may have a complementary role in expression of keratoconus phenotypes in KC6.

The variants in the VSX1 exon 3 obtained by PCR-sequencing and whole exome sequencing showed a concordance of >75%. The 2 SNPs, A182A *(pvalue 0.026*.OR3.14(CI 95%, 1.14- 7.60), P237P (*pvalue0.0.12* OR 3.61(CI 95%, 1.31-9.94) shows higher risk for KC in Family controls having these 2 VSX1 variants. With third variant R217H (*p value0.0.004*,Inverse Ratio OR=11.58 is 0.086,(CI 95%, ,4.21-31.88) suggests low risk ,and perhaps "protective role" of this gene variant against KC in Family controls.

CHAPTER 5 SUMMARY AND CONCLUSION

5.1 Clinical Keratoconus

This study has been a challenging journey of discovery for a Malaysian ophthalmologist. From an intense desire to help patients with keratoconus (KC) improve their vision, an attempt was made to gather useful information from clinical observations, ophthalmic examinations, and state-of-the art surgical procedures on the eye. In addition, potential risk factors including genetic predisposition was studied with a questionnaire survey and detection of genes previously reported to be associated with KC. Despite a relatively small cohort of KC patients, their family members and convenience non-KC controls, it was possible to identify eye-rubbing as an important behavioral risk factor, demonstrate the effectiveness of crosslinking followed by using RGPs for correcting, maintaining vision and preventing further visual deterioration,. It was also possible to associate the occurrence of KC with previously reported and unreported variants in the *VSX1* gene. Besides this, the small pilot study using Exome Sequencing on just 5 KC and 19 Family members, also served to give new light and understanding into the possibility of multiple genes being involved in the aetiopathogenesis of keratoconus.

The clinical study clearly showed that the diagnosis of KC could be easily missed owing to the varied clinical presentations of the disease. In the early stages, the disease was often indicated only by the patient's inability to achieve useful and correctable vision with spectacles after refraction. As the disease advanced, there would be progressive distortion of vision and further,

gradual loss of visual acuity. However, KC per se, was seldom the cause of total blindness. In all patients, early detection and early treatment resulted in almost normal vision and this improved the quality of life for patients, when they were no longer “visually-handicapped”. It was gratifying to see young KC patients resuming their studies to become engineers, scientists, doctors, and executives, with further education and training.

The KC population in this study reflected most of the characteristics of KC populations described in literature. The disease was twice as common in men than in women; the mean age was around 25 years; the corneal steepness was variable, but within the range of 46-72 dioptres. Although the KC cohort was small, the ethnic composition ratio of 6:3:1 for Indians, Malays and Chinese was consistent with the high proportion of KC among Indians reported elsewhere in literature. This possible ethnic bias deserves further future investigations.

Advanced technology has enabled early detection and treatment of KC. Since 2005, treatment with crosslinking (CXL) in KC cornea has helped to halt KC progression, and, together with the use of Rigid Gas Permeable (RGP) lenses, has improved corneal molding and optimized as well as maximized PostCXL vision.

In this study, the EPI-OFF Dresden Protocol was applied for all KC corneas resulting in success in every case. CXL halted and prevented further corneal thinning and reduced Kmax and astigmatism. With RGP or semiscleral lenses, patients were able to achieve up to 80 - 95% normal vision maintained

for at least six to ten years. Moreover, when the cornea was measured one year post CXL, it was found to be thicker than what it was immediately post-CXL.

This increase in thickness is likely to be due to the RGP lens flattening and remolding the corneal surface, resulting in the thickness being redistributed.

Although many ophthalmic surgeons perform either LASIK or PRK to correct myopia and astigmatism before CXL, these refractive procedures were not performed on the KC patients in this study. This important preventive and prophylactic decision was taken by the author , very early on. The reason was there is always the possibility that patients could have KC progression, and keratectasia after the Laser refractive procedure, due to iatrogenic-induced thinning of .the cornea, as part of the management to correct the refractive error. In addition, once the KC progression as halted , and refractive power was stabilized , safer procedures like INTACS corneal inserts, and Phakic IOLs are good alternatives. In fact once, the KC patient reaches beyond 50 years of age, Clear Lensectomy with IOL Implantation is definitely the answer, as patient will receive his final correction, and good vision as after Cataract Surgey.

5.1.1 Risk Factors- Role of Allergy and Atopy in Keratoconus

This study, through the questionnaire findings support the importance of eye rubbing as a major risk factor in the development of KC among Malaysians. It is known that intense eye rubbing is often practiced in Indian and Asian cultures such as Pakistanis and Iranians, among whom, it occurs spontaneously before sleep, on awakening and through the day, in response to emotional stress or ocular irritation or fatigue (Shetty, 2017). This response can also be due to dryness and itching or can be psychogenic and compulsive (Hawkes, 2014).

Eye rubbing has been related to higher KC rates in these cultures. It presumably disrupts the anterior corneal mosaic to cause loss of surface epithelium, disruption of the corneal clarity due to fibrous reactions and weakening of the biomechanical strength of the anterior lamellae of the stroma.

In this study, eye rubbing was a habit and practice for itchy eyes. KC cases had eye rubbing as well, and more severe rubbing in 78.6% of KC cases, in contrast to Family controls(50%) and Normal controls (58.3%) . This seems to indicate the importance of the mechanical contact in eye rubbing, resulting in loss of anterior mosaic cells., as proven by Koyabashi's confocal microscopy studies.

In the final analysis, of this study, eye rubbing KC seems to be 4.85 times increased risk compared to normal controls, Eye itchiness is 4.39 times increased risk and eye wateriness is 19.8% as a risk factor in KC cases in comparison to normal controls.

In contrast, significant association was not established with eye itchiness, redness, and nose block, although there was a significant association with asthma as well as skin and food allergy.

5.1.2 Corneal Biomechanical Features and CXL

This study suggests that 'the natural history of KC (evolution from a normal shape to the KC is associated with progressive modification of the cornea's biomechanical properties, reflected in decreased CH and CRF, and the increase in the difference between CH and CRF (Saad, *et al.*, 2018).

Corneal Hysteresis (CH) is reduced temporarily in the post-CXL KC eye. The exact time period, has still to be established. This however, enables the

KC cornea to become regularised and become a biomechanically stronger. Through this study, it can be seen that “time” is a very important factor for the cornea to regain the weakened crosslinks , due to KC progression, and regain its biomechanical strength Thus, though biomechanical strength can be gained over time, “How much” in quantum remains as an unanswered question.

PostCXL Biomechanical changes in the cornea

Initially corneal thinning was observed Post-CXL , a finding that is consistent with the results of studies by Raiskup *et al*.(2011) and Tobaas *et al*. (2016). This study however, shows about six months to one year later, there was slight thickening of the cornea due to the regularisation of the crosslinked cornea, and also flattening by the RGPs. Overall, the Post-CXL group still had visual acuity of LogMAR 0.5 -1 (6/15- 6/60) because of residual astigmatism.

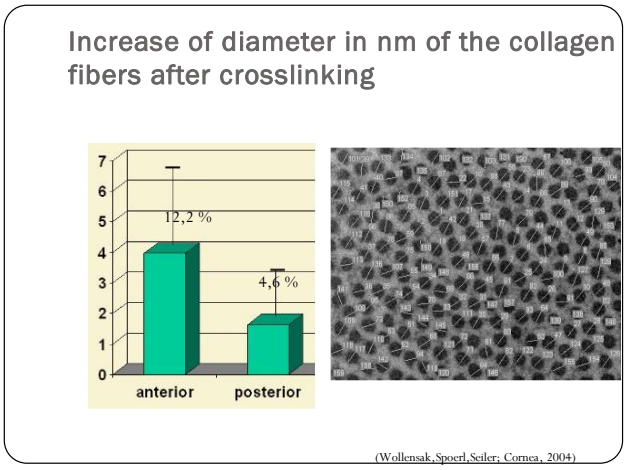

Figure 5. 1: Initially the collagen fibrils swell after CXL treatment , but by 6-2 months, may decrease back to original thickness
Source: Wollensak et al (2004)

Changes After CXL

Immediately - secondary to photochemical changes , there is corneal stiffness, resistance against enzymatic degradation , - shrinking temperature,- swelling behaviour

-24 hours to 15 weeks, -apoptosis of keratocytes,-migration of active keratocytes, -flattening of the cornea , -K-value and astigmatism changes, and appearance of the demarcation line.

After Crosslinking the corneal biomechanics change, over time and are clinically observed in the following manner,increase of tangential stiffness, remodeling of the cornea,flattening of the cornea,central displacement of the apex,regularisation of corneal surface,improvement of contact lens tolerance, improvement of itching sensations.

This study differs from all other studies, particularly in how vision is maximized with RGPs to give superior quality of vision, with all residual astigmatism corrected.

This study also attempted to emphasize the long-term lasting effect of CXL, through a review of 42 cases managed by the same ophthalmologist in two private eye centres. It showed that the CXL effectiveness is mainly to halt any further KC progression by improving corneal biomechanical interlamellar restructuring.

The cross-linked cornea behaves differently from the “virgin” progressive and untreated KC cornea. By the application of CXL, the progression is halted, owing to the increased crosslinks and rearrangement of the corneal lamellae that cause the cornea to stop thinning. The cornea instead becomes “regularized, as the apex of the cone migrates towards the centre of the cornea”. This is further enhanced and maximized especially vision-wise, as the patient begins wearing RGPs. The surface of the KC cornea is modified, and becomes even less astigmatic and more spherical. A sort of “corneal molding”

occurs as in Ortho-Keratology. In fact, astigmatism is observed to be reduced by as much as 3-5dioptres, more than any myopic reduction due to flattening caused by just RGP fitting.

Visual acuity improves and sharpens as the process of healing continues. This is stabilized by about 6 months to one year. This is still further maintained by the continual wearing of the RGPs as evidenced and recorded in this study.

5.1.3 The Effect of CXL On Progressive KC

In progressive KC, the tensile strength of the collagen fibrils reduces due to the progressive interlamellar slipping of collagen fibrils. The riboflavin used in the Riboflavin-Dextran solution acts as the photosensitizer to facilitate the crosslinking down to a corneal depth of at least 300u (Spoerle, 2003, Spaedea 2013).

The lastingness and effectiveness of CXL as first claimed by Wollensak, Spoerle, and Theo Seiler (2005), and later by Raiskup (2009), has of late been challenged by more recent researchers and clinicians. Arbelaez *et al*. (2009) claimed that the halting effect of CXL on KC could last only about one year under certain conditions and not longer. However, the lasting effect of CXL was evident in this Malaysian study. There were significant reductions in the Post-CXL K_{MAX} as described by Wollensak *et al*. (2005) and Wollensak and Spoerle (2003), that effectively reduced the myopic astigmatism in KC patients.

Previous studies also highlighted inconsistencies in the degree of corneal steepness reduction and spherical correction achieved in CXL. Wollensak *et al*. (2003), in a review of baseline patient characteristics, reported that eyes with

preoperative K_{Max} of 55 D or steeper were 5.4 times more likely to gain 2 D or more of K_{Max} flattening at 1 year after CXL.

Spaedea *et al*. (2016) in a review article, stressed the demarcation line (DL) in the stroma as the depth of crosslinking effect. Kymionis *et al.* (2009) compared the depth of stromal DL using both AS-OCT and confocal microscopy, studying the transition area between cellular and acellular zones. This however, showed there was no statistically significant difference between the 2 measurements.

Caporossi A (2005) in an Italian study of CXL treatment in 12 advanced KC cases, reported "the treatment has been associated with significant improvement in uncorrected visual acuity(UCVA) and best spectacle -corrected visual acuity (BSCVA) in all patients along with reduced corneal steepness and improvements in irregularity and coma." He further commented that crosslinking procedure is designed to address the fact that biomechanical resistance of keratoconic eyes is 50% lower than that of normal eys. Results from The Sienna study which included Dr Mazzotta, also observed an average 2-line increase in BSCVA and a 2.4 line improvement in UCVA. Even the mean Kmax readings was reduced by approximately 2dioptres.

However, Post-CXL ORA readings were consistent with Goldrich et al (2009) that the CH and CRF readings were not significantly different from the Pre-CXL readings. Instead CXL treatment seemed to give variable effects and results, more in the clinical changes in the astigmatism and the flattening of the cornea and reduction in myopic power in the post-CXL patients.

In summary, the average myopic astigmatism was reduced by at least 1-3.00 dioptres. But full visual potential was obtained in all cases who were able to adapt to KC RGP lenses and wear them successfully. The CXL halted the KC progression, and regularized the cornea, enabling the RGP to remain more centrally on the cornea. The result was stabilization and maintenance of the corneal thickness and refractive power in the KC patient.

Vinciguerra P et al (2012) found Mean UCVA and BSCVA at 2 years, were 0.58 ± 0.18 and 0.20 ± 0.09, respectively. The improvement in UCVA and BSCVA was significant throughout the postoperative follow-up ($P < .05$). Mean spherical equivalent refraction showed a significant decrease of 1.57 diopters (D) at 24 months ($P = .02$). Mean baseline simulated keratometry was 46.32 D in the flattest meridian and 51.48 D in the steepest meridian; at 2 years, the values were 45.30 D ($P = .04$) and 50.21 D ($P = .07$), respectively.

Vincegurerra R et al (2017) observed that his Post-CXL cases Post CXL reduction in Astigmatism is produced in both eyes in variable amounts. The mean change enhances the vision, but optimized correction is only possible with the wearing of RGPs. Post-CXL patients with additional correction with RGPs (Post-RGP Group) were able to achieve LogMAR 0.1 (6/7.5 - 6/6) vision. Once the Post-RGP group used the KC-RGP Lenses the irregular astigmatic corneal surface becomes aspheric. The visual acuity in the Post-CXL group finally gained about 95% vision with the RGPs.

Many different researchers and eminent ophthalmologists continue to innovate CXL into their practices. One application especially in the field of refractive surgery, CXL has become of important usage in Iatrogenic

kerectactesia, due to LASIK surgery, on high myopic patients. These highly myopic cases already have thinner compromised corneae, and CXL strengthens the biomechanics again.

This study has shown the effectiveness of CXL in KC treatment and has shown how it is the gold standard for stabilizing and halting the progression of KC. In addition, the important role of RGPs and Semisclerals is again seen. Together with other optical devices like phakic IOLs and intracorneal rings in suitable cases, almost 100% visual recovery is possible.

This study differs from all other studies, particularly in how vision is maximized with RGPs to give superior quality of vision, with all residual astigmatism corrected.

This study also attempted to emphasize the long-term lasting effect of CXL, through a review of 42 cases managed by the same ophthalmologist in two private eye centres.. It showed that the CXL effectiveness is mainly to halt any further KC progression by improving corneal biomechanical interlamellar restructuring.

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KC. In addition, the important role of RGPs and Semisclerals is again seen. Together with other optical devices like phakic IOLs and intracorneal rings in suitable cases, almost 100% visual recovery is possible.

This study also attempted to emphasize the long-term lasting effect of CXL, through a review of 42 cases managed by the same ophthalmologist in two private eye centres. It showed that the CXL effectiveness is mainly to halt any further KC progression by improving corneal biomechanical interlamellar restructuring. Furthermore, the results of this study also stress the importance of RGP contact lenses giving an 'additive effect" and "superior quality of vision" after CXL therapy

5.1.4 Conclusions on Clinical Keratoconus

There is still much to be learnt and understood, especially in the ever-evolving techniques and protocol modification changes in CXL. However, corneal CXL of the KC cornea with the Dresden Protocol has proven its efficiency and efficacy. The superior quality of vision attained by the smooth anterior surfaces of optical devices like RGPs and semisclerals complement and overcome the PostCXL residual astigmatism, thus enhancing and maximizing vision in KC management.

Clinically, the visual handicaps and astigmatic distortions in KC patients have been overcome by this combined therapy of CXL and RGP lenses. With this treatment regime modality available to all KC patients, they can face the future with confidence and enjoy a better quality of life with better and maximized vision.

5.2 Candidate Genes

The Genetics study was an attempt to find a genetic basis for KC in the patients recruited in this study. As reported by other researchers, three variants in the *VSX1* gene were found in KC patients using targetted gene PCR-sequencing. Although these variants were found in family and normal controls as well, with SNPSTATs analysis, it was possible to show a significantly increased (3-4 x) probability of KC development in patients who harboured the A182A(rs12480307) and p.P237P(rs56157240) variants and a possible protective effect of the third variant, R217H (rs6138482). It was interesting to note that A182A and P237P gene variants always appear as a pair, in cases and controls. However, the significance of occurrence in this manner is not fully understood. These results need to be confirmed with further studies and on larger populations.

The use of target gene PCR sequencing enabled the screening of all KC cases and controls, but. the information derived was limited to only a few selected candidate genes, namely VSX1, SOD1 and COL4A3. Both SOD1 and COL4A3 both yielded poor or negative results.

5.2.1 Summary and Discussion

It is conclusively seen through this study that Keratoconus has a genetic aetiology, among other risk factors.. It is possible however that KC is a polygenic disease with different genes independently inherited and differently expressed. Possible future research with Whole Exome Sequencing combined with Whole Genome Sequencing will enable this to be validated.

Independent replication of association between any 2 SNPs and Keratoconus, can support the association of their loci with the risk of disease

development. Genes definitely have a role in Keratoconus aetiology. However, further research needs to be done, to understand the role of genetic factors involved. For all VSX1 Gene variants, results seem to support an "additive genetic model", since secondary analysis under assumption of different models of inheritance, (dominant or recessive) failed to substantially improve the strength of association.

Four targeted Gene Variants were comparatively studied in KC cases and three control groups based on findings in other published studies.

This Malaysian Study shows similarity with Liscova Petal's study with findings from a cohort of Czech KC patients in 2017. This study has also revealed the fact that whether, minor (recessive) or major (dominant) allele, the Gene variant p.R217H (rs 6138482) seems to be in higher frequency in Normal controls, than in KC. The OR of 0.086 with pvalue <0.05, indicates perhaps this VSX1 variant has a protective role.

The presence of P237P with A182A as a pair, whenever one presents, the other also is present, may pose both as complementary, and give the higher risk for KC aetiopathogenesis. In contrast, the Family controls the OR 3.410.086, CI 95%(0.0313 – 0.237) and p value< 0.0001., seems to indicate that this variant may be a risk factor for KC development. In fact

The presence of both of the SNPs, A182A (rs 12480307) and p.P237P (rs56157240) which seem to be present in higher proportion in KC patients. They appeared always in a pair together, indicating perhaps a "strong linkage association" as seen in the haplotype block, wherin two Haplotype alleles were in association. This was proven through Haploview association software.

In this study, two of these SNPs, p.A182A and p.P237P were found more

frequently in Keratoconus subjects (p value= 0.012). Wheras p.R217H was more common in Normal controls than keratoconus, cases .All the VSX1 SNPs conformed to HWE (p>0.05). disequilibrium.

In Association test above this revealed that p.A182A(rs12480307) and p.P237P (rs56157240) were significantly associated with Keratoconus (pvalue 0.001). Also, it is seen that KC patients carrying the mutant allele for A182A and P237P were approximately 3.16 - 4 times higher than that of controls.

Lingkage Analysis and association studies, are two different important approaches to identify novel gene Association studies focus on the relation between a specific allele and the disease/trait within a population. The independent replication of association between any SNP and KC can support the association of the SNP with the risk of disease development.

In this study, four previously much researched *VSX*1Exon3 gene variants [p.R217H(rs6138482), p.P237P(rs56157240) p.A182A(12480307) and IVS64-24 C>T (201363715)] were studied in different control groups. No separate analyses were done for ethnicity and gender as the number of KC cases were too small. None of the four variants were statistically linked to pathogenicity when KC cases were compared to control groups.

Association studies showed the allele in the gene variant p.R217H (rs6138482), whether it is a major (dominant) or minor (recessive) allele, to be present more frequently in normal controls than in KC or family. This suggests a “protective role” of the variant., as the OR was 0.086 and pvalue <0.05. On the other hand, the other two SNPs, A182A (rs 12480307) and p.P237P (rs56157240) were present together three to four times more frequently in KC

patients than controls (pvalue 0.001). This could be an indication of a “strong linkage association” between KC and this pair of SNPs.

For these *VSX*1Exon3 variants, the results seemed to support an “additive genetic model”, since secondary analysis under assumption of different models of inheritance (dominant or recessive) failed to substantially improve the strength of association) (Wang and Pang, 2019) This study shows similarity with that of Liscova *et al*. (2017) on a cohort of Czech KC patients.

5.2.2 Conclusion on Candidate Genes

In conclusion, the results and findings of this study, raise confidence in the relevance of Gene variant’s association, in the aetiopathogenesis of Keratoconus.

The genotype and allele frequency of each identified polymorphism in *VSX*1Exon3 was compared between the KC disease cohort and different control cohorts. Disappointingly, allele frequencies of these identified SNPs were found in similar frequency, in KC cases and Family control groups., but differed from Normal controls. Hence, no association with pathogenicity can be established. This result was similar to that of Tanwar’s, who found that four similar SNPs linked to KC.

The observations in this study corroborated those of other researchers who reported a lack of evidence for the existence of pathogenic variations in the *VSX*1Exon3. More investigations on other *VSX*1 variants and other genes are needed to further understand the genetic basis of KC.

5. 3 Exome Sequencing

VSX1 remains as the best characterised keratoconus gene but only accounts for rare cases. Other candidate genes however play a role like in corneal dystrophy genes such as ZEB1 and TGFBI and collagen genes. Family-based studies have recently led to the identification of the MIR184 gene for keratoconus with cataract and to the DOCK9 gene in a family with isolated keratoconus. Numerous other linkages have been reported and new sequencing technologies are set to rapidly expand the number of identified keratoconus genes in these regions.

Similarly, recent genome-wide association studies in case-controlled cohorts have identified common variations in and around HGF, RAB3GAP1 and LOX as candidate risk factors for keratoconus. These gene identifications are starting to reveal the molecular aetiology of keratoconus but despite recent progress, there remain numerous genetic risk factors in this complex disease. (Burdon KP et al 2013)

5. 3.1 Genome wide association studies

Genome wide association studies, GWAS, is a wide ranging, unbiased approach to genetic studies. However, controversy still exists in research for a major gene involvement in keratoconus aetiology. Abu-Amero et al (2011) analysed VSX1 mutations and confirmed these genetic changes are not involved in keratoconus. More recently, studies show that keratoconus corneas have oxidative stress signs together with high mitochondrial DNA damage. This finding involves SOD1 as a possible causative gene.

The gene mutations in *SOD1* exon2 and *COL4A3* exon 17 were those reported by Stabuc-Silih et al. (2010).

Even more recently again, findings suggest micro-RNA may be implicated in aetiopathogenesis of keratoconus Stabuc-Silih (2010). Thus, the prospective areas of research seem to be to explore mitochondrial genes and micro-RNA. Again, possibly proteomic profiles in KC corneas and tears have shown, expression of several proteins, possibly having some role in KC etiology. Still KC remains in its enigmatic position, as we are unable to confirm its detail etiology comprehensively.

Xiayan Xu et al - (2020) has now gone to research further into the extracellular-matrix (ECM)-related genes, contributing to sporadic patients among Han Chinese population in China.

5.3.2 Summary and Discussion In Exome Sequencing

In this study, a small pilot study for of participants including 5 probands, and 19 Family members.WES helped to confirm as well as uncover newer variants and their coding position

The results were variable. VSX1 gene variants results were confirmed for all 42 KC cases as well as the controls. Also SOD1 exon2 was also confirmed as only present in one KC28 patient, and his mother.

COL4A3 gene variant was the gene that gave a very surprising result. The targeted variant at Exon 17, was found only in…small proportion, four Family members. In contrast another four variants were found at 4 other exon coding position, namely exons 25 and exon 52. It is that though COL4A3 Exon 17 was chosen for PCR sequencing, none of the 24 KC and family members had the variant. Unlike in WES, the COL4A3 Exon25 yielded more results in the same group though COL4A3 Exon 17 was chosen for PCR sequencing, none of

the 24 KC and family members had the variant. Unlike in WES, the COL4A3 Exon25 yielded more results in the same group.

Whole Exome Sequencing (WES) is only sequencing the part of the genome that contains protein-coding regions of genes in a genome.

The identified variants were annotated to predict the effect or function of an individual SNP using SNP annotation tools. One of the fundamental levels of variant annotation includes categorizing each variant based on its relationship to coding sequences in the genome, and how it may change the coding sequence and affect the gene product. The variant can be categorized into four different effect classes nonsense, missense, silent,

Three *VSX1*variants, A182A (rs 1248030307), p.P237P(rs 56157240) and R217H (rs6138482) were identified by WES. These *VSX1* SNPs locations based on the publication from Tanwar *et al.* (2010), were identical to those found by PCR-sequencing. The concordance of *VSX1* variants between PCR-sequencing and WES was >75%.

