Underlying genetic mechanisms of hereditary dystrophies in retina and cornea

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We don't grow when things are easy. We grow when we face challenges.

Anonymous

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Original papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

I. **Jonsson F***, Byström B*, Davidsson AE, Backman LJ, Kellgren TG, Tuft SJ, Koskela T, Rydén P, Sandgren O, Danielson P, Hardcastle AJ, Golovleva I.
Mutations in collagen, type XVII, alpha 1 (*COL17A1*) cause Epithelial Recurrent Erosion Dystrophy (ERED).
Hum Mutat. 2015 Apr; 36(4):463-73.

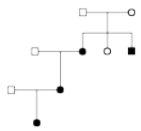
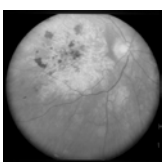
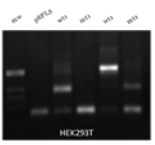
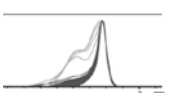
II. **Jonsson F**, Burstedt MS, Sandgren O, Norberg A, Golovleva I.
Novel mutations in *CRB1* and *ABCA4* genes cause Leber congenital amaurosis and Stargardt disease in a Swedish family.
Eur J Hum Genet. 2013 Nov; 21(11):1266-71.

III. **Jonsson F***, Westin IM*, Österman L, Burstedt MS, Golovleva I.
ABCA4 intronic variants c.4773+3A>G and c.5461-10T>C cause Stargardt disease due to defective splicing
Manuscript

IV. Köhn L*, Burstedt MS*, **Jonsson F**, Kadzhaev K, Haamer E, Sandgren O, Golovleva I.
Carrier of R14W in carbonic anhydrase IV presents Bothnia dystrophy phenotype caused by two allelic mutations in *RLBP1*.
Invest Ophthalmol Vis Sci. 2008 Jul; 49(7):3172-7.

* These authors contributed equally to the work.

Thesis at a glance

	Aim	Material & Methods	Results		Conclusion
I	To identify the genetic cause of epithelial recurrent erosion corneal dystrophy (ERED)	Five families with ERED APEX, SNP array, WES, ddPCR, IHC		A novel variant was found in the *COL17A1* gene	*COL17A1* is a genetic cause of ERED in a large Swedish family and, possibly worldwide
II	To identify the genetic causes of Leber congenital amarosis (LCA) and Stargardt disease	One family with variable phenotype in affected individuals APEX, SNP array, Sanger sequencing PCR-RFLP		A novel stop *CRB1* mutation segregates in LCA. Two splice mutations present in Stargardt patients	LCA and Stargardt in the same family caused by mutations in *CRB1* and *ABCA4*
III	To demonstrate functional role of intronic *ABCA4* splice mutations	HEK293T ARPE-19 *In vitro* splice assay, site-directed mutagenesis RT-PCR		*ABCA4*, c.5461-10C>T and c.4773+3A>G cause exon skipping	c.5461-10C>T and c.4773+3A>G causes Stargardt disease
IV	To explain Bothnia Dystrophy (BD) phenotype in heterozygous carriers of *RLBP1* c.700C>T	10 BD patients heterozygous for *RLBP1* mutation APEX, dHPLC Sanger sequencing PCR-RFLP		*RLBP1* c.677T>A and c.700C>T were allelic. One BD patient carried *CAIV* variant known to cause RP17	BD is caused by two mutations in *RLBP1* gene. *CAIV* c.40C>T is a benign variant in northern Sweden