From the whole exome sequencing results, the mutations appear to be random within the group. Table 4.51 lists the single nucleotide variants (SNVs)g.9339G>A, that was identified from the exome sequencing data of the 24 individuals within the *SOD1* gene in the present study. (Table 4.48)
The mutation found in *COL4A3* exon25 (p.P574) (rs28381984) is interesting. It was present in 4/5 KC (80%) but only in 60% (10/15) in family controls. Perhaps it has a complementary role with other genes. It is conclusively seen through this study that Keratoconus has a genetic aetiology, among other risk factors. It

is possible however that KC is a polygenic disease with different genes independently inherited and differently expressed.

Mutation found in COL4A3 Exon25 (p.P574) (rs28381984) however, is interesting. It was present in 4 members of the family of the proband K6 (4/4:100%). 4KC (4/4:100%) but only 60% (10/15) of Family controls.

The SNPs locations in VSX1 are similar to those in the PCR-sequencing results:Three VSX1variants, A182A (rs 1248030307), p.P237P(rs 56157240) and R217H (rs6138482) were identified.

In this study, KC cases and different non-KC control groups were examined by targeted gene PCR-sequencing and whole exome sequencing. Selection was based on gene mutations previously reported, associated with the KC phenotype. The overall results showed some association and linkiage of KC with some of these mutations.

There is however, no association of KC with rare or novel mutations, not reported before in medical literature. Overall, the variants in the *VSX1* exon 3 obtained by PCR-sequencing and whole exome sequencing showed a concordance of >75%. The 2 SNPs, A182A [(.OR3.14 (CI 95%, 1.14- 7.60), pvalue 0.026] and P237P [(OR 3.61(CI 95%, 1.31-9.94) pvalue0.0.12] showed a higher risk for KC in family controls having these 2 *VSX1* variants. The third variant R217H (OR 0.086 [(CI 95%, 0.0313 – 0.237) p value<0.0.0001,] appeared to confer a lower risk and perhaps even a "protective role" against KC in family controls.

Whole-exome sequencing is a widely used next-generation sequencing (NGS) method that involves sequencing the protein-coding regions of the genome.

The human exome represents less than 2% of the genome, but contains ~85% of known disease-related variants,[1] making this method a cost-effective alternative to whole-genome sequencing.

Exome sequencing using exome enrichment can efficiently identify coding variants across a broad range of applications, including population genetics, genetic disease, and cancer studies.

There are advantages in utilisation of Exome Sequencing in Genetic research. Variants can be identified across a wide range of applications, and coding regions can be comprehensively covered. It seems to be cost-effective, if large volume of research is anticipated. In fact whole-genome sequencing (4–5 Gb of sequencing per exome compared to ~90 Gb per whole human genome) can be done. WES in fact provides a much faster, easier data analysis WGS.

Exome sequencing is a cost-effective approach when whole-genome sequencing is not practical or necessary. Sequencing only the coding regions of the genome enables researchers to focus their resources on the genes most likely to affect the phenotype, and offers an accessible combination of turnaround time and price. Exome sequencing detects variants in coding exons, with the capability to expand targeted content to include untranslated regions (UTRs). DNA libraries can be prepared in as little as 1 day and require only 4–5 Gb of sequencing per exome.

5.3.3 Conclusion

It is conclusively seen through this study that Keratoconus has a genetic aetiology, among other risk factors. It is possible however that KC is a polygenic

disease with different genes independently inherited and differently expressed. Possible future research with Whole Exome Sequencing combined with Whole Genome Sequencing will enable this to be validated.

5.4 Limitations of this study

However, the limitations of this study, are the small sample size of actual Keratoconus cases, and even more so in the Ethnic composition of the study. There is unequal distribution in Ethnicity, though all participants and cases were from the Klang valley geographically.

It is thus aspired, that the future will provide more opportunities, to further expand this Keratoconus study. Also, Keratoconus as a disease is still uncommon in Malaysia or perhaps underdiagnosed. This may partly be due to lack of awareness of Keratoconus, among the public as well as eye-care personnel. More awareness can be created by screening programs with public talks on keratoconus and its diagnosis and treatment.

No pathogenic changes were detected, so only these four previously reported SNPs were studied (Tanwar et al 2010) Comparison of the genotype and allele frequency of each identified polymorphism was made, between the KC disease cohort and different control cohorts. However, allele frequencies of these identified SNPs were found in similar frequency, between KC cases and different control groups. This confirmed their non-pathogenecity.

In this study, other VSX1 gene variations such as p.D144E, p.L17P, p.N1S1S, pG160D, p.H244R, p.L159M, p.G160V, p.Q175H, p.R166W, p.H244R reported in other published papers were not explored or investigated. The lack of VSX1 pathogenic variations in a large number of unrelated sporadic keratoconus patients tend to omit its role, and corroborate the involvement of

other genetic, environmental or behavioral factors in the development of this complex disorder.

This study confirms findings of other studies done previously, that there is a lack of evidence of pathogenic variations in VSX1(Tanwar et al,2010, Burdon KP et al., 2011).Thus, this shows that there might be new genes and loci involved or other genetic or environmental factors. New technology has resulted in new investigative ways of research in Genetics.

This study is limited by the relatively small number of KC cases which precluded sub-analysis by gender, ethnicity and other factors that might be associated with gene variants. Although three genes were sequenced in this study, the number of *SOD*1Exon2 and *COL*4A3Exon 17, variants detected were too small for meaningful association studies. Hence, all comparative analysis was only conducted with the *VSX*1Exon3 variants.

Exome sequencing should be a powerful tool for the discovery of gene variants. The limited information from the exome sequencing in this study could be due to the sequencing being done only in a small number of patients and their family members. It could also be due to the KC phenotype being the result of gene variant expression and not the presence of the gene per se. Possibly, the inclusion of larger numbers of cases and non-family controls as well as the use of gene expression studies might give a more accurate picture of the genetic predisposition of KC in future study.

This limitation was partly overcome with whole exome sequencing that allowed the examination of all coding regions in the genome for the three candidate genes. Unfortunately, it was only possible to use this expensive

technology on a very small total number of 5KC patients and their 19 immediate family members.

The only additional and interesting information relevant to KC-associated gene variants was the discovery of the *COL4A3* exon25 variants in KC patients .in addition to 5 others. Nevertheless, the exome sequencing confirmed the absence of candidate gene variants in the *SOD1* exon2 gene.

This small WES study including 5KC patients and 19 family controls, whole exome sequencing for gene mutations previously reported to be associated with the clinical manifestation of KC. The overall results showed some linking of KC with some of these mutations.

There is however, no association of KC with rare mutations not reported before in medical literature. Owing to the formidable cost of large volume PCR-sequencing, only 3 targeted genes, *VSX1*, *SOD1* and *COL4A3* with specific exons, were screened for their occurrence in all KC cases and controls but exome sequencing was done for only 5KC patients and 19 Family controls as a pilot study.

With whole exome sequencing, it was possible to look at a larger number of genes for previously identified gene variants. However, this exploration also failed to identify specific variants linked to KC individuals and their family members.

The variants found in KC patients appeared not to be dominant alleles conforming to Mendelian inheritance. A possible explanation for this observation is that the KC phenotype is linked not to the presence of a gene but to its expression. In heterozygous individuals, the alleles are different in each

copy of the gene. Assuming that both gene copies are equally expressed at any given time, a heterozygous parent may express the gene from the non-variant copy, while the heterozygous child expresses the gene from the variant copy (or vice versa), resulting in the child developing KC while the parent appears to be normal. If this was the case, future further investigations with RNA sequencing for gene expression might lead to a clearer understanding of the genetic determinants of KC.

It is important to consider that the variant *COL4A3* exon 25 may have a compleimentary role in the expression of the KC phenotype in KC6. This was also the case in the other 3 KC cases, namely, KC19, KC24 and KC28, with the exception of KC14.

However, in this study, in order to obtain acceptable conclusive results, sample size and HWE calculations were performed to guage the sample size power (Dupont WD et al, 2009) and the level of sample biasness (Salani et al, 2005).

5.5 FUTURE PERSPECTIVES AND RESEARCH

Future case control studies with adequate numbers and planning may contribute to further research and discoveries.

It is seen through this study KC has a genetic aetiology. It is possible however that KC is a polygenic disease with different genes independently inherited and differently expressed. Possible future research WES combined with WGS will enable this to be validated.

The observations in this study corroborated those of other researchers who reported a lack of evidence for the existence of pathogenic variations in the

*VSX*1Exon3. More investigations on other *VSX*1 variants and other genes are needed to further understand the genetic basis of KC.

5.5 Future Research Direction

Research to date has not identified any single major gene, though in some families, the inheritance pattern does suggest such a model. It is clear that Keratoconus is a complex disease, and multifactorial issues complicate its aetiology and even its genetic heritage.

Future research seems to point towards the ability and comprehensiveness of Next Generational Sequencing applications such as Whole genome and Whole Exome Sequencing. Possible future research with Whole Exome Sequencing combined with Whole Genome Sequencing will enable this to be validated.

From just a pilot study, with WES, involving 24 particpants, it was possible to confirm the presence and absence of candidate genes VSX1, SOD1 AND COL4A3 and their variants. In addition it was possible to link up the association of these gene variants to each other, and to partially asssess the important role each played as a risk factor, in the aetiogenesis of Keratoconus. Especially the Collagen genes, COL4A3 had 5 exons which had higher frequencies of these particular variants at Exon 25 and Exon 52.

In addition, two other genes seemed to be of importance Hepatocyte Growth Factor (HGF) (Burdon et al 2013) and TGF1 (Wang et al 2003). HGF and TGF1was present in higher frequencies in cases compared to controls. Future research can definitely involve these two. as they are Growth Factor

genes, and possibly act synergistically and do affect growth of the eyeball, and may contribute to keratoconus aetiopathogenesis.

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Deva Jenny (2019). How effective is Crosslinking in Keratoconus? A Malaysian Study. *Academia .edu*. e-published 2019.

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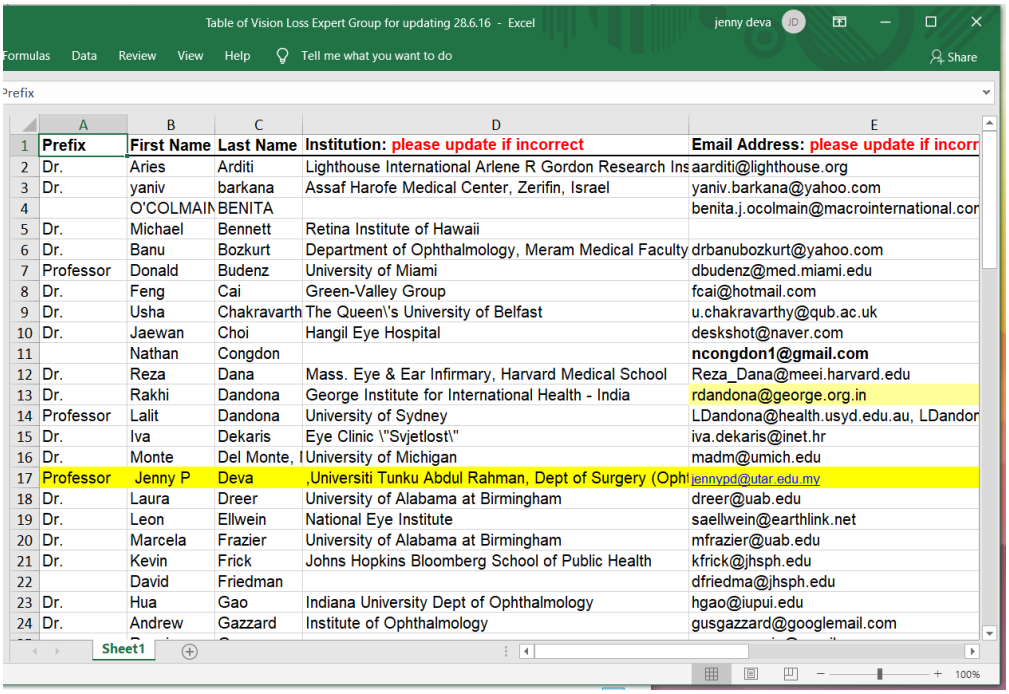
Member of Global Vision Loss Expert Group (VLEG)



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1. Jonas, J.B., George, R., Asokan, R., Flaxman, S.R., Keeffe, J., Leasher, J., Naidoo, K., Pesudovs, K., Price, H., Vijaya, L. and White, R.A., 2014. Prevalence and causes of vision loss in Central and South Asia: 1990–2010. *British Journal of Ophthalmology*, *98*(5), pp.592-598.

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3. Bourne, R., Steinmetz, J.D., Flaxman, S., Briant, P.S., Taylor, H.R., Resnikoff, S., Casson, R.J., Abdoli, A., Abu-Gharbieh, E., Afshin, A. and Ahmadieh, H., 2021. Trends in prevalence of blindness and distance and near vision impairment over 30 years: an analysis for the Global Burden of Disease Study. *The Lancet Global Health*, *9*(2), pp.e130-e143.

List of Conference Oral Presentations

Corneal changes of the Keratoconus Patients after UV-C3R treatment. 6-10months Malaysian Study. *3rd ICCL Zurich 2007.*

Corneal Crosslinking. Epithelium Off. *3rd International Congress of Corneal Cross Linking (ICCL). Zurich 2007.*

Case Report: Recurrence of keratoconus after Penetrating transplants.
7th International CXLCongress. . 9-10 December 2011 Zurich

Comparison of Preliminary Results From Crosslinked Keratoconus And Normal Corneae Using ORA, 9th CXL International Conference, Dublin 6-7th Dec 2013

Contact Lens Fitting for Keratoconus. Skills workshop. Annual Meeting. Tun Hussein Onn National Eye Hospital, Malaysia .2014

Genes and Environment: Preliminary Report on Keratoconus Research, *Human Ocular Genetics Seminar, FMHS, UTAR Sg Long, Oct 03, 2014*

Preliminary Report on Results of Screening School Children Between Ages Of 9-12years Of Age. *Utar Science Colloquium, Oct 18, 2014*

Comparison IOPcc, IOPg, CRF, CH And CCT of KC Post CXL With Controls, *10th International Congress Of Corneal Crosslinking, CBS, Zurich Switzerland, Dec 05, 2014 - Dec 06, 2014*

Superior Quality Of Vision After Crosslinking And Correction Of High Astigmatism With RGPs In Keratoconus, Asia Australia COPHyAA "Controversies In Ophthalmology", *Comtecmed.Com/COPHy/AA, Ho Chi Minh City, Vietnam, Feb 05, 2015 - Feb 08, 2015 Invited Speaker*

Screening School Children For Risk Factors In Keratoconus. APAO Congress Of Ophthalmology, 30th Apao Congress Of Ophthalmology. *20th Congress Of Chinese Ophthalmological Society at Guangzho, China, Apr 01, 2015 - Apr 04, 2015*

How Effective Is Crosslinking? Review Of 78 Cases. *12th International Crosslinking Conference.. Light For Sight at Zurich, Switzerland, Nov 29, 2017 - Dec 01, 2017*

Artificial Intelligence In Ophthalmology, *2nd Utar Biennial Scientific Conference, Jul 04, 2019 - Jul 06, 2019*

Genes and Environmental Risk Factors in Keratoconus. BIT's"WORLD 2015 DNA DAY" 26-28TH APRIL 2015 Nanjing, CHINA *BIT's Annual World Congress of Molecular & Cell Biology-201 Invited Speaker.*

Superior Quality of PostCXL Vision in Keratoconus with RGPs.". *Inaugural Asia Australian Congress." Controversies in Ophthalmology". COPHyAA. Ho Chi Minh City, Vietnam. 5-8th February 2015. Invited Speaker*

VSX1 paired gene variants A182A and p.P237P may be significantly associated with Keratoconus, wheras R217H may have a protective role. *UK Eye Genetics Group(EGG) 21st September 2021. Virtual Oral Presentation.*

List of Poster Presentations

第30届亚太眼科学会年会
THE 30TH ASIA-PACIFIC ACADEMY OF OPHTHALMOLOGY CONGRESS
暨中华医学会第二十次全国眼科学术大会
THE 20TH CONGRESS OF THE CHINESE OPHTHALMOLOGICAL SOCIETY
2015年 3月31日-4月4日 广州

SCREENING OF SCHOOL CHILDREN (9 -12 yrs) FOR KERATOCONUS, MYOPIA AND MYOPIC ASTIGMATISM

PROF. JENNY P DEVA MD, DEPT OF OPHTHALMOLOGY, UNIVERSITI TUNKU ABDUL RAHMAN , MALAYSIA

Purpose

1. To detect children who need to wear spectacles early to correct vision.
2. To prevent Ambylopia in those with Anisometropia

Methods

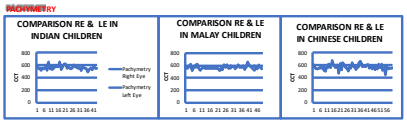
1. 40 children of 9 – 12yrs and of different ethnic (Indian, Malay and Chinese) groups were examined as follows: 2. Visual Acuity was taken with Snellens Chart . 3. Both eyes of each child were tested with Topography and Pachymetry. 4. ORA readings were also taken.

Results

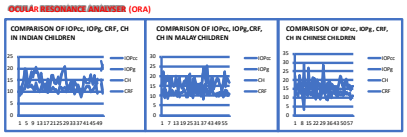
1. Indian Children seemed to have Higher Myopic Astigmatism (3-7DS)and Higher Keratometry Readings (Range45-50DS) while Chinese tended to have Lower Myopic Astigmatism but was more common in almost all.
2. Thickness of Cornea was in majority 500-600u, but about 2 Indian children had <500u.
3. All Myopic Astigmatism was correctable by glasses.
4. Only two Indian children had unilateral Ambylopia

COMPARISON OF RESULTS FOR INDIAN/ CHINESE /MALAY CHILDREN.

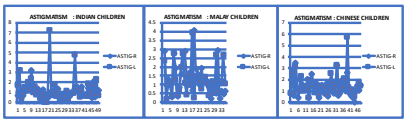
PACHYMETRY

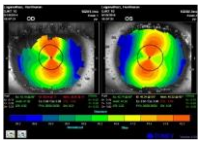


OCULAR RESONANCE ANALYSER (ORA)



ASTIGMATISM





TOPOGRAPHY

HIGH ASTIGMATISM WAS SEEN WITH STEEP CORNEA >45.0DS IN 25% INDIAN CHILDREN. 25% CHINESE CHILDREN HAD HIGH MYOPIA BUT MODERATE ASTIGMATISM(<3.00DS. ONLY 10% MALAY CHILDREN HAD MYOPIC ASTIGMATISM<2.00DS

VISUAL ACUITY

Refractive Errors were correctable in all children.
Only two Indian child had diminished vision of 6/18 despite spectacle correction.
They had Amylopia on one eye.

STUDY POPULATION : ETHNIC GROUPS:

CHINESE INDIAN MALAY

CONCLUSIONS

1. All children in this age group (9 -12 yrs) do not show the characteristics of Keratoconus.
2. However, High Myopic Astigmatism and Steeper cornea was more common among Indian children compared To Chinese and Malay children at this age group.

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3. Rabinowitz YS. Keratoconus.Surv Ophthalmol 1998; 42: 297 -319.

4. . Georgiou T, Funnell CL, Cassels-Brown A, O'Conor R. *Influence of ethnic origin on the incidence of keratoconus and associated atopic disease in Asians and white patients* . Eye (Lond) 2004; 18:379-83.

5. .Ariela Gordon-Shaag, Michel Millodot, Elinar Shneor. *Review Article. The Epidemiology and Etiology of Keratoconus. International Journal of Keratoconus and Ectatic Corneal Diseases* . January- April 2012; 1(1): 7-15

COMPARISON OF PRELIMINARY RESULTS FROM CROSSLINKED KERATOCONUS AND NORMAL CORNEAE USING ORA

9TH CXL INTERNATIONAL CONFERENCE, 6-7TH DECEMBER 2013, DUBLIN, REPUBLIC OF IRELAND.

JP Deva (jennypd@utar.edu.my)
Faculty of Medicine and Health Sciences, University Tunku Abdul Rahman, MALAYSIA

Introduction

Crosslinking (CXL) the keratoconus cornea has been the means to a effective stabilisation, regularisation and visual rehabilitation for keratoconus patients in the last decade. The corneal lamellae have realigned at molecular level as a result of CXL. Corneal Hysteresis (CH) and Corneal Resistance Factor (CRF) have become important indicators of the improved Biomechanical system of the post-CXL cornea using the Ocular Response Analyser (ORA).

Purpose

To evaluate, validate, and confirm that the keratoconus cornea is biomechanically stronger and have stabilised visco-elastic structure after CXL treatment.

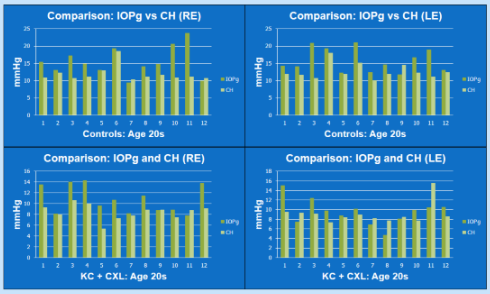
Methods

- 12 crosslinked Keratoconus (KC) patients and 12 Normal Controls were analysed using the ORA. These cases had CXL done 2- 3 years ago
- IOPcc, IOPg, WS, CH, CRF were measured and compared
- All keratoconus patients and controls were in the age group of 20-30 years
- While KC subjects were mostly Indian male, the Controls were mostly Chinese male which may pose a racial bias

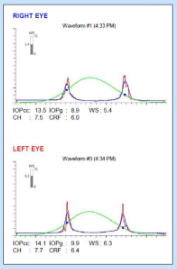
Conclusion

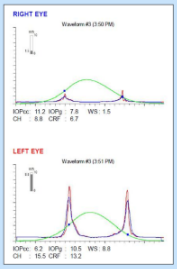
Corneal Hysteresis is reduced in post-CXL keratoconus eyes. This enables KC patient's cornea to become stabilised and regularise and become a biomechanically stronger structure.

Results



Figures: 1





Figures: 2a and 2b

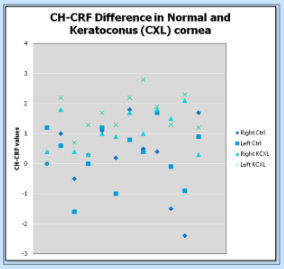


Figure: 3

Discussion

- Fig. 1: The graphs indicate that IOPg and CH have a similar relationship as in keratoconus.
- Fig. 2a: The 2 signal peaks indicate good response to CXL.
- Fig. 2b: The upper graph (RE) shows distorted signals indicating keratoconus is insufficiently treated. Post CXL. The lower graph (LE) shows a normal ORA Signal Analysis.
- Fig. 3: CH-CRF ratio: In normal cornea CH value is less than or near to CRF
- Higher CH value than CRF is present in keratoconus, even after CXL treatment indicating residual weakness in some advanced KC cases
- Though Corneal Hysteresis (CH) does not follow Young's Modulus of Elasticity, it is still a visco- elastic structure biomechanically. It is a measure of the difference in the inward and outward pressure in the dynamic bidirectional applanation process using ORA. Thus in post-CXL cornea, the reduced CH indicates the stronger biomechanics resulting from CXL treatment. and reconfirms that the visual outcome will be improved, the high astigmatism also reduced by the lamellae realignment.
- Further studies and larger study population groups will enable these preliminary observations to be substantiated further.

Main References from Ocular Response Analyzer Publication Abstracts

1. DH Glass, CJ Roberts, AS Litsky, PA Weber. A Viscoelastic Biomechanical Model of the Cornea Describing the effect of Viscosity and Elasticity on Hysteresis. *Investigative Ophthalmology & Visual Science*. 2008;49(9):3919-26.
2. K Kamiya, M Hagishima, F Fujimura, K Shimizu. Factors affecting Corneal Hysteresis in Normal eyes. *Graefe's Archive for Clinical and Experimental Ophthalmology*. 2008;246(10):1491-4.

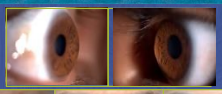
HOW EFFECTIVE IS CROSSLINKING IN KERATOCONUS (KC): A REVIEW OF 78 CASES

JENNY P DEVA MD (jennypd@utar.edu.my)

AIM

- To assess the clinical changes on Corneal Astigmatism, Visual Acuity and Quality of Life after CXL treatment

MALAYSIAN EXPERIENCE - STUDY POPULATION



INCLUSION CRITERIA

- Any newly detected Keratoconus Case
- Clear Corneas of >400u. After April Hypotonic R-D Solution was available for <400u
- No RGP contact lenses had been fitted yet
- The Worse eye (VA as well as Steeper K) of the two was selected for CXL treatment first
- Both eyes were fitted with Keratonus RGP lenses before the treatment
- The untreated eye (also better VA) serve as the Control for this study

EXCLUSION CRITERIA

- Advanced Keratoconus with Vogt's lines, corneal scarring, corneal oedema
- Corneal thickness <300u

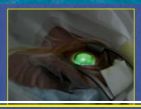
METHODOLOGY :DRESDEN PROTOCOL

- The apex of the cornea was cleared of epithelium down to Descemet's with a Toole's knife, or any blunt disposable blade
- 4-6 others similar small 2mm diameter round to oval epithelial windows were also removed
- This was to facilitate the diffusion of Riboflavin-Dextran solution anterior-posterior as well as laterally along the corneal layers

CXL TREATMENT WITH TIMED INSTILLATION OF RIBOFLAVIN DEXTRAN 2%(Thio Seiler/E. Spoerl)

- Regular 2min spaced Riboflavin-Dextran solution instillations were done for 20 mins
- Then the UV radiation unit (Peschke) was switched on
- However ,the Riboflavin-Dextran solution was continued at 5min intervals for 30 mins
- The UV-unit automatically switched off after 30 mins

CORNEAL EPITHELIAL WINDOWS STAINED WITH FLOURESCEIN



LIMITATIONS OF CORNEAL THICKNESS

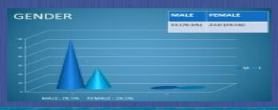
- Usual Riboflavin-Dextran solution is 2% for corneal thickness of 400u and above
- 1% Hypotonic-Riboflavin Dextran was made available since April 2007, for swelling the thinner cornea's <400U ,before CXL treatment Seiler T, "Collagen Cross-Linking in Thin Corneas"IROC ,2nd International ICCL, Zurich)

POST-OPERATIVE TREATMENT

- An Acuvue Disposable Soft Contact Lens is inserted at the end of the CXL - TT This Lens is removed after 24-48hrs, as soon as epithelium is complete.
- Gutt. Chloromycetin and Gutt. Dexamethasone drops are initially advised daily for one month (C Mazzotta, "Topical steroids are recommended in the postoperative of patients with higher risk of haze" 2nd ICCL ,2006). Maxitrol or Dexa ointment is nightly also for Haze reduction.
- Tear-Natural/Fresh eight minimums enhance epithelial healing and for lubrication for about 4 weeks. Gentle aCol is also used for about 2 weeks.
- In all cases Antiglaucoma eye drops were noted for two weeks.(Thiel M. "CCL increase IOP. "2nd ICCL2006.)

STUDY DEMOGRAPHIC DATA - ETHNICITY

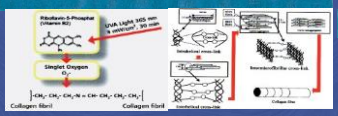
DEMOGRAPHIC DATA - SEX



DEMOGRAPHIC DATA - AGE

REFRACTIVE STATUS BEFORE TT WITH RGP OR CXL

THEORY



COLLAGEN FIBRILS BECOME ORGANISED AND LINKED SYSTEMATICALLY AGAIN RESULTING IN RECOVERY OF CORNEAL CLARITY

COLLAGEN FIBRIL REARRANGEMENT OCCURS, DUE TO INTERHELICAL AND INTRAMICROFIBRILLAR REORGANISATION AND REBONDING BY THE ULTRAVIOLET RADIATION OF WAVELENGTH 365u

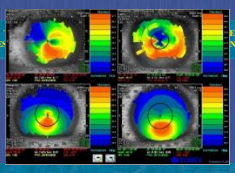
THIS IS ENHANCED AND POSSIBLE THROUGH THE PHOTO -CHEMICAL(-SENSITIVE) PROCESS, ENHANCED BY USING RIBOFLAVIN -DEXTRAN SOLUTIONS TO FACILITATE THE UV EFFECT THROUGH THE CORNEAL TISSUE TO AT LEAST 300u DEPTH(E Spoerdl).

RESULTS VISUAL ACUITY WITH RGPs

COMPARISON OF VISUAL IMPAIRMENTS / LogMAR

RESULTS CLINICAL OBSERVATION

- The K-RG Plens in at least 8 cases (8/13 eyes: 61.5%) became better centred
- The orbscan shows the cone apex also becomes more centred



TREATED LEFT EYE WITH CLS WELL-CENTRED

CLINICAL CHANGES OF THE CORNEA

- Two findings of other research are confirmed by this study

1 The cornea undergoes a process of "optical regularisation" the nature and process is still unknown

2 Optical pachymetry measures thinning of cornea after CXLinking *(Tobias Koller, Hans Peter Iseli et all, 2nd ICCL, Zurich 2006)*

RESULTS ON QUALITY OF LIFE BENEFITS DUE TO HAVING BETTER VISION

- ALL patients benefitted as follows:
- Have positive outlook on life
- Feel happier being able to see well
- Feel able to cope with work on a more competitive basis
- Feel more confident
- Feel more energetic
- Feel more able to achieve something

EFFICACY –RELATED BENEFITS

- ALL patients felt the following:
- Feel better able to concentrate
- Feel less depressed
- Feel less worried about the future

DISCUSSION & CONCLUSION 1

- Corneal Astigmatism is reduced by at least 2.0 dioptres.
- Corneal Cone Apex seems to move more centrally resulting in better centration of the K-RGP contact lens
- Visual Acuity and Quality are definitely improved
- The Psychological outlook and social attitudinal improvements was present in all patients
- Good counseling and encouragement of the patient goes a long way for the ophthalmologist's therapy and management of Keratoconus

DISCUSSION & CONCLUSION 2

- Keratoconus patients were mainly male (11:4)
- In this study there is a Indian bias in prevalence. Further research studies and larger population studies need to confirm the ratio of Malay, Indian and Chinese
- Further long-term longitudinal studies need to continue to confirm the permanence of the CXLinking effect to stop any further weakening of the cornea

CONCLUSION 3 :FUTURE PERSPECTIVES & POSSIBLE APPLICATIONS OF CXLINKING TREATMENT

- *Genetic studies* may reveal ancestry and hereditary influences on Keratoconus prevalence
- *Family Profile Studies* (Rabinowitz YS) will reveal the actual incidence of Keratoconus and Forme Fruste Keratoconus as well as High Astigmatism
- It is hoped that CXL treatment may become available as a *preventive and proactive* treatment for all Keratoconus patients in the near future

List of Courses

APAO Skills Instruction Course on Collagen Cross Linking(CXL), Beijing.2008

CXL Skills Transfer. 36th Annual Scientific Meeting Indonesian Ophthalmological Association. Manado .29th Sept-2nd oct. 2011

Keratoconus and the role of RGP KC lenses in Management of Keratoconus. Skills Transfer Workshop. 2nd -6th April 2014. World Congress of ophthalmology (WOC) Tokyo. Japan.

Crosslinking Procedure Skills Transfer Workshop. 2nd-6th April 2014.World Congress of Ophthalmology (WOC) Tokyo, Japan

List of Books

1) " You and Radial Keratotomy" ISBN:983-99635-2-8

2) "Laser Vision and Contact Lenses".ISBN:983-3392-00-8

3) Change My Heart, O Lord ISBN 978-983-3392-05-6

4) Change My Heart, O Lord (Amazon) ISBN-13: 978-148282924

List of Previous Research Projects (Since joining UTAR in 2012)

1. Prevalence of Underlining Risk Factors And Associated Genotypic Variants Of Keratoconus(Kc) In Malaysian School Going Children (9-12years Of Age), *UTARRF*, June 2013 - June 2014.

2. Keratoconus – The Disease. Self-funded.Research June 2013-2018

List of Awards
Distinguished Service Award APAO 1998. Nepal

LIST OF APPENDICES

A List of Chemical Solutions

Stock Solutions:

1. Cold TRIS-EDTA(TE) solution. 10/10Mm

2. Stock EDTA solution, pH8.0, 0.5M

3. SDS solution, 10% w/v

4. Proteinase K solution, 10mg/ml

5.Phenol-chloroform mixture, at 1:1 volume ratio

6. TRIS-buffered phenol

7. TE ($T_{100}E_{10}$) solution, 10X

8. TE ($T_{100}E_{1}$) solution, 1X

9. Chloroform-isoamyl alcohol mixture (CHISAM; or chloroform-3-methyl-1-butanol), at 24:1 volume ratio

10. NaCl solution, 4M

11. Cold Ethanol, 70%

12. Working Primers

13. EtBr solution, 0.5µg/ml

14. TRIS-boric acid-EDTA(TBE) buffer, 0.5X

15.Stock TBE buffer, 10X

16. Agarose gel, 1.5%

17. Salt-stimulated assay buffer

18. Stock TRIS-Cl solution, Ph8.0, 1M

19. Concentrated NaOH solution, 10M

20. $CaCl_2$ solution, 1M

21. Diluted DNA for quantification: purity and concentration analysis

22. Working DNA solution between 200-400ng/µl.

B Stock Chemicals

1. Calbiochem Tris (hydroxymethyl) aminomethane (TRIS)

Base, molecular biology grade (EMD Bioscience Inc., La Jolla, (CA92039, USA)

2. Titriplex® III (EDTA) for analysis (Merk KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

3. Calbiochem® Sodium n-dodecyl sulphate (SDS) , molecular biology grade (EMD Biosciences Inc., La Jolla, CA 92039, (USA).

4. Proteinase K from *Tritirachium album*, lyophilized powder for molecular biology (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103, USA)

5. Clorox® solution , for disinfection and cleaning (Clorox Headquarters, 1221 Broadway, Oakland CA 94612, USA)

6. 8-hydroxyquinolin, for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany

7. EMPROVE® for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany

8. EMSURE® for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany

9.Isopopyl alcohol (2-isopropanol or propan-2-ol) for analysis (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103, USA)

10. Isoamyl alcohol, for analysis (Sigma-Aldrich, 3050 Spruce St., St. Louis, MO 63103, USA)

11. Sodium chloride (NaCl) for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

12. Absolute ethanol, for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

13. GoTaq® Green Master Mix, 2X, 100 reaction units (Promega Corp., 2800 Woods, Hollow Road, Madison, W1 53711, USA).

14.Agarose powder, LE analytical grade (Promega Corp., 2800 Woods, Hollow Road, Madison, WI 53711, USA)

15. AMRESCO® ethidium bromide (EtBr) 10mg/ml, biotechnology grade (AMRESCO LLC, 6681 Cochran Rd, Solon, OH 44139, USA)

16. Calbiochem® Boric acid, molecular biology grade (EMD Bioscience Inc., La Jolla, CA 92039, USA)

17. DNA Ladder: 100bp and low molecular range (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA)

18. Restriction endonucleases (Res) , various units and types: *HpyCh*4III, *Alw*i, *Nla*III and *Bst*UI (New England Biolabs Inc. 240 County Road, Ipswich, Massachusetts 01938, USA)

19 10X NE Buffer No. 4 RFLP analysis (New England Biolabs Inc. 240 County Road, Ipswich, Massachusetts 01938, USA)

20. 100µg /ml bovine serum albumin for RFLP analysis (New England Biolabs Inc. 240 County Road, Ipswich, Massachusetts 01938, USA)

21. QIA quick Gel Extraction Kit (QIAGEN GmbH, QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany)

22. Hydrochloric Acid (HCL, 6M) for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

23. Sodium hydroxide (NaOH) , pellets for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

24. Calcium chloride ($CaCl_2$) for analysis (Merck KGaA, Feldbergstraβe 80, Darmstadt 64293, Germany)

25. Paraoxon-ethyl (paraoxon substrate) density of 1.274 g/ml at 25^0C (Sigma-Aldrich 3050 Spruce St., St. Louis , MO 63103, USA)

26. Diazinon-O-analog (diazoxon substrate) (Chem Service Inc., 660 Tower Lane, West Chester, PA 19381, USA)

C List of Materials

The Materials required in this study are as listed below:

1. PS3000 PureShield™ gloves; powder -free, polymer coated latex, non-sterile (Kossan Rubber Industries Bhd., Wisma Kossan, Lot 782, Jalan Sungai Putus, Off Batu 3, ¾ Jalan Kapar, 42100 Klang, Selangor, Malaysia).

2. Cotton wool balls

3. Alcohol swabs:70% isopropyl alcohol (Becton-Dickson, Franklin Lakes, NJ 07417, USA)

4. Vacutainer system: butterfly needles, K2-EDTA and heparinised vacutainer tubes (Becton-Dickson, Franklin Lakes, NJ 07417, USA)

5.Non-filtered pipette tips (Labcon, 3700 Lakeville Highway, Petaluma, CA 94954, USA)

6. Filtered pipette tips (Axgen Inc., Central Avenue, Union City, CA 94857, USA)

7. Falcon tubes: 15ml and 50ml tubes (Becton-Dickson, Franklin Lakes, NJ 07417, USA)

8. Terumo® 10cc/ml syringes and 21G x1" Needles: sterile, non-toxic, non-pyrogenic, latex-free (Terumo Corp., Tokyo, Japan)

9. Minisart syringe filter ,hydrophilic (Sartorius Stedim Biotech, 37070 Goettingen, Germany)

10. Quartz cuvettes: 600µl (PerkinElmer Inc., 940 Winter St., Waltham, Massachusetts 02451, USA)

11. Plastic cuvettes: 1ml. transparent (Sarstedt, Aktiengesellschaft & Co., North Rhine-Westphalia, D-51588 Numbrecht, Germany)

12. Plastic tubes: 0.2ml (Thermo Fisher Scientific, 81 Whyman St., Waltham, Massachusetts 02451, USA)

13.Plastic tubes : 0.5ml and 1.5ml (Labcon, 3700 Lakeville Highway, Petaluma, CA 94954, USA)

14. Laboratory Films: Parafilm® "M"(Pechiney Plastic Packaging, Chicago, II 60631, USA)

15. Sterile, single use carbon steel surgical blades and scalpels (CE 0197 certified)

16. Optifix® 10ml bottle-top chemical dispenser (Sigma-Aldrich 3050 Spruce St., St. Louis, MO 63103, USA)

17. Schott Duran® screw caps and graduated laboratory glass and amber bottles (nLabware SCHOTT North America, Inc. Corporate Office 555 Taxter Road Elmsford, NY 10523, USA)

18. Whatman FTA Cards for collection of finger prick blood in 4 cells . CAT No. W8120205. Active ingredient : Sodium Lauryl Sulfate. 4.1% Wt/Wt. Other ingredients 95%. GE Healthcare Life Sciences, , Little Chalfont, Buckinghamshire HP7 9NA, UK. GE Healthcare UK Limited, Amersham Place.

D List of Equipment

1. Bio-microscope Slit-Lamp: Model BP900® LED powered and AT900® (Haag-Streit AG, Gartenstadtstrasse 10, 3098 Koeniz, Switzerland

2. Pentacam and topography machine: Zeiss Visante ^{TM} OCT model 1000 (Carl Zeiss Meditec Inc, Dublin, CA 94568, USA)

3.Keratometer : Zeiss ATLAS^{TM} model 900 (Carl Zeiss Meditec Inc, Dublin, CA 94568, USA)

4. Pipette: Gilson Pipetman® Neo (Gilson S.A.S., 19, Avenue des Entrpreneurs, BP 145, F-955400 Villiers-le-Bei, France

5. Kelvinator® -20^0 C freezer (Electrolux, S:t Goransgatan 143, Stadshagen Stockholm, Sweden)

6. Heraeus -80^0 C bio-freezer (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA)

7. Standard table top centrifuge machines: Eppendorf type 5417C and Eppendorf type 5417R, 30wells. (Eppendorf AG, Barkausenweg 1, 22339 Hamburg, Germany)

8. Mass centrifuge machines, variable temperature: Eppendorf type 5810R (Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany); Thermo Scientific model ST16R (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA)

9. Mini-centrifuge: model CF-5, six wells (Daihan Scientific Co. Ltd., 187-1, Wolsong-ri, Jijeong-myeon, Wonju-si, Gangwon-do, South Korea)

10. Vortex machine: Vortex Genie® 2 model G-560E (Scientific Industries Inc., 70 Orville Dr 1, Bohemia, NY 1176, USA)

11. Autoclave machine: Hiclave ^{TM} HVE-50 (Hirayama Manufacturing Corp., 4-14-4-7, Hatchobori, Chuo-Ku, Tokyo 104-0032., Japan)

12. Eurotherm drying oven: NR range , set at 60^0 C (Carbolite Ltd. ,Parson Lane,Hope, Hope Valley, S33 6RB, UK)

13. Electrolux® microwave oven (Electrolux, St Goransgatan 143, Stadsshagen Stockholm, Sweden).

14. Water bath, set at 37^0C : Shel-Lab® model 1235 (Sheldon Manufacturing Inc., 300 N 26th Ave, Cornelius, OR 97113, United States)

15.Heated water bath, variable temperature: model Heto AT110 (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA)

16. Millipore pure water synthesis machine: model Rios 16 (Merck Millipore, Feldberggraβe 80 , Darmstadt 64293, Germany)

17. MilliQ ultrapure water synthesis machines: model MQ Synthesis (Merck Millipore, Feldberggraβe 80 , Darmstadt 64293, Germany)

18. Digital pH meter: model pH 525 (WTW Wissenschaftlich-Technische Werkstatten GmbH, Dr.-Karl-Slevogt-Straβe 1, D-82362 Weilheim, Germany)

19. Hot plate stirrer: model PMC 502C-2 (Thermo Fisher Scientific, 81 wyman St., Waltham, Massachusetts 02451,USA)

20. Weighing machine: Type B02002-S (Mettler Toledo, 1900 Polaris Parkway, Columbus, OH, 43240 USA)

21. Fume hood: built-in general purpose laboratory and chemical storage cabinet (Iryas Inc. (M) Sdn Bhd , Wisma Iryas, Lot 2 Jalan SS13/6B, Subang Jaya Industrial Estate, 47500 Petaling Jaya, Selangor Darul Ehsan, Malaysia),

22.Laminar flow hood cabinets: model HWS 1500 (Iryas Inc. (M) Sdn Bhd, Wisma Iryas,

Lot 2 Jalan SS13/6B, Subang Jaya Industrial Estate, 47500 Petaling Jaya, Selangor Darul Ehsan, Malaysia),

23. Shaking incubator: model type AK 85 (Infors HT, Rittergasse 27, CH-4103 Bottmingen/ Basel, Switzerland)

24. Spectrophotometer: Lambda Bio 25 UV/Vis spectrophotometer (PerkinElmaer Inc., 940 Winter St., Waltham, Massachusetts 02451, USA

25.Thermal cycler: models ABI VERITI and ABI PCR2720 (Applied Biosysatems, 850 Lincoln Centre Drive, Foster) City, CA 94404, USA),

26. Agarose gel electrophoresis system and gel cast: model Owl Easycast™ B1 (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451 USA)

27. Power supply forgel electrophoreses system: model Power PAC 300 (Bio-Rad, 1000 Alfred Nobel Drive, Hercules, California 94547, USA)

28. UV trans-illuminator Model ECX-F20.M (Vilber Lourmat, B P .66 Torey - Z.I. Sud, 77202 Marne La Vallee – Cedex 1, France).

29. Gel documentation system: model MTV- 12V6HE- R(RS-485) (Major Science, 19959 Sea Gull Way , Saratoga, CA 95070, USA)

30. Water jet pump: model Heto SUE 3 series (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA)

31. Ice FLAKER: model Scotsman AF-10 (Hubbard Systems, 106 Claydon Business Park, Gt Blakenham, Ipswich, Suffolk, IP6 ONL., UK)

32. DNA Analyser; Applied Biosystems model 3730x1 (Thermo Fisher Scientific, 81 Wyman St., Waltham, Massachusetts 02451, USA).

33. Crosslinking Equipment PERSCHKE PXL Platinum 330. 365nm wavelength. Peschke Trade GmbH. Boesch 67. 6331 Huenenberg, Switzerland (Pachymeter included)

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E Preparation of Working Solutions

1. CONCENTRATED NaOH solution, 10M

40g solid formof MaOH pellets (Merck KGaA, Germany) was weighed in an autoclaved laboratory glass bottle and dissolved in 80ml ddH_2O. The solution was stirred vigorously on magnetic stirrer before ddH_2O was used to top up until a final volume of 100ml was achieved. The solution was kept at room temperature, away from potential heat sources.

2. STOCK TRIS-CI solution, Ph 8.0, 1M

121.1g powdered form of TRIS (Merck KGaA, Germany) was weighed and dissolved in 800ml ddH_2O . The solution was stirred vigorously on magnetic stirrer, and adjusted to Ph 8.0 with 6M HCl. Then ddH_20 was used to top up the solution until a final volume of 1L was achieved. The stock was filtered and autoclaved before usage. The solution was stored at room temperature.

3.Stock buffered EDTA solution (NaEDTA $2H_2O$), Ph8.0., 0.5M

186.17g solid form of EDTA (Merck KGaA, Germany) was weighed anddissolved in 800ml ddH_2O. The solution was stirred vigorously on magnetic stirrer and adjusted to pH8.0 with 10M NaOH. Then, ddH_2O was used to top up solution until a final volume of 1L was achieved. The stock was filtered and autoclaved before use. The solution was stored at room temperature.

4. Cold TRIS-EDTA (TE) solution , 10/10Mm

10 ml of 1M TRIS and 20ml of 0.5M EDTA were mixed. Then ddH_2O used to top up until a final volume of 1L was achieved. The 10/10Mm TRIS-EDTA was filtered and autoclaved before use. The solution was stored at 4^0C .

5. SDS solution, 10% w/v

10g powdered form of SDS (EMB Biosciences Inc., USA) was weighed and dissolved in 80ml of ddH_2O . The solution was stirred vigorously on magnetic stirrer before ddH_2O was used to top up until a final volume of 100ml was achieved. The solution was filtered before use.

6. Proteinase K solution, 10mg/ml

100mg of Proteinase K lyophilized powder (Sigma-Aldrich, USA) was weighed and dissolved in 10ml of MiliQ water. The solution was then aliquoted into 100µl in several 1.5ml plastic tubes, and were kept in -20^0C freezer until further usage.

7.TE ($T_{100}E_{10}$) solution, 10X

50ml of 1M TRIS and 10ml of 0.5M EDTA was mixed. Then ddH_20 was used to top up until a final volume of 500ml was obtained. The 10X TE solution was autoclaved before use. The solution was stored at room temperature.

8. TE ($T_{10}E_1$) solution , IX

5ml of IM TRIS and 1ml of 0.5M EDTA were mixed. Then , ddH_2O was used to top up until a final volume of 500ml was obtained. The 1X TE solution was autoclaved before use. The solution was stored at room temperature.

9.TRIS-buffered phenol

Phenol (Merck KGaA, Germany) was allowed to liquefy into 1L in its original amber bottle, which was immersed in a water bath. at a temperature of 68^{o}C. The subsequent buffereing steps were performed:

i) 500ml of phenol was transferred into an autoclaved 1L amber bottle. The remaining unused , unbuffered phenol were kept in the 4^{0}C cold room until TRIS-buffered phenol was needed to be prepared again.

ii) 0.5g of 8-hydroxiquinoline was added into 500ml phenol.

iii) Phenol was mixed with equal volume of 10X TE ($T_{100}E_{10}$) solution, pH8.0.

iv)Mixture was shaken vigorously for about 15 minutes. T he mixture was allowed to separate overnight in the fume hood.

v) The following day , the mixture was separated into two layers. TE layer was situated above while phenol layer was situated below. The upper layer was removed by an aspirator which was powered by ac water jet pump.

vi) The bufferening steps was from iii) until v) and also repeated three times: once with 10X TE ($T_{100}E_{10}$) and subsequent two times with 1XTE ($T_{10}E_{1}$) solution.

After buffering steps were performed, a bottle-top dispenser was used to close the anber bottle before it was placed in a polystyrene box. The buffered phenol was stored in the cold room at 4^{0}C.

10.Phenol-chloroform mixture, at 1:1 volume ratio.

A volume of 500ml buffered phenol was mixed with an equal volume of chloroform(Merck KGaA, Germany) at 1:1 volume ratio, in an amber bottle. The mixture was kept at 4^0C in the cold room until further usage.

11.Chloroform-isoamyl alcohol mixture (CHISAM; or chloroform-3-methyl-1-1-butanol) at 24:1 volume ratio.