Abstract

Inherited retinal and corneal dystrophies represent a group of disorders with great genetic heterogeneity. Over 250 genes are associated with retinal diseases and 16 genes are causative of corneal dystrophies. This thesis is focused on finding the genetic causes of corneal dystrophy, Leber congenital amaurosis (LCA), Stargardt disease and retinitis pigmentosa in families from northern Sweden. By whole exome sequencing a novel mutation, c.2816C>T, p.Thr939Ile, in Collagen Type XVII, Alpha 1 chain, *COL17A1*, gene was identified in several families with epithelial recurrent erosion dystrophy (ERED). We showed that the COL17A1 protein is expressed in the basement membrane of the cornea, explaining the mutation involvement in the corneal symptoms. We could link all the families in this study to a couple born in the late 1700s confirming a founder mutation in northern Sweden. Our finding highlights role of COL17A1 in ERED and suggests screening of this gene in patients with similar phenotype worldwide. Furthermore the genetic causes in several retinal degenerations were identified. In one family with two recessive disorders, LCA and Stargardt disease, a novel stop mutation, c.2557C>T, p.Gln853Stop, was detected in all LCA patients. In the Stargardt patients two intronic variants, the novel c.4773+3A>G and c.5461-10T>C, were detected in the *ABCA4* gene. One individual was homozygous for the known variant c.5461-10T>C and the other one was compound heterozygote with both variants present. Both variants, c.4773+3A>G and c.5461-10T>C caused exon skipping in HEK293T cells demonstrated by *in vitro* splice assay, proving their pathogenicity in Stargardt disease. Finally, in recessive retinitis pigmentosa, Bothnia Dystrophy (BD), we identified a second mutation in the *RLBP1* gene, c.677T>A, p.Met226Lys. Thus, BD is caused not only by common c.700C>T variant but also by homozygosity of c.677T>A or compound heterozygosity. Notably, known variant, c.40C>T, p.R14W in the *CAIV* gene associated with a dominant retinal dystrophy RP17 was detected in one of the compound BD heterozygote and his unaffected mother. This variant appears to be a benign variant in the population of northern Sweden.

In conclusion, novel genetic causes of retinal dystrophies in northern Sweden were found demonstrating the heterogeneity and complexity of retinal diseases. Identification of the genetic defect in *COL17A1* in the corneal dystrophy contributes to understanding ERED pathogenesis and encourages refinement of IC3D classification. Our results provide valuable information for future molecular testing and genetic counselling of the families.

Populärvetenskaplig sammanfattning

Det finns flera ärftliga sjukdomar som drabbar ögats näthinna och hornhinna. Sjukdomar som drabbar näthinnan delas upp efter vilken fotoreceptorcell som påverkas först, tappar eller stavar. Tappar ger oss färgseende och synskärpa medan stavar hjälper oss att se i mörker. Ett samlingsnamn på sjukdomar med nattblindhet och skador på synfältet är Retinitis pigmentosa (RP). De kliniska varianterna av RP är många, allt från att man föds med en grav synnedsättning till att man får en lätt synnedsättning vid hög ålder. Samma variant av sjukdom kan även uttryckas på skiftande sätt hos olika individer. Ett hundratal arvsanlag kan orsaka RP. Man tror att runt 4-5000 individer i Sverige har någon form av RP men mörkertalet kan vara stort. Sjukdomen är vanligare i norra Sverige än i övriga delar av landet.

De ärftliga sjukdomar som drabbar hornhinnan delas upp efter vilket av hornhinnans lager som är påverkat. Även här finns många inblandade arvsanlag och för flera dystrofier är den ärftliga orsaken fortfarande okänd. Syftet med våra studier har varit att hitta den ärftliga orsaken till sjukdom i familjer med en näthinne- eller hornhinnesjukdom.

DNA har under årens lopp samlats och sparats från norrländska patienter, och ibland från anhöriga, tillhörande familjer som visat tecken på en misstänkt ärftlig ögonsjukdom. De familjer där ärftlig orsak till sjukdom var okänd, har sedan studerats ingående, både i kliniken och i en laboratoriemiljö med syfte att finna både det bakomliggande arvsanlaget och beskriva hur sjukdomen yttrar sig i patienten.

I **Arbete I** undersökte vi familjer med en ärftlig förekomst av sårbildningar på hornhinnan. Sårepisoderna debuterade i tidig ålder. Ett skov av sårbildning var väldigt smärtsamt och individerna upplevde kraftig ljuskänslighet i det drabbade ögat. Efter ett par dagar läkte såren på hornhinnan ut och en problemfri tid inföll till nästa skov. I tonåren-uppstod skoven alltmer sällan till att oftast helt upphöra vid 20 års ålder. Vid 40 års ålder–drabbades däremot många av synproblem pga en ojämn hornhinna orsakad av ärr efter sårläkning. Hos dessa individer kunde vi identifiera den ärftliga orsaken i kollagen genen, *COL17A1* som tidigare inte varit kopplat till ögonsjukdomar. Vi kunde påvisa att alla familjer i studien är kopplade till en gemensam förfader som bekräftar mutationen som en founder i vårt område.