A volume of 240ml chloroform (Merck KGaA, Germany) was mixed with 10ml of isoamyl alcohol (Sigma-Aldrich, USA) at 24:1 volume ratio . The mixture was kept at 4^0C in the cold room.

12.Clorox solution, 10%

100ml of Chlorox® solution were diluted with 900ml of ddH_2O to top up to a final volume of 1L. The solution was used to decant lysed blood and placed in the fume blood overnight being before being removed the following day.

13.NaCl solution 4M

58.44g solid form of NaCl salt (Merck KGaA, (Germany) was dissolved in 200ml ddH_2O. The solution was stirred vigorously on magnetic stirrer before ddH_2O was used to top up to a final volume of 250ml was achieved. The solution was filtered, autoclaved and stored at room temperature before use.

14. Cold Ethanol, 70%

175ml of absolute alcohol ethanol (Merck KGaA, Germany) was mixed in an autoclaved laboratory glass bottle with ddH_2O until a final volume of 250ml was achieved. The solution was stored in -20^0C freezer until further usage.

15. EtBr solution, 0.5µg/ml

0.5µl of 10µg/ml EtBr (AMRESCO, USA) was mixed with ddH_2O in 1.5 ml plastic tube, until a final volume of 1 ml was achieved. The tube were wrapped with aluminium foil and kept in a drawer, from light exposure.

16. Stock TBE buffer, 10X

Bothe 108g of TRIS base (Merck KDaA, Germany) and 55g of boric acid (Merck KGaA, Germany) were dissolved in 40ml of 0.5M EDTA pH8.0. and top up with ddH_2O UPTO 800ML.The mixture was stirred vigorously on a magnetic stirrer. Then ddH_20 was used to top up the solution until final volume of 1L was achieved. The solution was filtered and autoclaved before use.

17. TRIS-boric acid- EDTA (TBE) buffer 0.5X

A 1:20 dilution was done using stock 10X TBE was mixed k 10X TBE and ddH_2O where 50ml of stock 10X TBE was mixed with ddH20 until final volume of 1L. The solution was kept in the -20^{0}C freezer

18. Agarose gel 1.5%

1.5 g of agarose powder (Promega, USA) was weighed and dissolved in 100ml of .f 0.5X TBE buffer. 3.2µl of 0.5µ g/ml EtBr was added in the solution before pouring it onto a gel cast.

19. Agarose gel, 3.0%

3.0g of agarose powder (Promega, USA) was weighed and dissolved in 100ml of .f 0.5X TBE buffer. 3.2µl of 0.5µ g/ml EtBr was added in the solution before pouring it onto a gel cast.

20.$CaCl_2$ solution, IM

2.94g powdered form of $CaCl_2$ (Merck KGaA, Germany) was dissolved in 15ml ddH_2O.

The solution was stirred on a magnetic stirrer before ddH_2O. was used to top up until a final volume of 20ml was achieved. The solution was filtered and autoclaved before use. The solution was stored at room temperature.

21. Salt-stimulated assay buffer

116.8g of NaCl salt (Merck KGaA, Germany) was dissolved in 800ml ddH2O. 100ml IM TRIS-CL, Ph 8.0 and 2.0ml of IM $CaCl_2$. Final concentration of NaCl, TRIS-Cl and $CaCl_2$ in the buffer were 2M, 0.1M and 2.0Mm respectively. The ddH2O was used to top up the buffer until a final volume of 1L was obtained. The buffer was.stored up to six months at room temperature.

22.Working Primers, 100pmol/ µl

The lyophilized solution of 25nmole primers was reconstituted with 250µl of ddH_2O into 100pmol/µl from the primer suppliers. The primers were aliquoted into 10µl in several 0.5 tubes and stored in -20^0C at lease overnight before use.

23. DNA for quantification analysis

6µl of stock DNA was pipetted into 594µl of ddH_2O , at a ratio of 1:100, in a quartz cuvette. The diluted DNA was analysed at 260nm, 280nm and 320nm in a apectrophotometer for quantification analysis.

24. Working DNA solution, 200 – 400ng/µl

Stock DNA with more than 400ng/µl was diluted with ddH_2O until a desired concentration of 400ng/µl was obtained. For stock DNA between 200ng/µl to 400ng/µl, no dilution was needed.

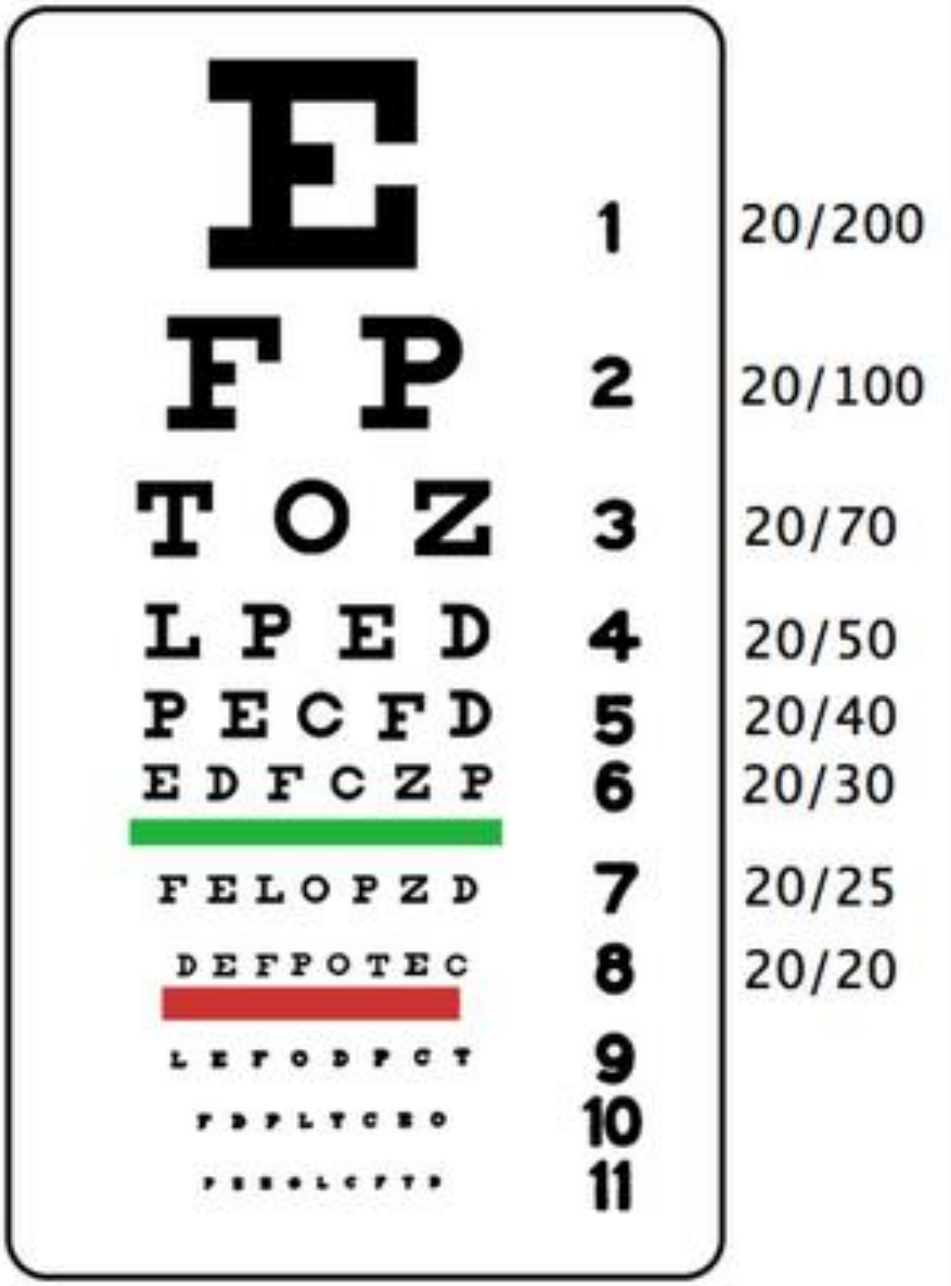
F1 Results of Keratometry KMAX

				Keratometry (in diopters)		Ultrasonic pachymetry (in µm)		Visual acuity in LogMar (PreCXL)		Visual acuity in LogMar PostCXL		Visual acuity in LogMar PostRGP		Keratometry (in diopters) RE			Keratometry (in diopters) LE		
Pt ID	Race	Age	Sex	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	K-MAX	K-MIN	ASTIG	K-MAX	K-MIN	ASTIG
KC1	C	32	F	47.33	55.2	542	489	0.5	1	0.3	0.6	0.1	0.5	47	45.5	1.5	61.2	59	2.2
KC2	M	28	M	55.59	56.39	435	509	1.5	1.2	1	1	0.2	0.2	61.3	55.4	5.9	61.3	48.5	12.8
KC3	M	17	M	44.47	45.2	449	362	1.8	0.6	Pk 0.5	0.3	0.3	0.1	68.7	68.7	8.7	55.3	45.3	8.9
KC4	I	32	M	65.6	48	466	488	1	1	1	0.8	0.3	0	66	54.1	11.9	50.7	46.6	4.5
KC5	M	19	F	45.32	45.31	430	500	0.6	0.2	0.4	0	0.2	0	58.49	50.15	8.34	45.4	44.31	1.11
KC6	I	21	M	47.23	59.71	555	608	0.9	0.5	0.2	0.4	0.1	0.1	55.6	46.1	9.5	52.8	48.9	3.9
KC7	M	16	M	48.7	55.14	507	476	0.5	1	0.3	0.5	0.2	0.2	61.2	48.5	9.7	62.9	50.9	7.8
KC8	I	32	F	52.2	48.25	482	534	0.9	0.5	0.7	0.2	0	0	53.03	50.72	2.31	50.1	45.85	4.27
KC9	I	29	M	58.81	51.76	523	519	1.5	0.3	1	0.1	0.1	0	65.39	55.3	9.93	44.8	41.4	3.37
KC10	M	17	M	48.61	50.58	328	329	1	1	0.6	0.4	0.1	0.1	47.25	44.6	2.65	49.5	44.5	4.6
KC11	I	19	M	50.1	47.9	365	415	0.8	0.3	0.5	0.2	0.2	0.2	53	43.6	9.4	49	42.9	6.1
KC12	I	22	M	48.2	44.61	444	481	0.6	0.2	0.4	0.1	0.1	0.1	48.4	45.7	2.7	44.9	44	0.8
KC13	I	30	F	47.78	54.24	583	512	0.8	0.9	0.3	0.3	0.1	0.1	46.6	42.4	4.2	51.6	46.9	4.7
KC14	M	26	M	54.37	52.6	456	462	1	1	0.6	0.6	0	0	56.9	49.6	7.3	53.1	49.6	3.5

				Keratometry (in diopters)		Ultrasonic pachymetry (in µm)		Visual acuity in LogMar (PreCXL)		Visual acuity in LogMar PostCXL		Visual acuity in LogMar PostRGP		Keratometry (in diopters) RE			Keratometry (in diopters) LE		
Pt ID	Race	Age	Sex	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	K-MAX	K-MIN	ASTIG	K-MAX	K-MIN	ASTIG
KC15	I	24	M	50.1	49	472	470	1	1	0.3	0.4	0	0	52.1	44	8.1	51.3	44	7.3
KC16	M	19	M	50.1	51.2	531	493	1	1	0.6	0.4	0.3	0.3	51.23	45.93	5.3	54.2	47.79	6.37
KC17	M	23	M	54.3	58.2	456	442	0.9	1.5	0.6	0.9	0.2	0.2	58.8	51.1	7.7	58.8	50.75	8.05
KC18	C	24	F	52.9	55.2	521	487	0.9	1	0.6	0.6	0.2	0.2	54.5	48.8	5.7	57.6	47.8	9.8
KC19	C	18	M	56.5	54.4	512	488	1	1	0.4	0.5	0.2	0.2	60.75	44.41	16.34	59.2	52.12	7.04
KC20	I	18	M	47.25	43.25	529	514	0	0.3	0	0.3	0	0.2	50.1	48.5	1.6	48.6	44.5	4.1
KC21	M	33	M	61.5	60.3	390	440	0.5	0.7	0.4	0.5	0.3	0.5	65.5	56.9	8.6	63.8	57.5	6.3
KC22	M	22	F	58.7	56.8	502	411	1	1	0.6	0.6	0.2	0.3	55	48	7	57.7	54	3.7
KC23	C	20	M	53.8	50.1	489	529	1.5	0.3	0.7	0.2	0.4	0.2	56.8	49.8	7	52.9	44.8	8.1
KC24	C	31	F	47.8	58	496	307	0.6	1	0.4	0.6	0.2	0.4	49.6	44.6	3	60.5	48.9	11.6
KC25	C	29	M	55.3	58.31	425	418	0.5	1.5	0.3	0.9	0.2	0.4	57.87	54.6	3.27	63.5	56.6	6.9
KC26	I	28	M	78.5	65	412	372	1.5	0.5	0.9	0.4	0.5	0.4	86.1	64	5.5	71	54.3	4.1
KC27	I	25	M	49.43	43.6	485	450	1	0.3	0.6	0.1	0	0	50.37	45.58	4.79	44.4	42.28	2.12
KC28	M	30	F	54.75	54.75	515	519	0.6	0.9	0.3	0.5	0.2	0.2	54.25	51	3.25	55.3	47.35	7.9
KC29	I	33	F	65.78	46.59	393	536	1.5	1	0.9	0.5	0.3	0.1	54.33	45.15	4.9	47.4	41.74	5.58
KC30	I	27	M	46.51	51.18	515	455	0	0.6	0	0.4	0.2	0.2	47.41	43.04	2.3	55.7	49.3	5.4
KC31	I	32	F	47.15	50	548	565	1	0.4	0.6	0.2	0.3	0.1	53.5	47.8	5.7	44.8	44	0.8
KC32	I	28	M	52.1	45.8	432	465	1	1	0.4	0.4	0.2	0.2	54.8	45.17	9.26	48.2	40.28	8.79

				Keratometry (in diopters)		Ultrasonic pachymetry (in µm)		Visual acuity in LogMar (PreCXL)		Visual acuity in LogMar PostCXL		Visual acuity in LogMar PostRGP		Keratometry (in diopters) RE			Keratometry (in diopters) LE		
Pt ID	Race	Age	Sex	OD	OS	OD	OS	OD	OS	OD	OS	OD	OS	K-MAX	K-MIN	ASTIG	K-MAX	K-MIN	ASTIG
KC33	I	35	F	45	45.25	581	549	0.2	1.5	0.2	0.9	0.1	0.3	46.7	41.5	5.2	44.4	42	2.4
KC34	I	22	F	48.2	51.5	410	416	0.9	0.6	0.4	0.4	0	0.2	50	49.6	0.4	54.8	51.75	3
KC35	I	18	M	55.7	49.4	449	549	0.9	0.3	0.6	0.2	0.1	0	52.93	43.8	8.6	49.6	44.95	4.44
KC36	I	14	M	48.4	44.9	502	573	0.8	0.2	0.5	0	0	0	47.6	46.1	1.5	45	44.3	0.66
KC37	I	19	M	45.8	50.7	574	522	0.2	0.7	0.2	0.2	0	0.1	45.35	44	1.35	50.5	47.4	3.1
KC38	I	24	M	45.98	45.19	489	471	1	0.7	0.4	0.3	0.1	0.1	45.75	43.8	1.95	46	43.7	2.3
KC39	M	19	F	45.6	44.8	508	518	0.6	0	0.3	0	0	0	42.8	42.1	0.7	43.6	42.1	1.1
KC40	I	20	M	44.1	43.5	494	489	0.8	0.8	0.3	0.3	0	0	46.8	42.7	4.1	46.7	41.4	5.3
KC41	M	33	F	48.73	63.07	453	414	0.8	1.5	0.6	1	0.1	0.4	53.2	44.1	9.1	59.8	49	10.7
KC42	I	25	M	48.7	59.6	486	327	0.3	1	0.2	0.8	0	0.4	50.4	47.3	3.1	54.9	52.7	2.2

F2 Snellen's Chart for Visual Assessment



F3 Questionnaires

Patient Recruit Information Sheet
Faculty of Medicine and Health Sciences,
University Tunku Abdul Rahman (UTAR)
Sg Long Campus, Kuala Lumpur, Malaysia

Prof. Jenny P Deva : 012-3987601

For office use only.

Recruit Index Number: _____

KERATOCONUS IN THE MALAYSIAN POPULATION: CLINICAL AND CORRELATIVE STUDIES OF THE GENOTYPES AND PHENOTYPES IN KERATOCONUS IN MALAYSIANS OF INDIAN ORIGIN

Name of propositus : _____

Name of relative and relationship : _____

Date of interview : _____

Diagnosis : Keratoconus ☐

Formefrustekeratoconus ☐ Pre-keratoconus ☐

High myopia + astigmatism (> -6.00 D) ☐

Moderate or low myopia + astigmatism (< -6.00 D) ☐

Normal ☐

Section A
Personal Specifications

(Please tick ✓ in the appropriate box.)

Name : _____

Mailing address : _____

_____

Telephone : (Home) _____ (Mobile) _____

Identity card no : _____ Sex : Male☐ Female☐

Date of birth : _____ Age : _____years

Marital status : _____ Occupation : _____

Ethnic group : _____ Education level : _____

Monthly collective household income: RM _____ per month

Section B
Medical History

Eye problem : Itchiness ☐ Redness ☐ Discharge ☐ NA ☐

Type of nose problem : Nose blockage☐ Nose drip ☐ NA ☐

1

Patient Recruit Information Sheet
Faculty of Medicine and Health Sciences,
University Tunku Abdul Rahman (UTAR)
Sg Long Campus, Kuala Lumpur, Malaysia

Prof. Jenny P Deva : 012-3987601

Nose problem	: Regular	☐	Seldom	☐	NA	☐			
Cough	: Spasmodic	☐	Prolonged	☐	Intense	☐	NA	☐	
Presence of recurrent Eyelid/ Eye itchiness	: Yes	☐	No	☐					
Presence of recurrent eyelid swelling	: Yes	☐	No	☐					
Eye-rubbing	: None ☐	Slightly ☐		Intense ☐	Very intense	☐			
Watering of eyes	: None ☐	+	☐	++	☐	+++	☐		
Skin allergy	: Yes	☐	No	☐					
Bronchial asthma	: Yes	☐	No	☐					
Food allergy	: Yes	☐	No	☐					
Medical condition	: _____								
Treatment by	: GP	☐	Specialist	☐	Self-medication	☐			
	: Herbal	☐	None	☐					
Medication	: Antihistamines	☐	Statins	☐					
	: Others	☐ _____		None	☐				
Who diagnosed KC	: Optician	☐	Optometrist	☐	Ophthalmologist	☐	NA	☐	
	: Details _____								
Who diagnosed myopic astigmatism	: Optician	☐	Optometrist	☐	Ophthalmologist	☐	NA	☐	
	: Details _____								
Age started wearing spectacles	: _____								
Age started wearing contact lenses/ RGPs/soft toric lenses	: _____								

Section C
Eyes

A. Visual Acuity

Without correction : RE _____ LE _____

With correction : RE _____ LE _____

Mode of correction : Spectacles ☐ Contact lenses ☐

Prof. Jenny P Deva : 012-3987601

Increase in spectacle power : Gradual ☐ Sudden ☐

What was the pattern of increase Slow/gradual or sudden : _____

B. Topography

	RE	LE
K-Reading	_____	_____
Pachymetry	_____	_____
Notes	_____	

Section D
Past History

History of sickness/ allergy : _____

History of ocular disease : _____

History of injury that affected vision : _____

Section E
Consanguinity in the Family

History of consanguinity : Yes ☐ No ☐ Don't know ☐

Section F
Racial Intermarriages

History of inter-racial marriage : Yes ☐ No ☐ Don't know ☐

Prof. Jenny P Deva : 012-3987601

Section G
Additional Notes

_____

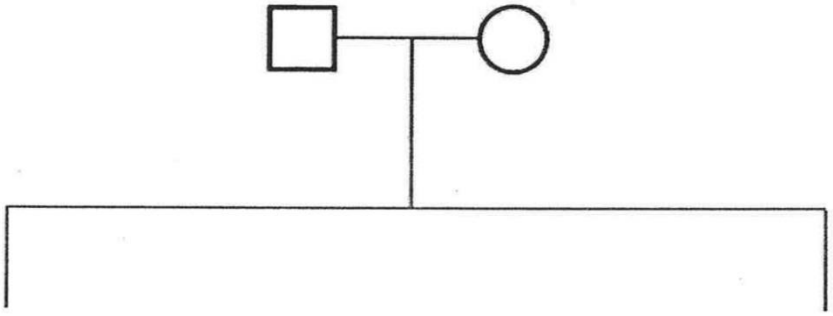
_____

_____

Thank you for taking the time to complete this questionnaire.

Taken by: _____

Family Tree



F4.1 Risk factors of The Eye and Allergy

Group * Eye redness

Crosstab

			EYERED		
			YES	NO	Total
GROUP	KC	Count	11	31	42
		% within GROUP	26.2%	73.8%	100.0%
		% within EYERED	14.9%	9.1%	10.1%
		% of Total	2.6%	7.5%	10.1%
	FAMILY	Count	25	101	126
		% within GROUP	19.8%	80.2%	100.0%
		% within EYERED	33.8%	29.5%	30.3%
		% of Total	6.0%	24.3%	30.3%
	NORMAL	Count	12	84	96
		% within GROUP	12.5%	87.5%	100.0%
		% within EYERED	16.2%	24.6%	23.1%
		% of Total	2.9%	20.2%	23.1%
	SCHOOL CHILDREN	Count	25	119	144
		% within GROUP	17.4%	82.6%	100.0%
		% within EYERED	33.8%	34.8%	34.6%
		% of Total	6.0%	28.6%	34.6%
Total		Count	74	342	416
		% within GROUP	17.8%	82.2%	100.0%
		% within EYERED	100.0%	100.0%	100.0%
		% of Total	17.8%	82.2%	100.0%

F4.2 Group * Eye discharge

Crosstab

			EYEDISCHARGE		
			YES	NO	Total
GROUP	KC	Count	21	21	42
		% within GROUP	50.0%	50.0%	100.0%
		% within EYEDISCHARGE	29.2%	6.1%	10.1%
		% of Total	5.0%	5.0%	10.1%
	FAMILY	Count	13	113	126
		% within GROUP	10.3%	89.7%	100.0%
		% within EYEDISCHARGE	18.1%	32.8%	30.3%
		% of Total	3.1%	27.2%	30.3%
	NORMAL	Count	4	92	96
		% within GROUP	4.2%	95.8%	100.0%
		% within EYEDISCHARGE	5.6%	26.7%	23.1%
		% of Total	1.0%	22.1%	23.1%
	SCHOOL CHILDREN	Count	33	111	144
		% within GROUP	22.9%	77.1%	100.0%
		% within EYEDISCHARGE	45.8%	32.3%	34.6%
		% of Total	7.9%	26.7%	34.6%
Total		Count	72	344	416
		% within GROUP	17.3%	82.7%	100.0%
		% within EYEDISCHARGE	100.0%	100.0%	100.0%
		% of Total	17.3%	82.7%	100.0%

F4.3 Group * Eyewatering

Crosstab

			EYEWATER		
			YES	NO	Total
GROUP	KC	Count	7	35	42
		% within GROUP	16.7%	83.3%	100.0%
		% within EYEWATER	8.8%	10.4%	10.1%
		% of Total	1.7%	8.4%	10.1%
	FAMILY	Count	40	86	126
		% within GROUP	31.7%	68.3%	100.0%
		% within EYEWATER	50.0%	25.6%	30.3%
		% of Total	9.6%	20.7%	30.3%
	NORMAL	Count	22	74	96
		% within GROUP	22.9%	77.1%	100.0%
		% within EYEWATER	27.5%	22.0%	23.1%
		% of Total	5.3%	17.8%	23.1%
	SCHOOL CHILDREN	Count	8	136	144
		% within GROUP	5.6%	94.4%	100.0%
		% within EYEWATER	10.0%	40.5%	34.6%
		% of Total	1.9%	32.7%	34.6%
Total		Count	80	336	416
		% within GROUP	19.2%	80.8%	100.0%
		% within EYEWATER	100.0%	100.0%	100.0%
		% of Total	19.2%	80.8%	100.0%

F4.4 Group * Noseblock

Crosstab

			NOSEBLK		
			YES	NO	Total
GROUP	KC	Count	8	34	42
		% within GROUP	19.0%	81.0%	100.0%
		% within NOSEBLK	12.1%	9.7%	10.1%
		% of Total	1.9%	8.2%	10.1%
	FAMILY	Count	28	98	126
		% within GROUP	22.2%	77.8%	100.0%
		% within NOSEBLK	42.4%	28.0%	30.3%
		% of Total	6.7%	23.6%	30.3%
	NORMAL	Count	16	80	96
		% within GROUP	16.7%	83.3%	100.0%
		% within NOSEBLK	24.2%	22.9%	23.1%
		% of Total	3.8%	19.2%	23.1%
	SCHOOL CHILDREN	Count	13	131	144
		% within GROUP	9.0%	91.0%	100.0%
		% within NOSEBLK	19.7%	37.4%	34.6%
		% of Total	3.1%	31.5%	34.6%
Total		Count	66	350	416
		% within GROUP	15.9%	84.1%	100.0%
		% within NOSEBLK	100.0%	100.0%	100.0%
		% of Total	15.9%	84.1%	100.0%

F4.5 Group * Foodallergy

Crosstab

			FOODALLERGY		
			YES	NO	Total
GROUP	KC	Count	15	27	42
		% within GROUP	35.7%	64.3%	100.0%
		% within FOODALLERGY	33.3%	7.3%	10.1%
		% of Total	3.6%	6.5%	10.1%
	FAMILY	Count	13	113	126
		% within GROUP	10.3%	89.7%	100.0%
		% within FOODALLERGY	28.9%	30.5%	30.3%
		% of Total	3.1%	27.2%	30.3%
	NORMAL	Count	17	79	96
		% within GROUP	17.7%	82.3%	100.0%
		% within FOODALLERGY	37.8%	21.3%	23.1%
		% of Total	4.1%	19.0%	23.1%
	SCHOOL CHILDREN	Count	0	144	144
		% within GROUP	0.0%	100.0%	100.0%
		% within FOODALLERGY	0.0%	38.8%	34.6%
		% of Total	0.0%	34.6%	34.6%
Total		Count	45	371	416
		% within GROUP	10.8%	89.2%	100.0%
		% within FOODALLERGY	100.0%	100.0%	100.0%
		% of Total	10.8%	89.2%	100.0%

Even Food allergy seems to pose as relatively significant in KC (35.7%) as a risk factor compared to other controls.

G1 Gene variants in the KC Group

R/N	*VSX1* Exon 3	*SOD1* Exon 2	*COL4A3* Exon 17
KC1	c.627+23G>A (het)		None
KC2	p.A182A; c.50424C>T;c.627+84T>A (all het)	None	
KC3	p.A182A; c.627+84T>A (all het)		
KC4	None		
KC5	None		
KC6	None	g.9339G>A	
KC8	None		
KC10	p.A182A;c.504-24C>T; c.627+84A (all het)		
KC11	c.627+23G>A (het)		
KC13	p.A182A; c.627+84 T>A (all het)		
KC14	c.627+23G>A (het)	None	
KC15	c.627+23G>A (homo)		
KC16	c.627+23G>A (homo)		
KC18	c.627+23G>A (homo)		
KC19	c.627+38C>A(novel), c.627+23G>A (all het)	g.9339G>A	
KC21	p.A182A; c.627+84 T>A (all het)		
KC22	None		
KC24	p.A182A; c.627+84T>A (all het)	g.9339G>A	
KC25	c.627+23G>A (homo)		
KC26	c.627+23G>A (het)	None	
KC27	c.627+23G>A (homo)		
KC28	c.627+23G>A (het)	None	
KC33	None		
KC35	None		
KC36	c.627+23G>A (homo)		
KC37	c.627+23G>A (het)		
KC38	None		
KC40	None		
KC44	c.627+23G>A		
KC45	c.627+23G>A (het)		
KC47	p.A182A; c.627+84T>A; c.627+23G>A (all het)		
KC48	c.627+23G>A		
KC49	p.A182A; c.627+84T>A		
KC50	None		
KC54	p.A182A; c.627+84T>A; c.627+23G>A; c.504-24C>T (all het)		
KC56	p.A182A; c.627+84T>A (all het)		
KC62	p.A182A;c.627+84T>A (all het)		
KC63	p.A182A; c.627+84T>A (all het)		

KC64	None		
KC66	None		
KC67	p.A182A; c.627+84T>A; c.627+23G>A		
KC68	p.A182A; c.627+84T>A (all het)		

*het: heterozygous; homo: homozygous

G2 Gene Variants in the Family Controls

R/N	*VSX1* Exon 3	*SOD1* Exon 2 deletion	*COL4A3* Exon 17
F1	None	Negative	
F2	None	Negative	
F3	None	Negative	
F4	p.A182A; c.627+84T>A (all het)	Negative	
F5	p.A182A; c.627+84T>A (all het)	Negative	
F6	None	Negative	
F7	None	Negative	
F8	None	Negative	
F9	None	Negative	
F10	None	Negative	
F11	None	Negative	
F12	None	Negative	
F13	None	Negative	
F14	None	Negative	
F15	None	Negative	
F16	None	Negative	
F17	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	
F18	None	Negative	
F19	None	Negative	
F20	None	Negative	
F21	None	Negative	
F22	None	Negative	
F23	None	Negative	
F24	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
F25	None	Negative	
F26	p.A182A; c.627+84T>A (all homo)	Negative	
F27	p.A182A; c.627+84T>A (all homo)	Negative	
F28	None	Negative	
F29	p.A182A; c.627+84T>A (all het)	Negative	
F30	p.A182A; c.627+84T>A (all homo)	Negative	
F31	p.A182A; c.627+84T>A (all het)	Negative	
F32	None	Negative	
F33	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	

F34	None	Negative	
F35	p.A182A; c.627+84T>A (all het)	Negative	
F36	None	Negative	
F37	None	Negative	
F38	None	Negative	
F39	None	Negative	
F40	None	Negative	
F41	c.627+23G>A (homo)	Negative	
F42	None	Negative	
F43	None	Negative	
F44	None	Negative	
F45	c.627+23G>A (het)	Negative	
F46	None	Negative	
F47	c.627+23G>A (homo)	Negative	
F48	None	Negative	
F49	p.A182A; c.627+84T>A (all het)	Negative	
F50	None	Negative	
F51	None	Negative	
F52	None	Negative	
F53	None	Negative	
F54	None	Negative	
F55	None	Negative	
F56	None	Negative	
F57	p.A182A; c.627+84T>A (all het)	Negative	
F58	None	Negative	
F59	None	Negative	
F60	p.A182A; c.627+84T>A (all het)	Negative	
F62	c.627+23G>A (het)	Negative	
F63	None	Negative	
F64	None	Negative	
F65	None	Negative	
F66	None	Negative	
F67	None	Negative	
F68	None	Negative	
F69	None	Negative	
F70	c.627+23G>A (het)	Negative	
F71	None	Negative	
F72	None	Negative	
F73	None	Negative	
F74	None	Negative	
F75	c.627+23G>A (homo)	Negative	
F76	None	Negative	
F77	None	Negative	
F78	c.627+23G>A; c.627+59T>A	Negative	
F79	None	Negative	
F80	None	Negative	
F81	None	Negative	
F82	None	Negative	

F83	None	Negative	
F84	None	Negative	
F85	c.627+23G>A (het)	Negative	
F86	None	Negative	
F87	None	Negative	
F88	c.627+23G>A (het)	Negative	
F89	None	Negative	
F90	None	Negative	
F91	None	Negative	
F92	None	Negative	
F93	None	Negative	
F94	c.627+23G>A (homo)	Negative	
F95	None	Negative	
F96	p.A182A; c.627+84T>A (all het)	Negative	
F97	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	
F98	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	
F99	None	Negative	
F100	None	Negative	
F101	None	Negative	
F102	None	Negative	
F103	None	Negative	
F104	None	Negative	
F105	p.A182A; c.627+84T>A (all het)	Negative	
F106	p.A182A; c.627+84T>A (all het)	Negative	
F107	None	Negative	
F108	None	Negative	
F109	None	Negative	
F110	None	Negative	
F111	None	Negative	
F112	None	Negative	
F113	None	Negative	
F114	None	Negative	
F115	None	Negative	
F116	None	Negative	
F117	None	Negative	
F118	None	Negative	
F119	None	Negative	
F120	None	Negative	
F121	c.627+23G>A (het)	Negative	
F122	None	Negative	
F123	None	Negative	
F124	None	Negative	
F125	None	Negative	
F126	p.A182A, c.627+84T>A (all het)	Negative	
F127	None	Negative	

*het: heterozygous; homo: homozygous

G3 Gene variants in the Normal Controls

R/N	*VSX1* Exon 3	*SOD1*Exon 2 deletion	*COL4A3* Exon 17
C1	c.627+23G>A (het)	Negative	
C2	c.627+23G>A (het)	Negative	
C3	None	Negative	
C4	c.627+23G>A (het)	Negative	
C5	None	Negative	
C6	None	Negative	
C7	p.A182A; c.627+84T>A (all het)	Negative	
C8	c.627+23G>A (homo)	Negative	
C9	c.627+23G>A (homo)	Negative	
C10	c.627+23G>A (homo)	Negative	
C12	c.627+23G>A (het)	Negative	
C13	c.627+23G>A (het)	Negative	
C14	c.627+23G>A (het)	Negative	
C15	c.627+23G>A (het)	Negative	
C16	None	Negative	
C17	None	Negative	
C18	c.627+23G>A (hetero)	Negative	
C19	None	Negative	
C20	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	
C21	c.627+23G>A (het)	Negative	
C22	p.A182A; c.627+84T>A; c.627+23G>A (all het)	Negative	
C23	c.627+23G>A (het)	Negative	
C24	c.627+23G>A (homo)	Negative	
C25	None	Negative	
C26	c.627+23G>A (het)	Negative	
C27	None	Negative	
C28	None	Negative	
C29	None	Negative	
C30	c.627+23G>A (het)	Negative	
C31	c.627+23G>A (het)	Negative	
C32	None	Negative	
C33	c.627+23G>A (het)	Negative	
C34	None	Negative	
C35	c.627+23G>A (het)	Negative	
C36	c.627+23G>A (het)	Negative	
C37	c.627+23G>A (homo)	Negative	
C38	c.627+23G>A (homo)	Negative	
C39	c.627+23G>A (het)	Negative	
C40	None	Negative	
C41	None	Negative	
C42	c.627+23G>A (het)	Negative	
C43	c.627+23G>A (het)	Negative	
C44	c.627+23G>A (het)	Negative	

C45	None	Negative	
C46	None	Negative	
C47	None	Negative	
C48	None	Negative	
C49	None	Negative	
C50	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
C51	c.627+23G>A (homo)	Negative	
C52	c.627+23G>A (het)	Negative	
C53	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
C54	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
C55	None	Negative	
C56	c.627+23G>A (het)	Negative	
C57	c.627+23G>A (het)	Negative	
C58	None	Negative	
C59	None	Negative	
C60	None	Negative	
C61	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
C62	c.627+23G>A (het)	Negative	
C63	c.627+23G>A (homo)	Negative	
C64	None	Negative	
C65-I	c.627+23G>A (het)	Negative	
C66-I	c.627+23G>A (homo)	Negative	
C67-I	c.627+23G>A (homo)	Negative	
C68-I	c.627+23G>A (homo)	Negative	
C69-I	None	Negative	
C70-I	c.627+23G>A (het)	Negative	
C71-I	c.627+23G>A (het)	Negative	
C72-I	None	Negative	
C73-I	c.627+23G>A (het)	Negative	
C74-I	None	Negative	
C75-I	p.A182A; c.627+84T>A (all het)	Negative	
C76-I	None	Negative	
C77-I	p.A182A; c.627+84T>A; c.504-24C>T (all het)	Negative	
C78-I	c.504-24C>T (het)	Negative	
C79-I	c.504-24C>T (het)	Negative	

C80-I	None	Negative	
C81-I	None	Negative	
C82-I	None	Negative	
C83-I	None	Negative	
C84-I	None	Negative	
C85-I	c.504-24C>T (het)	Negative	
C86-I	None	Negative	
C87-I	p.A182A; c.627+84T>A (all het)	Negative	
C88-I	p.A182A; c.627+84T>A (all het)	Negative	
C89-I	p.A182A; c.627+84T>A (all het)	Negative	
C90-I	c.627+23G>A (homo)	Negative	
C91-I	c.627+23G>A (homo)	Negative	
C92-I	c.627+23G>A (homo)	Negative	
C93-I	c.627+23G>A (homo)	Negative	
C94-I	c.627+23G>A (homo)	Negative	
C95-I	None	Negative	
C96-I	None	Negative	
C97-I	c.627+23G>A (homo)	Negative	
C98	c.627+23G>A (het)	Negative	

*het: heterozygous; homo: homozygous

G4 Gene Variants in School Children (Chinese)

School Children	*VSX1* Exon 3 Variants	*SOD1* Variants	*SOD1* Exon 2 Deletion	*COL4A3* Variants	*COL4A3* homozygous /heterozygous
SM01	c.627+23G>A	None	Negative	None	-
SM02	c.627+23G>A	None	Negative	None	-
SM03	c.627+23G>A	None	Negative	None	-
SM04	c.627+23G>A	None	Negative	None	-
SM05	c.627+23G>A	None	Negative	None	-
SM06	None	None	Negative		
SM07	c.627+23G>A	None	Negative	None	-
SM08	None	None	Negative		
SM09	None	None	Negative		
SM10	None	None	Negative	None	-
SM11	None	None	Negative	None	-
SM12	c.627+23G>A	None	Negative		
SM13	c.627+23G>A	None	Negative	None	-
SM14	c.627+23G>A	None	Negative		
SM15	p.A182A, c.627+84T>A	None	Negative		
SM16	c.627+23G>A	None	Negative		
SM17	p.A182A, c.627+84T>A	None	Negative		
SM18	c.627+23G>A	None	Negative		
SM19	None	None	Negative		
SM20	None	None	Negative	None	-
SM21	None				
SM22	None				
SM23	None				
SM24	p.A182A, c.627+84T>A, c.504-24C>T				
SM25					
SM26	\				
SM27	p.A182A, c.627+84T>A				
SM28					
SM29	c.627+23G>A				
SM30	c.627+23G>A				
SM31	None				
SM32	None				
SM33	None				
SM34	None				
SM35	None				
SM36	c.627+29A>G?				
SM37	c.627+23G>A				
SM38	None				

SM39					
SM40	c.504-24C>T				
SM41	None				
SM42	None				
SM43	c.627+23G>A				
SM44	p.A182A, c.627+84T>A				
SM45	None				
SM46	c.627+23G>A				
SM47	c.627+23G>A				
SM48	c.627+23G>A				
SM49	c.627+23G>A				
SM50	None				
SM51	c.627+29A>G				
SM52	None				
SM53	c.627+23G>A				
SM54	c.627+23G>A				
SM55	None	None	Negative	Yes	p.D326Y (het*)
SM56		None	Negative	None	-
SM57		None	Negative	None	-
SM58		None	Negative	None	-
SM59		None	Negative		
SM60		None	Negative	Yes	p.D326Y (het*)
SM61		None	Negative		

*het: heterozygous

G5 Gene Variants in School Children (Malay)

School Children	*VSX1* Exon 3 Variants	*SOD1* Variants	*SOD1* Exon 2 Deletion	*COL4A3* Variants	*COL4A3* homozygous /heterozygous
BTHO1	c.627+23G>A	None	Negative		
BTHO2	c.504-24C>T, p.A182A, c.627+84T>A	None	Negative		
BTHO3	c.627+23G>A	None	Negative		
BTHO4	c.627+23G>A				
BTHO5	c.627+23G>A				
BTHO6	c.627+23G>A	None	Negative	None	-
BTHO7	c.627+23G>A				
BTHO8					
BTHO9		None	Negative	None	-
BTHO10					
BTHO11					
BTHO12	c.627+23G>A				
BTHO13	c.627+23G>A				
BTHO14	p.A182A, c.627+84T>A				
BTHO15	c.627+23G>A				
BTHO16	c.627+23G>A				
BTHO17	None				
BTHO18	p.A182A, c.627+23G>A, c.627+84T>A				
BTHO19	c.504-24C>T, p.A182A, c.627+23G>A, c.627+84T>A				
BTHO20					
BTHO21	None				
BTHO22	None				
BTHO23	None				
BTHO24	c.627+23G>A				
BTHO25	None				
BTHO26	None				
BTHO27					
BTHO28	None				
BTHO29	None				
BTHO30	c.627+23G>A				
BTHO31	None				
BTHO32	None				
BTHO33	None				
BTHO34	None				
BTHO35	None				
BTHO36					
BTHO37					
BTHO38					
BTHO39	None				

BTHO40	None				
BTHO41	None				
BTHO42	c.627+23G>A				
BTHO43	c.627+23G>A				
BTHO44	None	None	Negative	None	-
BTHO45	c.627+23G>A	None	Negative	None	-
BTHO46	None	None	Negative	None	-
BTHO47	c.627+23G>A	None	Negative		
BTHO48	c.627+23G>A	None	Negative	None	-
BTHO49	None	None	Negative	None	-
BTHO50	None	None	Negative		
BTHO51	c.627+23G>A	None	Negative	None	-

G6 Gene Variants in School Children (Indian)

School Children	*VSX1* Exon 3 Variants	*SOD1* Variants	*SOD1* Exon 2 Deletion	*COL4A3* Variants	*COL4A3* homozygous /heterozygous
ST1	None	None	Negative		
ST2	None	None	Negative		
ST3	c.627+23G>A	None	Negative		
ST4	c.627+23G>A	None	Negative		
ST5					
ST6	c.627+23G>A				
ST7	c.627+23G>A				
ST8	c.627+23G>A				
ST9	p.A182A, c.627+84T>A				
ST10	p.A182A, c.627+84T>A				
ST11	c.627+23G>A				
ST12	p.A182A, c.627+84T>A				
ST13	None	None	Negative		
ST14	None	None	Negative		
ST15	p.A182A, c.627+84T>A	None	Negative		
ST16	None	None	Negative		
ST17	None	None	Negative		
ST18	c.627+23G>A	None	Negative		
ST19	None	None	Negative		
ST20	p.A182A, c.627+84T>A	None	Negative		
ST21	None	None	Negative		
ST22	None	None	Negative		
ST23	None	None	Negative		
ST24	None	None	Negative		
ST25	c.627+23G>A	None	Negative		
ST26	p.A182A, c.627+84T>A, c.627+76G>T?	None	Negative		
ST27	p.A182A, c.623+23G>A, c.627+84T>A, c.504-24C>T				
ST28	p.A182A, c.627+84T>A				
ST29	c.627+23G>A				
ST30	c.627+23G>A				
ST31					
ST32					
ST33	p.A182A, c.627+84T>A				
ST34	p.A182A, c.627+84T>A				
ST35	None				
ST36	None				
ST37	p.A182A, c.627+84T>A				
ST38	p.A182A, c.627+84T>A, c.504-24C>T				
ST39	c.627+23G>A				
ST40	c.627+23G>A				

ST41					
ST42					
ST43		None	Negative	None	-
ST44		None	Negative	None	-
ST45		None	Negative	None	-
ST46		None	Negative	None	-
ST47		None	Negative	None	-
ST48		None	Negative		
ST49		None	Negative	No	-
ST50		None	Negative		

H List of software and parameter that is used for NGS analysis (EXOME SEQUENCING)

Sequencing reads mapping to reference genome

BWA-MEM (version: 0.7.8-r455)

Options	Values
gapOpenPenalty	6
clipPenalty	5
Additional	
gapExtensionPenalty	1
matchScore	1
readSingletonPenalty	17

Removal of PCR/optical duplicatesREF:7.4.2

SAMtools (subprogram: rmdup; version: 0.1.19-44428cd) SAMtools (subprogram: filter; version:0.1.19-44428cd)

Options	Values
mapQ	30

SAMtools (subprogram: index; version: 0.1.19-44428cd)

mismatchPenalty	4

* Reference genome build used is hg19

Realignment around INDELsREF:7.4.3

GATK (subprogram: realign; version: 3.3-0-g37228af)

Options	Values
maxInMemory	200000
additional	-rfNotPrimaryAlignment --consensusDeterminationModel USE_READS -l ERROR
LOD	0.4

Base quality recalibration

GATK (subprogram: recal; version: 3.3-0-g37228af)

Options	Values
additional	--disable_indel_quals -rfBadCigar -l ERROR
covariates	ReadGroupCovariate,QualityScoreCovariate,ContextCovariate,CycleCovariate

* dbSNP138 corresponding to reference genome build hg19 was used

Variant identification / variant calling 7.5.1

GATK (subprogram: HaplotypeCaller; version: 3.3-0-g37228af)

Options	Values
genotyping_mode	DISCOVERY
stand_emit_conf	10
stand_call_conf	30
ERC	GVCF

GATK (subprogram: GenotypeGVCFs; version: 3.3-0-g37228af)

Options	Values
Dbsnp	dbsnp_hg19_138.vcf

Variant annotation and Counting

Variant annotation

SnpEff (vcersion: 4.2)

Options	Values
o	gatk

GATK (subprogram: VariantAnnotator; version: 3.3-0-g37228af)

Opti s	Val s

Variant Counting

SnpSift (subprogram: caseControl; version: 4.2)

I Slit-Lamp Findings of Cornea of KC Patients

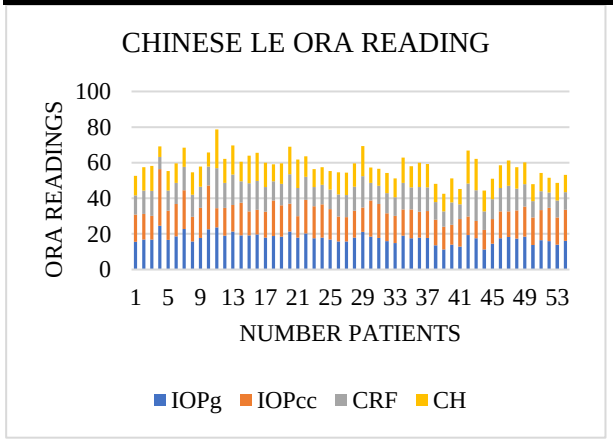
				Munsen sign		Vogt's striae		Hydrops		Scarring	
Pt ID	Race	Age	Sex	OD	OS	OD	OS	OD	OS	OD	OS
KC1	C	32	F	/	/	/	/	x	x	x	x
KC2	M	28	M	/	/	/	/	x	x	x	x
KC3	M	17	M	/	/	x	/	/	/	/	x
KC4	I	32	M	/	/	/	/	x	x	/	x
KC5	M	19	F	/	/	/	x	x	x	x	x
KC6	I	21	M	/	/	/	/	x	x	x	x
KC7	M	16	M	/	/	/	/	x	x	x	x
KC8	I	32	F	/	/	/	/	x	x	x	x
KC9	I	29	M	/	/	/	/	x	x	x	x
KC10	M	17	M	/	/	/	/	x	x	x	x
KC11	I	19	M	/	/	/	x	x	x	x	x
KC12	I	22	M	/	/	/	/	x	x	x	x
KC13	I	30	F	/	/	/	/	x	x	x	x
KC14	M	26	M	/	/	/	/	x	x	x	x
KC15	I	24	M	/	/	/	x	x	x	x	x
KC16	M	19	M	/	/	x	x	x	x	x	x
KC17	M	23	M	/	/	/	/	x	x	x	x
KC18	C	24	F	/	/	/	/	x	x	x	x
KC19	C	18	M	/	/	/	/	x	x	x	x
KC20	I	18	M	/	/	/	/	x	x	x	x
KC21	M	33	M	/	/	x	x	x	x	x	x
KC22	M	22	F	/	/	/	x	x	x	x	x
KC23	C	20	M	/	/	x	x	x	x	x	x
KC24	C	31	F	/	/	/	/	x	x	x	x
KC25	C	29	M	/	/	/	/	x	x	x	x
KC26	I	28	M	/	/	/	/	/	x	/	x
KC27	I	25	M	/	/	/	x	x	x	/	x
KC28	M	30	F	/	/	x	/	x	x	x	x
KC29	I	33	F	/	/	/	/	x	x	x	x
KC30	I	27	M	/	/	/	/	x	x	x	x
KC31	I	32	F	/	/	/	/	x	x	x	x
KC32	I	28	M	/	/	/	x	x	x	x	x
KC33	I	35	F	/	/	/	/	x	x	x	x
KC34	I	22	F	/	/	x	x	x	x	x	x
KC35	I	18	M	/	/	/	/	x	x	x	x
KC36	I	14	M	/	/	/	/	x	x	x	x
KC37	I	19	M	/	/	/	/	x	x	x	/
KC38	I	24	M	/	/	/	/	x	x	x	x
KC39	M	19	F	/	/	/	/	x	x	x	/
KC40	I	20	M	/	/	/	/	x	x	x	x