I **Arbete II** undersökte vi en familj där flera individer hade fått diagnosen Leber kongenitala amaurosis (LCA). I samma familj fanns även två individer

med Stargardt sjukdom. Hos individerna med LCA fann vi en ny förändring i *CRB1* i dubbel uppsättning. Denna förändring gjorde att hela arvsanlaget inte fungerade normalt och på så sätt bildades ett icke-funktionellt protein. Hos individerna med Stargardt sjukdom fann vi två mutationer i *ABCA4* genen. En förändring var ny medan den andra rapporterats i hög frekvens hos Stargardt patienter. I **Arbete III** visar vi att dessa två förändringar orsakar en avläsningsrubbning när arvsanlaget ska bilda ett protein.

I **Arbete IV** undersökte vi patienter med Bothnia Dystrofi, en RP variant som är vanlig i norra Sverige. Sjukdomen är recessiv men flera patienter visade samma sjukdomsbild trots att de endast bar förändringen i *RLBP1* genen i enkel uppsättning. Hos dessa individer kunde vi påvisa ytterligare en förändring i samma gen. Ett bifynd i detta arbete var att en av patienterna även bar en rubbning i *CAIV* genen som är kopplad till en dominant RP variant. En frekvensanalys gjordes som visade att hela 4% av vår friska norrländska population bar denna förändring.

Resultaten som presenteras i denna avhandling har bidragit till att öka kunskapen om vilka arvsanlag och förändringar som kan förekomma och vara defekta hos patienter med ärftliga näthinne- och hornhinnesjukdomar. Den ärftliga orsaken och mekanismen bakom sjukdomen ger ovärderlig kunskap för att utarbeta nya behandlingsmetoder som angriper själva grundproblemet och inte bara symtomen. Dessa fynd är även en förutsättning för att kunna ge patienterna korrekt långtidsprognos och bedöma risken för att kommande barn drabbas.

Abbreviations

ACMG	The American College of Medical Genetics and Genomics
AD	autosomal dominant
AR	autosomal recessive
APEX	arrayed primer extension
BD	Bothnia dystrophy
BM	basement membrane
CD	corneal dystrophy
CNV	copy number variation
COD	cone dystrophy
CORD	cone-rod dystrophy
CSNB	congenital stationary night blindness
ddPCR	digital droplet polymerase chain reaction
dHPLC	denaturing high performance liquid chromatography
DNA	deoxyribonucleic acid
ERED	epithelial recurrent erosion dystrophy
IC3D	International Committee for classification of Corneal Dystrophies
IHC	immunohistochemistry
INL	inner nuclear layer
IRD	inherited retinal disease
LCA	Leber congenital amaurosis
LOH	loss of heterozygosity

MGB	miner groove binder
mRNA	messenger ribonucleic acid
mt	mutant type
ONL	outer nuclear layer
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
ROH	regions of homozygosity
RP	retinitis pigmentosa
RPE	retinal pigment epithelium
SNP	single nucleotide polymorphism
STGD	Stargardt disease
UPD	uniparental disomy
VUS	variant of uncertain significance
WES	whole exome sequencing
wt	wild type

Genes:

ABCA4	ATP Binding Cassette Subfamily A, Member 4
CAIV	Carbonic Anhydrase 4
COL17A1	Collagen Type XVII, Alpha 1 Chain
CRB1	Crumbs 1, Cell Polarity Complex Component, former known as Crumbs Homolog 1
RLBP1	Retinaldehyde Binding Protein 1

Introduction

The eye and its functions

The Eye

The eye is a complex organ that detects, focuses, and converts light into images that are then perceived by the brain. The eye contains three major components:

i. the outer layer consists of the cornea and the sclera;
ii. the middle layer consists of the uvea, which includes the middle vascular layer – the iris, the lens, the ciliary body, and the choroid;
iii. the innermost layer of the eye is the retina (Figure 1).

This complexity leads to a wide range of diseases that can affect any of the eye layers and cause visual loss (Fernandez and Normann, 1995). This thesis focuses on the cornea and the retina.

The Cornea

The cornea, the transparent cover at the front of the eye, provides about two-thirds of the eye's focusing power. The remaining one-third is provided by the lens. The cornea may look clear and seems to lack substance but is, in fact, a highly organised tissue with five layers, each layer with an important function (Figure 1). Although the cornea is transparent, there are many biochemical and structural similarities between the skin and the cornea.

The epithelium, the cornea's outermost surface, consists of 5-7 layers of cells (Kaufman, et al., 1998). The primary function of these cells is to protect the eye from foreign objects, to absorb oxygen and nutrients from tears, and to distribute those to the inner layers. The layers of the epithelium are constantly undergoing mitosis and have a turnover time of about seven days. The epithelium is filled with thousands of tiny nerve endings that make the cornea extremely sensitive (Goyal and Hamrah, 2016). The epithelial cells are connected to an epithelial **basement membrane** (BM). Located beneath the BM, the **Bowman's layer** consists of collagen fibres. If the Bowman's layer is injured, scars can form that could result in vision loss.

The stroma, the thickest layer of the cornea, consists mostly of collagen and water. The collagen in the stroma gives the cornea its elasticity, strength, and shape, all important aspects for the cornea's light-conducting transparency.

The Descemet's membrane, found on the posterior side of the stroma, is composed of collagen fibres produced by cells in the endothelial layer. This structure can easily repair itself if injured.

The endothelium, the cornea's innermost layer, is important for keeping the cornea clear. Its primary function is to pump excess fluid out of the stroma. In a healthy eye, the fluid that moves into the cornea and the fluid that is pumped out of the cornea are in perfect balance. The endothelial layer is very delicate; if injured, the cells in this layer cannot be repaired or replaced (Ljubimov, et al., 2002).

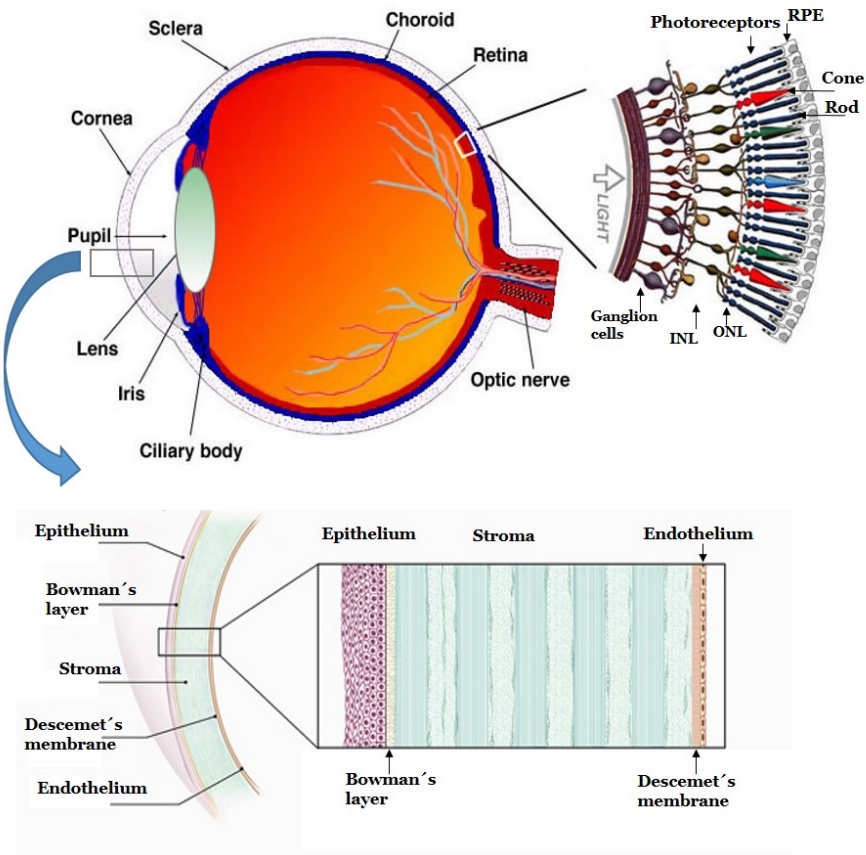


Figure 1. A cross-section of the human eye with a schematic enlargement of the retina and the corneal layers. Modified and printed with permission from National Eye Institute, National Institutes of Health (NEI/NIH).