KC41	M	33	F	/	/	/	/	x	x	/	x
KC42	I	25	M	/	/	/	/	x	x	/	x

J ORA Reading for Study Groups

LE CHINESE SCHOOL FREQUENCY

		IOPg	IOPcc	CRF	CH
N	Valid	54	54	54	54
	Missing	0	0	0	0
Mean		17.58	15.89	12.54	12.21
Median		17.75	15.35	12.30	11.70
Std. Deviation		2.79	3.58	2.88	2.88
Variance		7.76	12.79	8.30	8.28
Range		13.40	22.00	15.70	15.70
Minimum		11.20	9.80	6.80	6.00
Maximum		24.60	31.80	22.50	21.70



RE INDIAN SCHOOL FREQUENCY

		IOPg	IOPcc	CH	CRF
N	Valid	48	48	48	48
	Missing	0	0	0	0
Mean		14.77	14.41	11.19	11.44
Median		14.65	14.10	11.20	11.50
Std. Deviation		2.83	3.02	1.27	1.33
Variance		8.03	9.12	1.61	1.76
Range		12.60	12.00	6.80	6.40
Minimum		8.30	8.70	7.70	8.80
Maximum		20.90	20.70	14.50	15.20

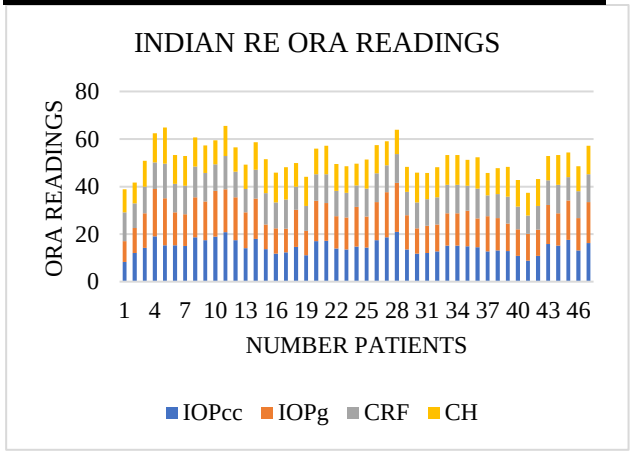


TABLE 5B: LE INDIAN SCHOOL FREQUENCY

		IOPg	IOPcc	CRF	CH
N	Valid	48	48	48	48
	Missing	0	0	0	0
Mean		14.77	14.40	11.19	11.44
Median		14.65	14.10	11.20	11.50
Std. Deviation		2.83	3.02	1.27	1.33
Variance		8.03	9.12	1.61	1.76
Range		12.60	12.00	6.80	6.40
Minimum		8.30	8.70	7.70	8.80
Maximum		20.90	20.70	14.50	15.20

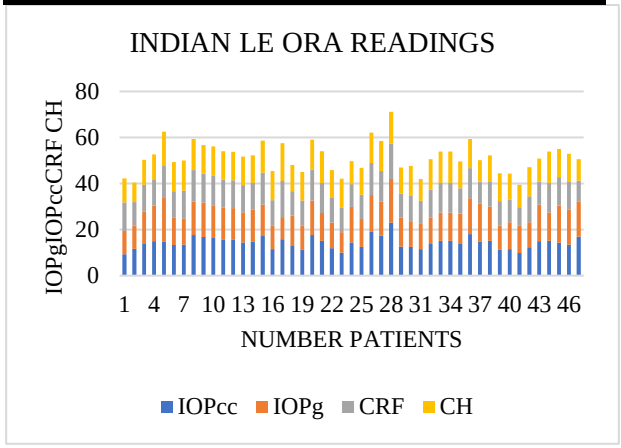


TABLE 6A: RE MALAY SCHOOL FREQUENCY

		IOPg	IOPcc	CRF	CH
N	Valid	52	52	52	52
	Missing	0	0	0	0
Mean		17.13	16.45	11.69	11.33
Median		16.60	15.90	11.60	11.30
Std. Deviation		3.12	3.09	1.72	1.62
Variance		9.73	9.56	2.94	2.64
Range		13.50	13.00	7.10	7.50
Minimum		11.90	11.30	8.90	7.90
Maximum		25.40	24.30	16.00	15.40

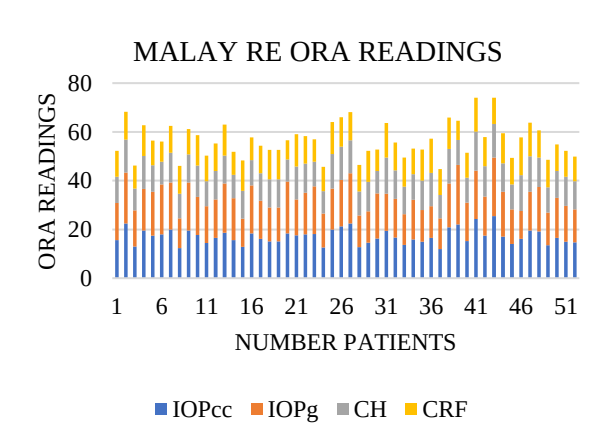
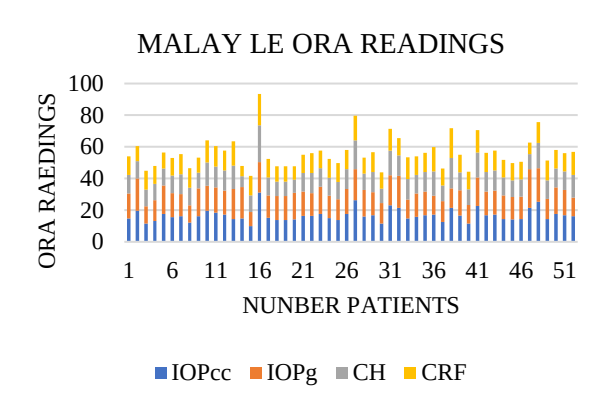


TABLE 6B: LE MALAY SCHOOL FREQUENCY

		IOPg	IOPcc	CRF	CH
N	Valid	52	52	52	52
	Missing	0	0	0	0
Mean		16.69	15.49	12.19	12.03
Median		16.15	15.20	11.70	11.80
Std. Deviation		3.96	2.99	2.79	2.34
Variance		15.67	8.95	7.77	5.46
Range		21.20	15.60	16.30	13.40
Minimum		9.90	8.90	6.90	6.50
Maximum		31.10	24.50	23.20	19.90



K Frequency of allele in different study groups

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
1	KC44	1-KC	C	F	32	A/A	T/T	A/A	C/C
2	KC48	1-KC	M	M	28	G/A	T/T	A/A	C/C
3	KC1	1-KC	M	M	17	G/A	T/T	A/A	C/C
4	KC10	1-KC	I	M	32	G/G	T/A	A/G	C/T
5	KC11	1-KC	M	F	19	G/A	T/T	A/A	C/C
6	KC13	1-KC	I	M	21	G/G	T/A	A/G	C/C
7	KC14	1-KC	M	M	16	G/A	T/T	A/A	C/C
8	KC15	1-KC	I	F	32	A/A	T/T	A/A	C/C
9	KC16	1-KC	I	M	29	A/A	T/T	A/A	C/C
10	KC18	1-KC	M	M	17	A/A	T/T	A/A	C/C
11	KC19	1-KC	I	M	19	G/A	T/T	A/A	C/C
12	KC2	1-KC	I	M	22	G/G	T/A	A/G	C/T
13	KC21	1-KC	I	F	30	G/G	T/A	A/G	C/C
14	KC22	1-KC	M	M	26	G/G	T/T	A/A	C/C
15	KC24	1-KC	I	M	24	G/G	T/A	A/G	C/C
16	KC25	1-KC	M	M	19	A/A	T/T	A/A	C/C
17	KC26	1-KC	M	M	23	G/A	T/T	A/A	C/C
18	KC27	1-KC	C	F	24	A/A	T/T	A/A	C/C
19	KC28	1-KC	C	M	18	G/A	T/T	A/A	C/C
20	KC3	1-KC	I	M	18	G/A	T/A	A/A	C/C
21	KC33	1-KC	M	M	33	G/G	T/T	A/A	C/C
22	KC35	1-KC	M	F	22	G/G	T/T	A/A	C/C
23	KC36	1-KC	C	M	20	A/A	T/T	A/A	C/C
24	KC37	1-KC	C	F	31	G/A	T/T	A/A	C/C
25	KC38	1-KC	C	M	29	G/G	T/T	A/A	C/C
26	KC4	1-KC	I	M	28	G/G	T/T	A/A	C/C
27	KC40	1-KC	I	M	25	G/G	T/T	A/A	C/C
28	KC45	1-KC	M	F	30	G/A	T/T	A/A	C/C
29	KC47	1-KC	I	F	33	G/A	T/A	A/G	C/C
30	KC49	1-KC	I	M	27	G/G	T/A	A/G	C/C
31	KC5	1-KC	I	F	32	G/G	T/T	A/A	C/C
32	KC50	1-KC	I	M	28	G/G	T/T	A/A	C/C
33	KC54	1-KC	I	F	35	G/A	T/A	A/G	C/T
34	KC56	1-KC	I	F	22	G/G	T/A	A/G	C/C
35	KC6	1-KC	I	M	18	G/G	T/T	A/A	C/C
36	KC62	1-KC	I	M	14	G/G	T/A	A/G	C/C
37	KC63	1-KC	I	M	19	G/G	T/A	A/G	C/C
38	KC64	1-KC	I	M	24	G/G	T/T	A/A	C/C
39	KC66	1-KC	M	F	19	G/G	T/T	A/A	C/C
40	KC67	1-KC	I	M	20	G/A	T/A	A/G	C/C
41	KC68	1-KC	M	F	33	G/G	T/A	A/G	C/C
42	KC8	1-KC	I	M	25	G/G	T/T	A/A	C/C
43	PreKC1	0-PreKC	I	M	8	G/G	T/T	A/A	C/C
44	PreKC2	0-PreKC	I	F	12	G/G	T/A	A/G	C/C
45	PreKC3	0-PreKC	I	F	26	G/G	T/A	A/G	C/C
46	PreKC4	0-PreKC	I	F	58	G/G	T/T	A/A	C/C
47	PreKC5	0-PreKC	I	M	8	G/G	T/A	A/A	C/C
48	PreKC6	0-PreKC	I	F	32	G/G	A/A	G/G	C/C
49	PreKC7	0-PreKC	I	M	21	G/A	T/A	A/G	C/C
50	PreKC8	0-PreKC	I	M	13	G/G	T/T	A/A	C/C
51	F1	0-FAMILY	M	F	31	G/G	T/T	A/A	C/C
52	F2	0-FAMILY	I	M	48	G/G	T/T	A/A	C/C
53	F3	0-FAMILY	I	F	41	G/G	T/T	A/A	C/C
54	F4	0-FAMILY	I	M	18	G/G	T/A	A/G	C/C
55	F5	0-FAMILY	I	M	14	G/G	T/A	A/G	C/C
56	F6	0-FAMILY	M	M	19	G/G	T/T	A/A	C/C
57	F7	0-FAMILY	M	M	51	G/G	T/T	A/A	C/C
58	F8	0-FAMILY	M	F	50	G/G	T/T	A/A	C/C
59	F9	0-FAMILY	M	M	22	G/G	T/T	A/A	C/C
60	F10	0-FAMILY	M	M	15	G/G	T/T	A/A	C/C

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
61	F11	0-FAMILY	M	M	8	G/G	T/T	A/A	C/C
62	F12	0-FAMILY	M	M	27	G/G	T/T	A/A	C/C
63	F13	0-FAMILY	M	F	29	G/G	T/T	A/A	C/C
64	F14	0-FAMILY	I	M	42	G/G	T/T	A/A	C/C
65	F15	0-FAMILY	I	F	40	G/G	T/T	A/A	C/C
66	F16	0-FAMILY	I	F	11	G/G	T/T	A/A	C/C
67	F17	0-FAMILY	I	M	44	G/A	T/A	A/G	C/C
68	F18	0-FAMILY	I	M	63	G/G	T/T	A/A	C/C
69	F19	0-FAMILY	I	F	57	G/G	T/T	A/A	C/C
70	F20	0-FAMILY	I	F	37	G/G	T/T	A/A	C/C
71	F21	0-FAMILY	I	M	41	G/G	T/T	A/A	C/C
72	F22	0-FAMILY	I	M	12	G/G	T/T	A/A	C/C
73	F23	0-FAMILY	I	M	8	G/G	T/T	A/A	C/C
74	F24	0-FAMILY	I	F	33	G/G	T/A	A/G	C/T
75	F25	0-FAMILY	C	M	50	G/G	T/T	A/A	C/C
76	F26	0-FAMILY	I	F	57	G/G	A/A	G/G	C/C
77	F27	0-FAMILY	I	F	14	G/G	A/A	G/G	C/C
78	F28	0-FAMILY	I	M	51	G/G	T/T	A/A	C/C
79	F29	0-FAMILY	I	F	48	G/G	T/A	A/G	C/C
80	F30	0-FAMILY	I	F	11	G/G	A/A	G/G	C/C
81	F31	0-FAMILY	I	M	17	G/G	T/A	A/G	C/C
82	F32	0-FAMILY	I	M	60	G/G	T/T	A/A	C/C
83	F33	0-FAMILY	I	F	21	G/A	T/A	A/G	C/C
84	F34	0-FAMILY	I	M	52	G/G	T/T	A/A	C/C
85	F35	0-FAMILY	I	F	49	G/G	T/A	A/G	C/C
86	F36	0-FAMILY	M	M	41	G/G	T/T	A/A	C/C
87	F37	0-FAMILY	C	F	68	G/G	T/T	A/A	C/C
88	F38	0-FAMILY	M	M	63	G/G	T/T	A/A	C/C
89	F39	0-FAMILY	M	F	29	G/G	T/T	A/A	C/C
90	F40	0-FAMILY	M	M	23	G/G	T/T	A/A	C/C
91	F41	0-FAMILY	C	F	24	G/G	T/T	A/A	C/C
92	F42	0-FAMILY	C	F	20	A/A	T/T	A/A	C/C
94	F44	0-FAMILY	M	M	51	G/G	T/T	A/A	C/C
95	F45	0-FAMILY	M	F	49	G/A	T/T	A/A	C/C
96	F46	0-FAMILY	M	M	16	G/G	T/T	A/A	C/C
97	F47	0-FAMILY	M	F	13	A/A	T/T	A/A	C/C
98	F48	0-FAMILY	I	F	56	G/G	T/T	A/A	C/C
99	F49	0-FAMILY	I	F	21	G/G	T/A	A/G	C/C
100	F50	0-FAMILY	M	F	41	G/G	T/T	A/A	C/C
101	F51	0-FAMILY	M	M	46	G/G	T/T	A/A	C/C
102	F52	0-FAMILY	M	M	12	G/G	T/T	A/A	C/C
103	F53	0-FAMILY	M	F	10	G/G	T/T	A/A	C/C
104	F54	0-FAMILY	M	M	7	G/G	T/T	A/A	C/C
105	F55	0-FAMILY	M	M	5	G/G	T/T	A/A	C/C
106	F56	0-FAMILY	M	M	25	G/G	T/T	A/A	C/C
107	F57	0-FAMILY	I	M	40	G/G	T/A	A/G	C/C
108	F58	0-FAMILY	I	F	39	G/G	T/T	A/A	C/C
109	F59	0-FAMILY	I	F	13	G/G	T/T	A/A	C/C
110	F60	0-FAMILY	I	M	12	G/G	T/A	A/G	C/C
111	F62	0-FAMILY	M	M	49	G/A	T/T	A/A	C/C
112	F63	0-FAMILY	M	F	21	G/G	T/T	A/A	C/C
113	F64	0-FAMILY	M	F	19	G/G	T/T	A/A	C/C
114	F65	0-FAMILY	I	M	56	G/G	T/T	A/A	C/C
115	F66	0-FAMILY	I	F	51	G/G	T/T	A/A	C/C
116	F67	0-FAMILY	I	M	21	G/G	T/T	A/A	C/C
117	F68	0-FAMILY	I	M	11	G/G	T/T	A/A	C/C
118	F69	0-FAMILY	C	M	52	G/G	T/T	A/A	C/C
119	F70	0-FAMILY	C	F	48	G/A	T/T	A/A	C/C
120	F71	0-FAMILY	I	M	6	G/G	T/T	A/A	C/C
121	F72	0-FAMILY	I	F	56	G/G	T/T	A/A	C/C
122	F73	0-FAMILY	I	F	32	G/G	T/T	A/A	C/C
123	F74	0-FAMILY	I	M	5	G/G	T/T	A/A	C/C
124	F75	0-FAMILY	M	F	54	A/A	T/T	A/A	C/C
125	F76	0-FAMILY	C	M	49	G/G	T/T	A/A	C/C
126	F77	0-FAMILY	C	F	50	G/G	T/T	A/A	C/C
127	F79	0-FAMILY	C	F	43	G/G	T/T	A/A	C/C
128	F80	0-FAMILY	C	F	15	G/G	T/T	A/A	C/C
129	F81	0-FAMILY	I	M	52	G/G	T/T	A/A	C/C
130	F82	0-FAMILY	I	F	45	G/G	T/T	A/A	C/C
131	F83	0-FAMILY	I	M	24	G/G	T/T	A/A	C/C

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
132	F84	0-FAMILY	M	F	50	G/G	T/T	A/A	C/C
133	F85	0-FAMILY	M	F	19	G/A	T/T	A/A	C/C
134	F86	0-FAMILY	C	F	49	G/G	T/T	A/A	C/C
135	F87	0-FAMILY	C	F	27	G/G	T/T	A/A	C/C
136	F88	0-FAMILY	C	M	25	G/A	T/T	A/A	C/C
137	F89	0-FAMILY	C	M	58	G/G	T/T	A/A	C/C
138	F90	0-FAMILY	C	M	28	G/G	T/T	A/A	C/C
139	F91	0-FAMILY	M	M	17	G/G	T/T	A/A	C/C
140	F92	0-FAMILY	M	F	51	G/G	T/T	A/A	C/C
141	F93	0-FAMILY	M	M	17	G/G	T/T	A/A	C/C
142	F94	0-FAMILY	C	F	48	A/A	T/T	A/A	C/C
143	F95	0-FAMILY	C	M	28	G/G	T/T	A/A	C/C
144	F96	0-FAMILY	I	M	45	G/G	T/A	A/G	C/C
145	F97	0-FAMILY	I	F	41	G/A	T/A	A/G	C/C
146	F98	0-FAMILY	I	F	66	G/A	T/A	A/G	C/C
147	F99	0-FAMILY	I	M	46	G/G	T/T	A/A	C/C
148	F100	0-FAMILY	I	F	44	G/G	T/T	A/A	C/C
149	F101	0-FAMILY	I	F	15	G/G	T/T	A/A	C/C
150	F102	0-FAMILY	I	F	12	G/G	T/T	A/A	C/C
151	F103	0-FAMILY	I	M	57	G/G	T/T	A/A	C/C
152	F104	0-FAMILY	I	F	53	G/G	T/T	A/A	C/C
153	F105	0-FAMILY	I	M	58	G/G	T/A	A/G	C/C
154	F106	0-FAMILY	I	F	80	G/G	T/A	A/G	C/C
155	F107	0-FAMILY	I	F	30	G/G	T/T	A/A	C/C
156	F108	0-FAMILY	I	F	55	G/G	T/T	A/A	C/C
157	F109	0-FAMILY	I	F	58	G/G	T/T	A/A	C/C
158	F110	0-FAMILY	I	F	52	G/G	T/T	A/A	C/C
159	F111	0-FAMILY	I	M	60	G/G	T/T	A/A	C/C
160	F112	0-FAMILY	I	M	26	G/G	T/T	A/A	C/C
161	F113	0-FAMILY	I	F	50	G/G	T/T	A/A	C/C
162	F114	0-FAMILY	I	F	22	G/G	T/T	A/A	C/C
163	F115	0-FAMILY	I	M	19	G/G	T/T	A/A	C/C
164	F116	0-FAMILY	I	F	11	G/G	T/T	A/A	C/C
165	F117	0-FAMILY	I	M	40	G/G	T/T	A/A	C/C
166	F118	0-FAMILY	I	M	57	G/G	T/T	A/A	C/C
167	F119	0-FAMILY	I	F	50	G/G	T/T	A/A	C/C
168	F120	0-FAMILY	I	M	23	G/G	T/T	A/A	C/C
169	F121	0-FAMILY	M	M	49	G/A	T/T	A/A	C/C
170	F122	0-FAMILY	M	F	47	G/G	T/T	A/A	C/C
171	F123	0-FAMILY	I	M	52	G/G	T/T	A/A	C/C
172	F124	0-FAMILY	I	F	48	G/G	T/T	A/A	C/C
173	F125	0-FAMILY	I	F	37	G/G	T/T	A/A	C/C
174	F126	0-FAMILY	I	M	37	G/G	T/A	A/G	C/C
175	F127	0-FAMILY	C	M	28	G/G	T/T	A/A	C/C
176	ST1	0-CHILDREN	I	F	10	G/G	T/T	A/A	C/C
177	ST2	0-CHILDREN	I	M	9	G/G	T/T	A/A	C/C
178	ST3	0-CHILDREN	I	F	9	G/A	T/T	A/A	C/C
179	ST10	0-CHILDREN	I	F	10	G/G	T/A	A/G	C/C
180	ST11	0-CHILDREN	I	F	10	A/A	T/T	A/A	C/C
181	ST12	0-CHILDREN	I	F	11	G/G	A/A	G/G	C/C
182	ST13	0-CHILDREN	I	F	11	G/G	T/T	A/A	C/C
184	ST15	0-CHILDREN	I	M	10	G/G	T/A	A/G	C/C
185	ST16	0-CHILDREN	I	F	9	G/G	T/T	A/A	C/C
186	ST17	0-CHILDREN	I	M	10	G/G	T/T	A/A	C/C
187	ST18	0-CHILDREN	I	M	11	G/A	T/T	A/A	C/C
188	ST19	0-CHILDREN	I	F	10	G/G	T/T	A/A	C/C
189	ST20	0-CHILDREN	I	F	10	G/G	A/A	G/G	C/C
190	ST21	0-CHILDREN	I	M	11	G/G	T/T	A/A	C/C
191	ST22	0-CHILDREN	I	F	12	G/G	T/T	A/A	C/C
192	ST23	0-CHILDREN	I	M	12	G/G	T/T	A/A	C/C
193	ST24	0-CHILDREN	I	F	11	G/G	T/T	A/A	C/C
194	ST25	0-CHILDREN	I	M	10	A/A	T/T	A/A	C/C
195	ST26	0-CHILDREN	I	M	10	G/G	A/A	G/G	C/C
196	ST27	0-CHILDREN	I	F	10	G/G	T/A	A/G	C/T
197	ST28	0-CHILDREN	I	F	10	G/G	T/A	A/G	C/C
198	ST29	0-CHILDREN	I	F	9	A/A	T/T	A/A	C/C
199	ST30	0-CHILDREN	I	F	10	G/A	T/T	A/A	C/C
200	ST33	0-CHILDREN	I	F	10	G/G	T/A	A/G	C/C
201	ST34	0-CHILDREN	I	M	11	G/G	T/A	A/G	C/C
202	ST35	0-CHILDREN	I	M	9	G/G	T/T	A/A	C/C

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
203	ST36	0-CHILDREN	I	F	10	G/G	T/T	A/A	C/C
204	ST37	0-CHILDREN	I	F	12	G/G	T/A	A/G	C/C
205	ST38	0-CHILDREN	I	F	12	G/G	T/A	A/G	C/T
206	ST39	0-CHILDREN	I	F	10	G/A	T/T	A/A	C/C
207	ST40	0-CHILDREN	I	F	12	G/A	T/T	A/A	C/C
208	BTHO1	0-CHILDREN	M	F	10	A/A	T/T	A/A	C/C
209	BTHO2	0-CHILDREN	M	F	10	G/G	A/A	A/G	C/T
210	BTHO3	0-CHILDREN	M	F	9	G/A	T/T	A/A	C/C
211	BTHO4	0-CHILDREN	M	F	9	G/A	T/T	A/A	C/C
212	BTHO5	0-CHILDREN	M	F	9	G/A	T/T	A/A	C/C
213	BTHO6	0-CHILDREN	M	M	10	G/A	T/T	A/A	C/C
214	BTHO7	0-CHILDREN	M	M	9	A/A	T/T	A/A	C/C
215	BTHO12	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
216	BTHO13	0-CHILDREN	M	M	12	G/A	T/T	A/A	C/C
217	BTHO15	0-CHILDREN	M	M	10	G/A	T/T	A/A	C/C
218	BTHO16	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
219	BTHO17	0-CHILDREN	M	M	11	G/G	T/T	A/A	C/C
220	BTHO18	0-CHILDREN	M	M	12	G/A	T/A	A/G	C/C
221	BTHO19	0-CHILDREN	M	M	11	G/A	T/A	A/G	C/T
222	BTHO21	0-CHILDREN	M	M	9	G/G	T/T	A/A	C/C
223	BTHO22	0-CHILDREN	M	F	9	G/G	T/T	A/A	C/C
224	BTHO23	0-CHILDREN	M	F	10	G/G	T/T	A/A	C/C
225	BTHO24	0-CHILDREN	M	F	10	G/A	T/T	A/A	C/C
226	BTHO25	0-CHILDREN	M	M	9	G/G	T/T	A/A	C/C
227	BTHO26	0-CHILDREN	M	M	9	G/G	T/T	A/A	C/C
228	BTHO28	0-CHILDREN	M	M	10	G/G	T/T	A/A	C/C
229	BTHO29	0-CHILDREN	M	F	10	G/G	T/T	A/A	C/C
230	BTHO30	0-CHILDREN	M	M	10	A/A	T/T	A/A	C/C
232	BTHO32	0-CHILDREN	M	F	11	G/G	T/T	A/A	C/C
233	BTHO33	0-CHILDREN	M	M	11	G/G	T/T	A/A	C/C
234	BTHO34	0CHILDREN	M	F	11	G/G	T/T	A/A	C/C
235	BTHO35	0-CHILDREN	M	F	10	G/G	T/T	A/A	C/C
236	BTHO39	0-CHILDREN	M	F	10	G/G	T/T	A/A	C/C
237	BTHO40	0-CHILDREN	M	M	10	G/G	T/T	A/A	C/C
238	BTHO41	0-CHILDREN	M	M	9	G/G	T/T	A/A	C/C
239	BTHO42	0-CHILDREN	M	M	9	A/A	T/T	A/A	C/C
240	BTHO43	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
241	BTHO44	0-CHILDREN	M	M	11	G/G	T/T	A/A	C/C
242	BTHO45	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
243	BTHO47	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
244	BTHO48	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
245	BTHO49	0-CHILDREN	M	M	11	G/G	T/T	A/A	C/C
246	BTHO50	0-CHILDREN	M	M	11	G/G	T/T	A/A	C/C
247	BTHO51	0-CHILDREN	M	M	11	G/A	T/T	A/A	C/C
248	SM01	0-CHILDREN	C	F	11	A/A	T/T	A/A	C/C
249	SM02	0-CHILDREN	C	M	10	A/A	T/T	A/A	C/C
250	SM03	0-CHILDREN	C	M	9	A/A	T/T	A/A	C/C
251	SM04	0-CHILDREN	C	M	10	G/A	T/T	A/A	C/C
252	SM05	0-CHILDREN	C	F	11	G/A	T/T	A/A	C/C
253	SM06	0-CHILDREN	C	F	10	G/G	T/T	A/A	C/C
254	SM07	0-CHILDREN	C	F	11	G/G	T/T	A/A	C/C
256	SM09	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
257	SM10	0-CHILDREN	C	F	11	G/G	T/T	A/A	C/C
258	SM11	0-CHILDREN	C	F	10	G/G	T/T	A/A	C/C
259	SM12	0-CHILDREN	C	M	10	G/A	T/T	A/A	C/C
260	SM13	0-CHILDREN	C	F	9	A/A	T/T	A/A	C/C
261	SM14	0-CHILDREN	C	M	11	G/A	T/T	A/A	C/C
262	SM15	0-CHILDREN	C	F	11	G/G	T/A	A/G	C/C
263	SM16	0-CHILDREN	C	M	12	G/A	T/T	A/A	C/C
264	SM17	0-CHILDREN	C	M	11	G/G	T/A	A/G	C/C
265	SM18	0-CHILDREN	C	F	10	G/A	T/T	A/A	C/C
266	SM19	0-CHILDREN	C	F	12	G/G	T/T	A/A	C/C
267	SM20	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
268	SM21	0-CHILDREN	C	M	10	G/G	T/T	A/A	C/C
269	SM22	0-CHILDREN	C	F	10	G/G	T/T	A/A	C/C
270	SM23	0-CHILDREN	C	F	12	G/G	T/T	A/A	C/C
271	SM24	0-CHILDREN	C	M	11	G/G	T/A	A/G	C/T
272	SM27	0-CHILDREN	C	F	11	G/G	T/A	A/G	C/C
273	SM29	0-CHILDREN	C	M	10	G/A	T/T	A/A	C/C
274	SM30	0-CHILDREN	C	F	11	G/A	T/T	A/A	C/C

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
275	SM31	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
276	SM32	0-CHILDREN	C	F	11	G/G	T/T	A/A	C/C
277	SM33	0-CHILDREN	C	F	10	G/G	T/T	A/A	C/C
278	SM34	0-CHILDREN	C	F	11	G/G	T/T	A/A	C/C
280	SM36	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
281	SM37	0-CHILDREN	C	F	12	G/A	T/T	A/A	C/C
282	SM38	0-CHILDREN	C	F	11	G/G	T/T	A/A	C/C
283	SM40	0-CHILDREN	C	M	9	G/G	T/T	A/A	C/T
284	SM41	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
285	SM42	0-CHILDREN	C	M	11	G/G	T/T	A/A	C/C
286	SM43	0-CHILDREN	C	F	12	G/A	T/T	A/A	C/C
287	SM44	0-CHILDREN	C	F	10	G/G	T/A	A/G	C/C
288	SM45	0-CHILDREN	C	M	12	G/G	T/T	A/A	C/C
289	SM46	0-CHILDREN	C	F	10	G/A	T/T	A/A	C/C
290	C3	0-CONTROL	I	M	39	G/G	T/T	A/A	C/C
291	C4	0-CONTROL	I	F	37	G/G	T/T	A/A	C/C
292	C7	0-CONTROL	I	F	21	G/G	T/A	A/G	C/C
293	C14	0-CONTROL	I	F	23	G/A	T/T	A/A	C/C
294	C16	0-CONTROL	I	F	23	G/G	T/T	A/A	C/C
295	C29	0-CONTROL	I	F	22	G/G	T/T	A/A	C/C
296	C34	0-CONTROL	I	F	24	G/G	T/T	A/A	C/C
297	C41	0-CONTROL	I	F	22	G/G	T/T	A/A	C/C
298	C45	0-CONTROL	I	M	22	G/G	T/T	A/A	C/C
299	C50	0-CONTROL	I	M	55	G/G	T/A	A/G	C/T
300	C52	0-CONTROL	I	F	28	G/A	T/T	A/A	C/C
301	C53	0-CONTROL	I	F	45	G/G	T/A	A/G	C/T
302	C54	0-CONTROL	I	F	43	G/G	T/A	A/G	C/T
304	C61	0-CONTROL	I	F	41	G/G	T/A	A/G	C/T
305	C62	0-CONTROL	I	F	39	G/A	T/T	A/A	C/C
306	C63	0-CONTROL	I	M	8	A/A	T/T	A/A	C/C
307	C64	0-CONTROL	I	F	16	G/G	T/T	A/A	C/C
308	C72-I	0-CONTROL	I	M	59	G/G	T/T	A/A	C/C
309	C77-I	0-CONTROL	I	M	51	G/G	T/A	A/G	C/T
310	C85-I	0-CONTROL	I	F	48	G/G	T/T	A/A	C/T
311	C86-I	0-CONTROL	I	F	30	G/G	T/T	A/A	C/C
312	C87-I	0CONTROL	I	M	53	G/G	T/A	A/G	C/C
313	C88-I	0-CONTROL	I	F	52	G/G	T/A	A/G	C/C
314	C89-I	0-CONTROL	I	F	49	G/G	T/A	A/G	C/C
315	C96-I	0-CONTROL	I	F	42	G/G	T/T	A/A	C/C
316	C97-I	0-CONTROL	I	F	40	A/A	T/T	A/A	C/C
317	C98	0-CONTROL	I	F	15	G/A	T/T	A/A	C/C
318	C5	0-CONTROL	C	F	53	G/A	T/T	A/A	C/C
319	C8	0-CONTROL	C	F	21	A/A	T/T	A/A	C/C
320	C9	0-CONTROL	C	F	22	A/A	T/T	A/A	C/C
321	C10	0-CONTROL	C	M	21	A/A	T/T	A/A	C/C
322	C12	0-CONTROL	C	F	21	G/A	T/T	A/A	C/C
323	C13	0-CONTROL	C	F	23	G/A	T/T	A/A	C/C
324	C15	0-CONTROL	C	F	23	G/A	T/T	A/A	C/C
325	C17	0-CONTROL	C	M	21	G/G	T/T	A/A	C/C
326	C18	0-CONTROL	C	M	21	G/A	T/T	A/A	C/C
328	C20	0-CONTROL	C	F	21	G/A	T/A	A/G	C/C
329	C21	0-CONTROL	C	F	21	G/G	T/T	A/A	C/C
330	C22	0-CONTROL	C	F	22	G/A	T/A	A/G	C/C
331	C23	0-CONTROL	C	F	21	G/A	T/T	A/A	C/C
332	C24	0-CONTROL	C	F	21	A/A	T/T	A/A	C/C
333	C25	0-CONTROL	C	M	20	G/G	T/T	A/A	C/C
334	C26	0-CONTROL	C	F	21	G/A	T/T	A/A	C/C
335	C27	0-CONTROL	C	F	26	G/G	T/T	A/A	C/C
336	C28	0-CONTROL	C	F	22	G/G	T/T	A/A	C/C
337	C30	0-CONTROL	C	M	22	G/A	T/T	A/A	C/C
338	C31	0-CONTROL	C	M	21	G/A	T/T	A/A	C/C
339	C32	0-CONTROL	C	M	22	G/G	T/T	A/A	C/C
340	C33	0-CONTROL	C	M	21	G/A	T/T	A/A	C/C
341	C35	0-CONTROL	C	F	22	G/A	T/T	A/A	C/C
342	C36	0-CONTROL	C	F	22	G/A	T/T	A/A	C/C
343	C37	0-CONTROL	C	M	22	A/A	T/T	A/A	C/C
344	C38	0-CONTROL	C	F	22	A/A	T/T	A/A	C/C
345	C39	0-CONTROL	C	F	21	G/A	T/T	A/A	C/C
346	C42	0-CONTROL	C	F	22	G/A	T/T	A/A	C/C
347	C43	0-CONTROL	C	F	22	G/A	T/T	A/A	C/C

	ID	STATUS	Race	Gender	Age	rs6138482	rs56157240	rs12480307	rs201363715
348	C44	0-CONTROL	C	F	22	G/A	T/T	A/A	C/C
349	C47	0-CONTROL	C	M	20	G/G	T/T	A/A	C/C
350	C48	0-CONTROL	C	F	22	G/G	T/T	A/A	C/C
352	C65-I	0-CONTROL	C	M	36	G/A	T/T	A/A	C/C
353	C66-I	0-CONTROL	C	F	42	A/A	T/T	A/A	C/C
354	C67-I	0-CONTROL	C	M	24	A/A	T/T	A/A	C/C
355	C68-I	0-CONTROL	C	M	48	A/A	T/T	A/A	C/C
356	C69-I	0-CONTROL	C	F	42	G/G	T/T	A/A	C/C
357	C70-I	0-CONTROL	C	M	14	G/A	T/T	A/A	C/C
358	C71-I	0-CONTROL	C	M	9	G/A	T/T	A/A	C/C
359	C73-I	0-CONTROL	C	M	57	G/A	T/T	A/A	C/C
360	C74-I	0-CONTROL	C	F	58	G/G	T/A	A/G	C/C
361	C76-I	0-CONTROL	C	F	55	G/G	T/T	A/A	C/C
362	C78-I	0-CONTROL	C	M	60	G/G	T/T	A/A	C/T
363	C79-I	0-CONTROL	C	F	47	G/G	T/T	A/A	C/T
364	C80-I	0-CONTROL	C	M	69	G/G	T/T	A/A	C/C
365	C81-I	0-CONTROL	C	F	66	G/G	T/T	A/A	C/C
366	C82-I	0-CONTROL	C	F	33	G/G	T/T	A/A	C/C
367	C83-I	0-CONTROL	C	M	36	G/G	T/T	A/A	C/C
368	C84-I	0-CONTROL	C	F	37	G/G	T/T	A/A	C/C
369	C90-I	0-CONTROL	C	M	17	A/A	T/T	A/A	C/C
370	C94-I	0-CONTROL	C	F	41	A/A	T/T	A/A	C/C
371	C95-I	0-CONTROL	C	M	8	G/G	T/T	A/A	C/C
372	C1	0-CONTROL	M	F	33	G/A	T/T	A/A	C/C
373	C2	0-CONTROL	M	M	36	G/A	T/T	A/A	C/C
374	C6	0-CONTROL	M	F	34	G/G	T/T	A/A	C/C
376	C46	0-CONTROL	M	F	22	G/G	T/T	A/A	C/C
377	C51	0-CONTROL	M	F	22	A/A	T/T	A/A	C/C
378	C56	0-CONTROL	M	M	20	G/A	T/T	A/A	C/C
379	C57	0-CONTROL	M	M	18	G/A	T/T	A/A	C/C
380	C58	0-CONTROL	M	M	17	G/G	T/T	A/A	C/C
381	C59	0-CONTROL	M	M	17	G/G	T/T	A/A	C/C
382	C60	0-CONTROL	M	M	17	G/G	T/T	A/A	C/C
383	C91-I	0-CONTROL	M	M	49	A/A	T/T	A/A	C/C
384	C92-I	0-CONTROL	M	F	49	A/A	T/T	A/A	C/C
385	C93-I	0-CONTROL	M	F	17	A/A	T/T	A/A	C/C

L1 SNPStats results 42 KC & FAMILY (ALL RACES)

SNPStats results

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Descriptive statistics

Response variable: STATUS **Type:** categorical

	n	missing	unique
All subjects	167	0	2
STATUS=0-FAMILY	125 (74.85%)	---	---
STATUS=1-KC	42 (25.15%)	---	---

Covariate: Race **Type:** categorical

	n	missing	unique
All subjects	167	0	3
STATUS=0-FAMILY	125	0	3
STATUS=1-KC	42	0	3

	C	I	M
All subjects	25 (15%)	94 (56%)	48 (29%)
STATUS=0-FAMILY	19 (15%)	71 (57%)	35 (28%)
STATUS=1-KC	6 (14%)	23 (55%)	13 (31%)

Covariate: Gender **Type:** categorical

	n	missing	unique
All subjects	167	0	2
STATUS=0-FAMILY	125	0	2
STATUS=1-KC	42	0	2

	F	M
All subjects	77 (46%)	90 (54%)
STATUS=0-FAMILY	63 (50%)	62 (50%)
STATUS=1-KC	14 (33%)	28 (67%)

Covariate: Age **Type:** quantitative

	n	missing	unique	mean	.05	.10	.25	.50	.75	.90	.95
All subjects	167	0	55	32.98	11	13.6	19	29	48.5	55.4	58
STATUS = 0-FAMILY	125	0	54	35.8	10.2	12	19	40	50	57	59.6
STATUS = 1-KC	42	0	20	24.6	17	18	19	24	29.75	32	33

lowest: 5, 6, 7, 8, 10 highest: 60, 63, 66, 68, 80

Single-SNP analysis

SNP: rs6138482

Percentage of typed samples: 167/167 (100%)

rs6138482 allele frequencies (n=167)						
	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Allele	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
G	289	0.87	232	0.93	57	0.68
A	45	0.13	18	0.07	27	0.32

rs6138482 genotype frequencies (n=167)						
	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Genotype	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
A/A	11	0.07	4	0.03	7	0.17

G/A	23	0.14	10	0.08	13	0.31
G/G	133	0.8	111	0.89	22	0.52

rs6138482 exact test for Hardy-Weinberg equilibrium (n=167)						
	N11	N12	N22	N1	N2	P-value
All subjects	133	23	11	289	45	<0.0001
STATUS=0-FAMILY	111	10	4	232	18	0.0011
STATUS=1-KC	22	13	7	57	27	0.075

rs6138482 association with response STATUS (n=167, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-FAMILY	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	111 (88.8%)	22 (52.4%)	1.00			
	G/A	10 (8%)	13 (30.9%)	10.17 (3.36-30.78)	<0.0001	157.5	179.3
	A/A	4 (3.2%)	7 (16.7%)	16.43 (3.23-83.46)			
Dominant	G/G	111 (88.8%)	22 (52.4%)	1.00			
	G/A-A/A	14 (11.2%)	20 (47.6%)	11.58 (4.21-31.88)	<0.0001	155.8	174.5
Recessive	G/G-G/A	121 (96.8%)	35 (83.3%)	1.00			
	A/A	4 (3.2%)	7 (16.7%)	8.05 (1.82-35.70)	0.004	173.9	192.6
Overdominant	G/G-A/A	115 (92%)	29 (69%)	1.00			
	G/A	10 (8%)	13 (30.9%)	6.60 (2.35-18.52)	2e-04	168.6	187.4
Log-additive	---	---	---	5.54 (2.58-11.90)	<0.0001	157.8	176.5

SNP: rs56157240

Percentage of typed samples: 167/167 (100%)

rs56157240 allele frequencies (n=167)						
	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	297	0.89	227	0.91	70	0.83
A	37	0.11	23	0.09	14	0.17

rs56157240 genotype frequencies (n=167)						
	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	3	0.02	3	0.02	0	0
T/A	31	0.19	17	0.14	14	0.33
T/T	133	0.8	105	0.84	28	0.67

rs56157240 exact test for Hardy-Weinberg equilibrium (n=167)						
	N11	N12	N22	N1	N2	P-value
All subjects	133	31	3	297	37	0.43
STATUS=0-FAMILY	105	17	3	227	23	0.065
STATUS=1-KC	28	14	0	70	14	0.57

rs56157240 association with response STATUS (n=167, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-FAMILY	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	T/T	105 (84%)	28 (66.7%)	1.00			
	T/A	17 (13.6%)	14 (33.3%)	4.31 (1.53-12.11)	0.0067	174.1	196
	A/A	3 (2.4%)	0 (0%)	0.00 (0.00-NA)			
Dominant	T/T	105 (84%)	28 (66.7%)	1.00			
	T/A-A/A	20 (16%)	14 (33.3%)	3.61 (1.31-9.94)	0.012	175.8	194.5
Recessive	T/T-T/A	122 (97.6%)	42 (100%)	1.00			
	A/A	3 (2.4%)	0 (0%)	0.00 (0.00-NA)	0.15	180.1	198.8
Overdominant	T/T-A/A	108 (86.4%)	28 (66.7%)	1.00			
	T/A	17 (13.6%)	14 (33.3%)	4.61 (1.65-12.92)	0.0031	173.4	192.1
Log-additive	---	---	---	2.27 (0.95-5.45)	0.066	178.7	197.5

SNP: rs12480307

Percentage of typed samples: 167/167 (100%)

rs12480307 allele frequencies (n=167)						
	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	298	0.89	227	0.91	71	0.85

G	36	0.11	23	0.09	13	0.15

rs12480307 genotype frequencies (n=167)

	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	134	0.8	105	0.84	29	0.69
A/G	30	0.18	17	0.14	13	0.31
G/G	3	0.02	3	0.02	0	0

rs12480307 exact test for Hardy-Weinberg equilibrium (n=167)

	N11	N12	N22	N1	N2	P-value
All subjects	134	30	3	298	36	0.41
STATUS=0-FAMILY	105	17	3	227	23	0.065
STATUS=1-KC	29	13	0	71	13	0.57

rs12480307 association with response STATUS (n=167, adjusted by Race+Gender+Age)

Model	Genotype	STATUS=0-FAMILY	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	105 (84%)	29 (69%)	1.00			
	A/G	17 (13.6%)	13 (30.9%)	**3.75 (1.34-10.50)**	0.014	175.6	197.4
	G/G	3 (2.4%)	0 (0%)	0.00 (0.00-NA)			
Dominant	A/A	105 (84%)	29 (69%)	1.00	0.026	177.2	195.9
	A/G-G/G	20 (16%)	13 (30.9%)	**3.14 (1.14-8.60)**			
Recessive	A/A-A/G	122 (97.6%)	42 (100%)	1.00	0.15	180.1	198.8
	G/G	3 (2.4%)	0 (0%)	0.00 (0.00-NA)			
Overdominant	A/A-G/G	108 (86.4%)	29 (69%)	1.00	0.0073	174.9	193.7
	A/G	17 (13.6%)	13 (30.9%)	**4.02 (1.44-11.20)**			
Log-additive	---	---	---	2.04 (0.85-4.88)	0.11	179.6	198.3

SNP: rs201363715

Percentage of typed samples: 167/167 (100%)

rs201363715 allele frequencies (n=167)

	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
C	330	0.99	249	1	81	0.96
T	4	0.01	1	0	3	0.04

rs201363715 genotype frequencies (n=167)

	All subjects		STATUS=0-FAMILY		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
C/C	163	0.98	124	0.99	39	0.93
C/T	4	0.02	1	0.01	3	0.07

rs201363715 exact test for Hardy-Weinberg equilibrium (n=167)

	N11	N12	N22	N1	N2	P-value
All subjects	163	4	0	330	4	1
STATUS=0-FAMILY	124	1	0	249	1	1
STATUS=1-KC	39	3	0	81	3	1

rs201363715 association with response STATUS (n=167, adjusted by Race+Gender+Age)

Model	Genotype	STATUS=0-FAMILY	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	C/C	124 (99.2%)	39 (92.9%)	1.00	0.027	177.2	195.9
	C/T	1 (0.8%)	3 (7.1%)	**11.66 (1.08-125.77)**			

<<< Step 3: Customize analysis

SNPStats results

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Descriptive statistics

Response variable: STATUS **Type:** categorical

	n	missing	unique
All subjects	50	0	2
STATUS=0-PreKC	8 (16%)	---	---
STATUS=1-KC	42 (84%)	---	---

Covariate: Gender **Type:** categorical

	n	missing	unique
All subjects	50	0	2
STATUS=0-PreKC	8	0	2
STATUS=1-KC	42	0	2

	F	M
All subjects	18 (36%)	32 (64%)
STATUS=0-PreKC	4 (50%)	4 (50%)
STATUS=1-KC	14 (33%)	28 (67%)

Covariate: Age **Type:** quantitative

	n	missing	unique	mean	.05	.10	.25	.50	.75	.90	.95
All subjects	50	0	24	24.22	12.45	15.8	19	24	29.75	32.1	33
STATUS = 0-PreKC	8	0	7	22.25	8	8	11	17	27.5	39.8	48.9
STATUS = 1-KC	42	0	20	24.6	17	18	19	24	29.75	32	33

lowest: 8, 12, 13, 14, 16 highest: 31, 32, 33, 35, 58

Single-SNP analysis

SNP: rs6138482

Percentage of typed samples: 50/50 (100%)

rs6138482 allele frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	72	0.72	15	0.94	57	0.68
A	28	0.28	1	0.06	27	0.32

rs6138482 genotype frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	7	0.14	0	0	7	0.17
G/A	14	0.28	1	0.12	13	0.31
G/G	29	0.58	7	0.88	22	0.52

rs6138482 exact test for Hardy-Weinberg equilibrium (n=50)						
	N11	N12	N22	N1	N2	P-value
All subjects	29	14	7	72	28	**0.036**
STATUS=0-PreKC	7	1	0	15	1	1
STATUS=1-KC	22	13	7	57	27	0.075

rs6138482 association with response STATUS (n=50, adjusted by Gender+Age)							
Model	Genotype	STATUS=0-PreKC	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	7 (87.5%)	22 (52.4%)	1.00	0.092	46.8	56.4

	G/A	1 (12.5%)	13 (30.9%)	4.32 (0.46-40.40)			
	A/A	0 (0%)	7 (16.7%)	NA (0.00-NA)			
Dominant	G/G	7 (87.5%)	22 (52.4%)	1.00	0.047	45.7	53.3
	G/A-A/A	1 (12.5%)	20 (47.6%)	6.70 (0.73-61.14)			
Recessive	G/G-G/A	8 (100%)	35 (83.3%)	1.00	0.1	46.9	54.6
	A/A	0 (0%)	7 (16.7%)	NA (0.00-NA)			
Overdominant	G/G-A/A	7 (87.5%)	29 (69%)	1.00	0.26	48.3	56
	G/A	1 (12.5%)	13 (30.9%)	3.18 (0.35-29.23)			
Log-additive	---	---	---	5.36 (0.72-40.16)	0.032	45	52.7