The Retina

The retina is the sensory neural layer covering about 70% of the inner part of the eye globe (Figure 1). This layer has five types of neurons: ganglion cells, bipolar cells, horizontal cells, amacrine cells, and photoreceptors. The major route of information flow is called a direct three-neuron chain – from photoreceptor cell via bipolar cell to ganglion cell, from the light source to the optic nerve. The **retinal pigment epithelium** (RPE) is separated by Bruch´s membrane from the choroid, the vascular layer of the eye. The microvilli of the RPE contact the outer segments of **the photoreceptor cells**. The RPE supports the photoreceptor cells with direct transport of nutrients, removal of waste products, and transport and regeneration of visual pigment (Bonilha, et al., 2006).

The bodies of the photoreceptor cells are in the **outer nuclear layer** (ONL), and the cell bodies of bipolar cells are in the **inner nuclear layer** (INL), where the horizontal cells and the amacrine cells interact. The large axons of the ganglion cells form the optic nerve that carries information about retinal stimulation to the rest of the central nervous system.

Photoreceptors

Photoreceptors are responsible for converting light into nerve signals. In humans, there are two types of photoreceptor cells: **rods** and **cones**. Rods mediate vision at low light levels (scotopic vision), and cones mediate vision at high light levels (photopic vision) and are responsible for the perception of colour (Cook and Desplan, 2001). There are three types of cones and these types are labelled according to their maximum spectral sensitivity: red, green, and blue. This arrangement explains colour vision; we distinguish colours during daytime when the light intensity is high enough to stimulate the cones. A normal retina has about 120 million rods and six million cones (Adler, 1950). The central retina, the fovea, has the highest concentration of cones and the greatest visual acuity; this acuity decreases the closer to the periphery of the fovea (Koenekoop, 2009). In rods, the photopigment (opsin) is referred to as rhodopsin; in cones, the photopigment is referred to as photopsin. Both photopigments exist in a complex with a derivate of vitamin A, called retinal. Vitamin A is therefore an essential component of the photosensitive visual pigment in rods and cones and is very important for a good vision.

The visual process

The visual cycle consists of three parts. During the first step, known as phototransduction, light energy is converted into electrical signals in photoreceptor cells. During the second step, light initiates the isomerization of 11-*cis* retinal to all-*trans* retinal (photoisomerization) and this triggers a cascade of events resulting in transformation of an electric signal to the optic nerve and further to the brain where it is interpreted as an image. During the final step, known as the visual or retinoid cycle, regeneration of visual pigment takes place in the RPE (Saari, 2012).

An isomerisation of 11-*cis* retinal to all-*trans* retinal occurs upon light absorption. For all-*trans* retinal to participate again in the visual process, reverse isomerisation to the 11-*cis* retinal is needed. Initially, all-*trans* retinal released from the opsin protein is reduced to all-*trans* retinol and then transported back to the RPE to be recharged. The all-*trans* retinol is first esterified by lecithin-retinol acyltransferase (LRAT) to all-*trans* retinyl ester. The all-*trans* retinyl ester can then be converted to 11-*cis* retinol by the isomerohydrolase retinol pigment epithelium-specific protein (RPE65). The final oxidation by 11-*cis* retinol dehydrogenase (11-*cis* RDH) converts the 11-*cis* retinol to 11-*cis* retinal, which is shuttled back to the rod outer segment and forms a new functional visual pigment when conjugated with an opsin (Gonzalez-Fernandez, et al., 2015). Any defect in proteins or enzymes crucial for the visual cycle can lead to different types of retinal malfunctions and degenerations.

Dystrophies affecting the eye function

The corneal dystrophies

A **corneal dystrophy** (CD) is a condition in which one or more layers of the cornea affect corneal clarity. It is a heterogeneous group of diseases with a wide range of clinical manifestations depending on different entities, with prognosis variable from minimal vision defect to corneal blindness (Klintworth, 2009). In 2008, an international committee published a classification system for CDs (IC3D) (Weiss, et al., 2008). Before 1970, CDs were identified and characterized almost exclusively by their clinical appearance and some entities was presented as a single family. Therefore, it was difficult to separate different dystrophies from one another. Genetic analyses revolutionized the knowledge of the CDs and now makes it possible to refine classification inaccuracies. This classification was revised in 2015 (Weiss, et al., 2015). The CD classification is mainly based on what layers of

the cornea that are affected and the classifications are grouped per these affected layers (Table 1).

Table 1. The IC3D classification with categories and genes.