SNP: rs56157240

Percentage of typed samples: 50/50 (100%)

rs56157240 allele frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	80	0.8	10	0.62	70	0.83
A	20	0.2	6	0.38	14	0.17

rs56157240 genotype frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	1	0.02	1	0.12	0	0
T/A	18	0.36	4	0.5	14	0.33
T/T	31	0.62	3	0.38	28	0.67

rs56157240 exact test for Hardy-Weinberg equilibrium (n=50)						
	N11	N12	N22	N1	N2	P-value
All subjects	31	18	1	80	20	0.66
STATUS=0-PreKC	3	4	1	10	6	1
STATUS=1-KC	28	14	0	70	14	0.57

rs56157240 association with response STATUS (n=50, adjusted by Gender+Age)						
Model	Genotype	STATUS=0-PreKC	STATUS=1-KC	OR (95% CI)	P-value	AIC BIC
Codominant	T/T	3 (37.5%)	28 (66.7%)	1.00		
	T/A	4 (50%)	14 (33.3%)	0.42 (0.08-2.22)	0.085	46.7 56.2
	A/A	1 (12.5%)	0 (0%)	0.00 (0.00-NA)		
Dominant	T/T	3 (37.5%)	28 (66.7%)	1.00	0.17	47.7 55.3
	T/A-A/A	5 (62.5%)	14 (33.3%)	0.33 (0.07-1.64)		
Recessive	T/T-T/A	7 (87.5%)	42 (100%)	1.00	0.049	45.7 53.4
	A/A	1 (12.5%)	0 (0%)	0.00 (0.00-NA)		
Overdominant	T/T-A/A	4 (50%)	28 (66.7%)	1.00	0.47	49.1 56.7
	T/A	4 (50%)	14 (33.3%)	0.56 (0.12-2.69)		
Log-additive	---	---	---	0.26 (0.06-1.15)	0.066	46.2 53.9

SNP: rs12480307

Percentage of typed samples: 50/50 (100%)

rs12480307 allele frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	82	0.82	11	0.69	71	0.85
G	18	0.18	5	0.31	13	0.15

rs12480307 genotype frequencies (n=50)						
	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	33	0.66	4	0.5	29	0.69
A/G	16	0.32	3	0.38	13	0.31
G/G	1	0.02	1	0.12	0	0

rs12480307 exact test for Hardy-Weinberg equilibrium (n=50)						
	N11	N12	N22	N1	N2	P-value

All subjects	33	16	1	82	18	1
STATUS=0-PreKC	4	3	1	11	5	1
STATUS=1-KC	29	13	0	71	13	0.57

rs12480307 association with response STATUS (n=50, adjusted by Gender+Age)

Model	Genotype	STATUS=0-PreKC	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	4 (50%)	29 (69%)	1.00			
	A/G	3 (37.5%)	13 (30.9%)	0.60 (0.11-3.31)	0.12	47.4	56.9
	G/G	1 (12.5%)	0 (0%)	0.00 (0.00-NA)			
Dominant	A/A	4 (50%)	29 (69%)	1.00	0.35	48.7	56.4
	A/G-G/G	4 (50%)	13 (30.9%)	0.47 (0.09-2.32)			
Recessive	A/A-A/G	7 (87.5%)	42 (100%)	1.00	0.049	45.7	53.4
	G/G	1 (12.5%)	0 (0%)	0.00 (0.00-NA)			
Overdominant	A/A-G/G	5 (62.5%)	29 (69%)	1.00	0.81	49.5	57.2
	A/G	3 (37.5%)	13 (30.9%)	0.81 (0.16-4.18)			
Log-additive	---	---	---	0.34 (0.08-1.47)	0.15	47.5	55.1

SNP: rs201363715

Percentage of typed samples: 50/50 (100%)

rs201363715 allele frequencies (n=50)

	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Allele	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
C	97	0.97	16	1	81	0.96
T	3	0.03	0	0	3	0.04

rs201363715 genotype frequencies (n=50)

	All subjects		STATUS=0-PreKC		STATUS=1-KC	
Genotype	**Count**	**Proportion**	**Count**	**Proportion**	**Count**	**Proportion**
C/C	47	0.94	8	1	39	0.93
C/T	3	0.06	0	0	3	0.07

rs201363715 exact test for Hardy-Weinberg equilibrium (n=50)

	N11	N12	N22	N1	N2	P-value
All subjects	47	3	0	97	3	1
STATUS=0-PreKC	8	0	0	16	0	1
STATUS=1-KC	39	3	0	81	3	1

rs201363715 association with response STATUS (n=50, adjusted by Gender+Age)

Model	Genotype	STATUS=0-PreKC	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	C/C	8 (100%)	39 (92.9%)	1.00	0.4	48.9	56.5
	C/T	0 (0%)	3 (7.1%)	NA (0.00-NA)			

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Descriptive statistics

Response variable: STATUS **Type:** categorical

	n	missing	unique
All subjects	138	0	2
STATUS=0-CONTROL	96 (69.57%)	---	---
STATUS=1-KC	42 (30.43%)	---	---

Covariate: Race **Type:** categorical

	n	missing	unique
All subjects	138	0	3
STATUS=0-CONTROL	96	0	3
STATUS=1-KC	42	0	3

	C	I	M
All subjects	60 (43%)	51 (37%)	27 (20%)
STATUS=0-CONTROL	54 (56%)	28 (29%)	14 (15%)
STATUS=1-KC	6 (14%)	23 (55%)	13 (31%)

Covariate: Gender **Type:** categorical

	n	missing	unique
All subjects	138	0	2
STATUS=0-CONTROL	96	0	2
STATUS=1-KC	42	0	2

	F	M
All subjects	73 (53%)	65 (47%)
STATUS=0-CONTROL	59 (61%)	37 (39%)
STATUS=1-KC	14 (33%)	28 (67%)

Covariate: Age **Type:** quantitative

	n	missing	unique	mean	.05	.10	.25	.50	.75	.90	.95
All subjects	138	0	45	28.54	16	17	21	23	33.75	49	55
STATUS = 0-CONTROL	96	0	38	30.27	15.75	17	21	22	41	52.5	57.25
STATUS = 1-KC	42	0	20	24.6	17	18	19	24	29.75	32	33

lowest: 8, 9, 14, 15, 16 highest: 58, 59, 60, 66, 69

Single-SNP analysis

SNP: rs6138482

Percentage of typed samples: 138/138 (100%)

rs6138482 allele frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	185	0.67	128	0.67	57	0.68
A	91	0.33	64	0.33	27	0.32

rs6138482 genotype frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	24	0.17	17	0.18	7	0.17

G/A	43	0.31	30	0.31	13	0.31
G/G	71	0.51	49	0.51	22	0.52

rs6138482 exact test for Hardy-Weinberg equilibrium (n=138)						
	N11	N12	N22	N1	N2	P-value
All subjects	71	43	24	185	91	0.00088
STATUS=0-CONTROL	49	30	17	128	64	0.0052
STATUS=1-KC	22	13	7	57	27	0.075

rs6138482 association with response STATUS (n=138, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CONTROL	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	49 (51%)	22 (52.4%)	1.00			
	G/A	30 (31.2%)	13 (30.9%)	1.50 (0.53-4.25)	0.6	144.4	164.9
	A/A	17 (17.7%)	7 (16.7%)	1.78 (0.50-6.42)			
Dominant	G/G	49 (51%)	22 (52.4%)	1.00	0.33	142.5	160
	G/A-A/A	47 (49%)	20 (47.6%)	1.59 (0.62-4.07)			
Recessive	G/G-G/A	79 (82.3%)	35 (83.3%)	1.00	0.51	143	160.6
	A/A	17 (17.7%)	7 (16.7%)	1.49 (0.46-4.88)			
Overdominant	G/G-A/A	66 (68.8%)	29 (69%)	1.00	0.62	143.2	160.7
	G/A	30 (31.2%)	13 (30.9%)	1.28 (0.49-3.34)			
Log-additive	---	---	---	1.36 (0.73-2.52)	0.33	142.5	160

SNP: rs56157240

Percentage of typed samples: 138/138 (100%)

rs56157240 allele frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	250	0.91	180	0.94	70	0.83
A	26	0.09	12	0.06	14	0.17

rs56157240 genotype frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
T/A	26	0.19	12	0.12	14	0.33
T/T	112	0.81	84	0.88	28	0.67

rs56157240 exact test for Hardy-Weinberg equilibrium (n=138)						
	N11	N12	N22	N1	N2	P-value
All subjects	112	26	0	250	26	0.61
STATUS=0-CONTROL	84	12	0	180	12	1
STATUS=1-KC	28	14	0	70	14	0.57

rs56157240 association with response STATUS (n=138, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CONTROL	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	T/T	84 (87.5%)	28 (66.7%)	1.00	0.02	138	155.6
	T/A	12 (12.5%)	14 (33.3%)	4.05 (1.21-13.56)			

SNP: rs12480307

Percentage of typed samples: 138/138 (100%)

rs12480307 allele frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	251	0.91	180	0.94	71	0.85
G	25	0.09	12	0.06	13	0.15

rs12480307 genotype frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	113	0.82	84	0.88	29	0.69
A/G	25	0.18	12	0.12	13	0.31

rs12480307 exact test for Hardy-Weinberg equilibrium (n=138)						
	N11	N12	N22	N1	N2	P-value
All subjects	113	25	0	251	25	0.6
STATUS=0-CONTROL	84	12	0	180	12	1
STATUS=1-KC	29	13	0	71	13	0.57

rs12480307 association with response STATUS (n=138, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CONTROL	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	A/A	84 (87.5%)	29 (69%)	1.00	0.029	138.7	156.2
	A/G	12 (12.5%)	13 (30.9%)	**3.78 (1.11-12.79)**			

SNP: rs201363715

Percentage of typed samples: 138/138 (100%)

rs201363715 allele frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
C	265	0.96	184	0.96	81	0.96
T	11	0.04	8	0.04	3	0.04

rs201363715 genotype frequencies (n=138)						
	All subjects		STATUS=0-CONTROL		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
C/C	127	0.92	88	0.92	39	0.93
C/T	11	0.08	8	0.08	3	0.07

rs201363715 exact test for Hardy-Weinberg equilibrium (n=138)						
	N11	N12	N22	N1	N2	P-value
All subjects	127	11	0	265	11	1
STATUS=0-CONTROL	88	8	0	184	8	1
STATUS=1-KC	39	3	0	81	3	1

rs201363715 association with response STATUS (n=138, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CONTROL	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	C/C	88 (91.7%)	39 (92.9%)	1.00	0.98	143.4	161
	C/T	8 (8.3%)	3 (7.1%)	1.02 (0.18-5.73)			

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Descriptive statistics

Response variable: STATUS **Type:** categorical

	n	missing	unique
All subjects	156	0	2
STATUS=0-CHILDREN	114 (73.08%)	---	---
STATUS=1-KC	42 (26.92%)	---	---

Covariate: Race **Type:** categorical

	n	missing	unique
All subjects	156	0	3
STATUS=0-CHILDREN	114	0	3
STATUS=1-KC	42	0	3

	C	I	M
All subjects	48 (31%)	55 (35%)	53 (34%)
STATUS=0-CHILDREN	42 (37%)	32 (28%)	40 (35%)
STATUS=1-KC	6 (14%)	23 (55%)	13 (31%)

Covariate: Gender **Type:** categorical

	n	missing	unique
All subjects	156	0	2
STATUS=0-CHILDREN	114	0	2
STATUS=1-KC	42	0	2

	F	M
All subjects	71 (46%)	85 (54%)
STATUS=0-CHILDREN	57 (50%)	57 (50%)
STATUS=1-KC	14 (33%)	28 (67%)

Covariate: Age **Type:** quantitative

	n	missing	unique	mean	.05	.10	.25	.50	.75	.90	.95
All subjects	156	0	24	14.26	9	9	10	11	17	27.5	31.25
STATUS = 0-CHILDREN	114	0	4	10.45	9	9	10	10	11	12	12
STATUS = 1-KC	42	0	20	24.6	17	18	19	24	29.75	32	33

lowest: 9, 10, 11, 12, 14 highest: 30, 31, 32, 33, 35

Single-SNP analysis

SNP: rs6138482

Percentage of typed samples: 156/156 (100%)

rs6138482 allele frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
G	231	0.74	174	0.76	57	0.68
A	81	0.26	54	0.24	27	0.32

rs6138482 genotype frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	18	0.12	11	0.1	7	0.17

G/A	45	0.29	32	0.28	13	0.31
G/G	93	0.6	71	0.62	22	0.52

rs6138482 exact test for Hardy-Weinberg equilibrium (n=156)						
	N11	N12	N22	N1	N2	P-value
All subjects	93	45	18	231	81	**0.003**
STATUS=0-CHILDREN	71	32	11	174	54	**0.02**
STATUS=1-KC	22	13	7	57	27	0.075

rs6138482 association with response STATUS (n=156, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CHILDREN	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	G/G	71 (62.3%)	22 (52.4%)	1.00			
	G/A	32 (28.1%)	13 (30.9%)	0.00 (0.00-NA)	1	14	35.3
	A/A	11 (9.7%)	7 (16.7%)	0.00 (0.00-NA)			
Dominant	G/G	71 (62.3%)	22 (52.4%)	1.00			
	G/A-A/A	43 (37.7%)	20 (47.6%)	0.00 (0.00-NA)	1	12	30.3
Recessive	G/G-G/A	103 (90.3%)	35 (83.3%)	1.00			
	A/A	11 (9.7%)	7 (16.7%)	0.00 (0.00-NA)	1	12	30.3
Overdominant	G/G-A/A	82 (71.9%)	29 (69%)	1.00			
	G/A	32 (28.1%)	13 (30.9%)	0.00 (0.00-NA)	NA	12	30.3
Log-additive	---	---	---	0.00 (0.00-NA)	1	12	30.3

SNP: rs56157240

Percentage of typed samples: 156/156 (100%)

rs56157240 allele frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
T	275	0.88	205	0.9	70	0.83
A	37	0.12	23	0.1	14	0.17

rs56157240 genotype frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	4	0.03	4	0.04	0	0
T/A	29	0.19	15	0.13	14	0.33
T/T	123	0.79	95	0.83	28	0.67

rs56157240 exact test for Hardy-Weinberg equilibrium (n=156)						
	N11	N12	N22	N1	N2	P-value
All subjects	123	29	4	275	37	0.23
STATUS=0-CHILDREN	95	15	4	205	23	**0.014**
STATUS=1-KC	28	14	0	70	14	0.57

rs56157240 association with response STATUS (n=156, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CHILDREN	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	T/T	95 (83.3%)	28 (66.7%)	1.00			
	T/A	15 (13.2%)	14 (33.3%)	NA (0.00-NA)	1	14	35.3
	A/A	4 (3.5%)	0 (0%)	NA (0.00-NA)			
Dominant	T/T	95 (83.3%)	28 (66.7%)	1.00			
	T/A-A/A	19 (16.7%)	14 (33.3%)	NA (0.00-NA)	1	12	30.3
Recessive	T/T-T/A	110 (96.5%)	42 (100%)	1.00			
	A/A	4 (3.5%)	0 (0%)	NA (0.00-NA)	1	12	30.3
Overdominant	T/T-A/A	99 (86.8%)	28 (66.7%)	1.00			
	T/A	15 (13.2%)	14 (33.3%)	NA (0.00-NA)	1	12	30.3
Log-additive	---	---	---	NA (0.00-NA)	1	12	30.3

SNP: rs12480307

Percentage of typed samples: 156/156 (100%)

rs12480307 allele frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
A	277	0.89	206	0.9	71	0.85

G	35	0.11	22	0.1	13	0.15

rs12480307 genotype frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
A/A	124	0.79	95	0.83	29	0.69
A/G	29	0.19	16	0.14	13	0.31
G/G	3	0.02	3	0.03	0	0

rs12480307 exact test for Hardy-Weinberg equilibrium (n=156)						
	N11	N12	N22	N1	N2	P-value
All subjects	124	29	3	277	35	0.41
STATUS=0-CHILDREN	95	16	3	206	22	0.064
STATUS=1-KC	29	13	0	71	13	0.57

rs12480307 association with response STATUS (n=156, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CHILDREN	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
Codominant	A/A	95 (83.3%)	29 (69%)	1.00			
	A/G	16 (14%)	13 (30.9%)	NA (0.00-NA)	1	14	35.3
	G/G	3 (2.6%)	0 (0%)	NA (0.00-NA)			
Dominant	A/A	95 (83.3%)	29 (69%)	1.00			
	A/G-G/G	19 (16.7%)	13 (30.9%)	NA (0.00-NA)	1	12	30.3
Recessive	A/A-A/G	111 (97.4%)	42 (100%)	1.00			
	G/G	3 (2.6%)	0 (0%)	NA (0.00-NA)	1	12	30.3
Overdominant	A/A-G/G	98 (86%)	29 (69%)	1.00			
	A/G	16 (14%)	13 (30.9%)	NA (0.00-NA)	1	12	30.3
Log-additive	---	---	---	NA (0.00-NA)	1	12	30.3

SNP: rs201363715

Percentage of typed samples: 156/156 (100%)

rs201363715 allele frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Allele	Count	Proportion	Count	Proportion	Count	Proportion
C	303	0.97	222	0.97	81	0.96
T	9	0.03	6	0.03	3	0.04

rs201363715 genotype frequencies (n=156)						
	All subjects		STATUS=0-CHILDREN		STATUS=1-KC	
Genotype	Count	Proportion	Count	Proportion	Count	Proportion
C/C	147	0.94	108	0.95	39	0.93
C/T	9	0.06	6	0.05	3	0.07

rs201363715 exact test for Hardy-Weinberg equilibrium (n=156)						
	N11	N12	N22	N1	N2	P-value
All subjects	147	9	0	303	9	1
STATUS=0-CHILDREN	108	6	0	222	6	1
STATUS=1-KC	39	3	0	81	3	1

rs201363715 association with response STATUS (n=156, adjusted by Race+Gender+Age)							
Model	Genotype	STATUS=0-CHILDREN	STATUS=1-KC	OR (95% CI)	P-value	AIC	BIC
---	C/C	108 (94.7%)	39 (92.9%)	1.00			
	C/T	6 (5.3%)	3 (7.1%)	0.03 (0.00-NA)	1	12	30.3

<<< Step 3: Customize analysis

M Overall Results Summary of Variables

Variables	**Cases Mean (SD) / n (%)**	**Controls (Normal) Mean (SD) / n%**	**Controls (Family) Mean (SD) / n%**	**Controls (School Children) Mean (SD) / n%**
Age 15 – 20 y: Adolescence 21 – 30 y: Young adults 31 – 40 y: Adults	RANGE 15-40y 16 (38.10%) 20 (47.62%) 6 (14.28%)	RANGE 20- 40y	RANGE 11-50y	RANGE 9-12y
Gender Male Female	 28 (66.67%) 14 33.33(%)	 42 (%) 55 (%)	 72 (%) 55 (%)	 81 (%) 71 (%)
Race Malay Chinese Indian	 9 (21.43%) 5 (11.90%) 28 (66.67%)	 14(%) 53 (%) 30 (%)	 33(%) 21 (%) 71 (%)	 51 (%) 42 (%) 59 (%)
Eye-Rubbing Mild - moderate Severe	 33 (78.6%) 33 (78.6%)	 56 (40%) 81(84.4%)	 63(50%) 85(67.5%)	 40 (27.8%) 24 (16.7%)
Eye Itch Yes No	 15 (35.7%) 27 (64.3%)	 31 (32.3%) 65 (67.7%)	 53 (42.1%) 73(57.9%)	 19 (13.2%) 125 (86.8%)
Red-Eyed Yes No	 11 (26.2%) 31 (73.8%)	 12 (12.5%) 84 (87.5%)	 25 (19.8 %) 101 (80.2%)	 25 (17.4%) 119(82.6%)
Watery Eyes Yes No	 7 (16.7%) 35 (83.3%)	 22 (22.9%) 74 (77.1%)	 40 (31.7%) 86 (68.3%)	 8 (5.6%) 136 (94.4%)
Nose Blockage Yes No	 8 (19.0%) 34 (81.0%)	 16 (16.7%) 80 (83.3%)	 28(22.2 %) 98 (77.8%)	 13 (9.0%) 131 (91.0%)
Skin Allergy Yes No	 28 (66.7%) 14 (33.3%)	 23 (24%) 73 (76%)	 20 (15.9%) 106 (84.1%)	 18 (12.5%) 126 (87.5%)

Asthma Yes No	16 (38.1%) 26(61.9 %)	9.0 (9.4%) 87 (90.6%)	24 (19.0%) 102 (81.0%)	21 (14.6%) 123 (85.4%)
Food Allergy Yes No	13 (10.3%) 113 (89.7%)	17 (17.7%) 79 (82.3%)	13 (10.3%) 113 (89.7%)	0 (0%) 145 (100%)
Slit-lamp Findings Munson's RE: LE: Vogt's RE: LE: Hydrops RE: LE: Scarring RE: LE:	 84 (100%) 84 (100%) 11(2.50%) 9 (2.21%) 2 (3.57%) 2(3.57%) 6 (7.0%) 2 (3.57%)	 zero zero zero zero zero zero zero zero	 zero zero zero zero zero zero zero zero	 zero zero zero zero zero zero zero zero
K-Max Values TABLES :4.1.3.3 RE: < 45 Kmax: 45 – 50 Kmax: 51- 60 Kmax: 61-70 Kmax: LE: < 45 Kmax: 45 – 50 Kmax: 51- 60 Kmax: 61-70 Kmax:	 RE 4 (9.52%) 18 (42.86%) 16 (38.10%) 4 (9.52%) LE 4 (9.52%) 20 (47.62%) 16 (38.10%) 2 (4.76 %)	 RE MEAN44.32 STD 4.76 LE MEAN 45.30 STD 3.71	 RE MEAN44.05 STD 1.86 LE MEAN44.95 STD.2.75	 RE MEAN44.75 STD 1.43 LE MEAN44.38n STD 1.45
Intraocular Pressure Tables 2.19 – 2.26 Cases Controls	RE MEAN 12.32 STD 1.84 LE MEAN 6.91 STD 1.78	RE MEAN15.04 STD3.60 LE MEAN15.32 STD3.44	RE MEAN14.51 STD2.89 LE MEAN14.64 STD 2.72	RE MEAN17.27 STD4.898 LE MEAN16.70 S4.898TD
Corneal Readings(Mean) Tables 4.1.3.3 Cases & Controls Standard Deviation (STD)	MEAN 475.85µ STD4.898	MEAN 517.0µ STD5.29	MEAN 530.40µ STD5.14	MEAN 562µ STD4.89

Clinical features of KC pre- and post- CXL (Tables2.29, & 2.30) Fig. 2.31 & 2.32 GRAPHS Pre-CXL Visual Acuity: Slit Lamp: K-Max Value: ORA: Post-CXL: (Table 2.29 & 2.30) Fig.2.31 &2.32 Visual Acuity: Slit Lamp: K-Max Value: ORA:	n (%) n (%) n (%) n (%) n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%) n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%) n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%) n (%) n (%) n (%) n (%)
Candidate Genes VSX1: SOD1: COL4A3:	n (%) n (%) n (%)	n (%) n (%) n (%)	n (%) n (%) n (%)	n (%) n (%) n (%)
VSX1 Variants A182A: P237P: R217H: c.504 – 24 C>T:	14 (33.33%) 14 (33.33%) 18 42.85(%) 3 (7.14%)	12 (12.37%) 12 (12.37%) 49 (50.52%) 5 (5.15%)	20 (15.87%) 20 (15.87%) 11 (8.73%) 1 (0.79%)	23 (15.13%) 23 (15.13%) 53 (34.87%) 8 (5.26%)q
Alleles and genotypes of VSX1 Variants A182A: P237P: R217H: c.504 – 24 C>T:	**SNPsSTATS CASES ASSOCIATION TABLES CASES vs CONTROLS** n (%) n (%) n (%) n (%)	**NORMAL CONTROLS Tables 4.33-4.36** n (%) n (%) n (%) n (%)	**FAMILY Tables 4.29-4.32** n (%) n (%) n (%) n (%)	**SCHOOL CHILDREN Tables 4.33-4.36** n (%) n (%) n (%) n (%)
Variants Found in Various Candidate Genes VSX1Exon 3 A182A: P237P: R217H: c.504 – 24 C>T: SOD1 Exon 2 COL4A3exon17	n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%)	n (%) n (%) n (%) n (%)