The IC3D Classification	Category	Gene
Epithelial and subepithelial dystrophies		
Epithelial basement membrane dystrophy	C1	*TGFBI*
Epithelial recurrent dystrophies - Franceschetti CD - Dystrophia Smolandiensis - Dystrophia Helsinglandica	C3	?
Subepithelial mucinous CD	C4	?
Meesmann CD	C1	*KRT3, KRT12*
Lisch epithelial CD	C2	?
Gelatinous drop-like CD	C1	*TACSTD2*
Epithelial-stromal TGFBI dystrophies		
Reis-Bücklers CD	C1	*TGFBI*
Thiel-Behnke CD	C1	*TGFBI*
Lattice CD	C1	*TGFBI*
Granular CD type 1	C1	*TGFBI*
Granular CD type 2	C1	*TGFBI*
Stromal dystrophies		
Macular CD	C1	*CHST6*
Schnyder CD	C1	*UBIAD1*
Congenital stromal CD	C1	*DCN*
Flecks CD	C1	*PIKFYVE*
Posterior amorphous CD	C1	*KERA, LUM, DCN, EPYC*
Central cloudy dystrophy of François	C4	?
Pre-Descement CD	C1, C4	*STS*
Edothelial dystrophies		
Fuchs endothelial CD	C1, C2, C3	*COL8A2*
Posterior polymorphous CD	C1, C2	*COL8A2, ZEB1*
Congenital hereditary endothelial dystrophy	C1	*SLC4A11*
X-linked endothelial CD	C2	?

The first group, **epithelial and sub-epithelial dystrophies**, includes the dystrophies affecting the most superficial part of the cornea. The second

group includes all **epithelial and stromal dystrophies** caused by mutation in the transforming growth factor beta induced (*TGFBI*) gene, including the Reis-Bückler and Thiel-Behnke dystrophy. The remaining dystrophies affecting the stroma constitute the third group, **stromal dystrophies**. Finally, the last group, the **endothelial dystrophies**, includes dystrophies where the innermost corneal layer is affected. The most common in this group is Fuchs endothelial dystrophy (Vedana, et al., 2016).

A subgroup of the epithelial and sub-epithelial dystrophies is the **epithelial recurrent erosion dystrophies** (EREDs). In addition to the three variants listed, other families with recurring corneal erosions have been reported. These patients share some features with the existing ERED variants: dominant recurrent erosions, early age of onset and symptoms reduced with age (Holt, 1956; Wales, 1955; Valle, 1967).

A more modern way of classification is based on the knowledge of the dystrophies genetic cause. The different entities in this classification are also divided into four categories. The first category (**C1**) includes a well-defined CD in which the gene has been mapped and identified and mutations are known. The second category (**C2**) includes well-defined CDs that have been mapped to one or more specific chromosomal loci, but the genes are not identified. The third category (**C3**) includes well-defined CDs without mapped chromosomal locus. The fourth category (**C4**) is reserved for suspected, new, or previously documented CDs whose possibility to be a distinct entity is not yet convincing. As knowledge increases, the categories will be refined, possibly adding new categories or amending the original four categories. The latest edition of the IC3D identifies 22 CDs and mutations in 16 separate genes that cause corneal disease. These genes are *TGFBI*, *KRT3*, *KRT12*, *TACSTD2*, *CHST6*, *UBAID1*, *DCN*, *PIKFYVE*, *KERA*, *LUM*, *DCN*, *EPYC*, *STS*, *COL8A2*, *ZEB1*, and *SLC4A11* (Weiss, et al., 2015).

The retinal dystrophies

Inherited retinal diseases (IRDs) represent an overlapping group of clinically and genetically heterogeneous disorders with more than 250 known genes involved (http://www.sph.uth.tmc.edu/RetNet/) (Daiger, et al., 1998) (Figure 2). Visual impairment may vary from poor night vision or poor colour vision to loss of peripheral and/or central vision to complete blindness. The disease might be stationary or progressive when severity increases with age (Nash, et al., 2015). A disease can be syndromic or non-syndromic. The retinal dystrophies are divided into broad groups depending on the type of photoreceptors affected and the manifestations or degree of atrophy within the retina. These groups include rod-dominated diseases,

cone-dominated diseases, and retinal degenerations involving both rods and cones.

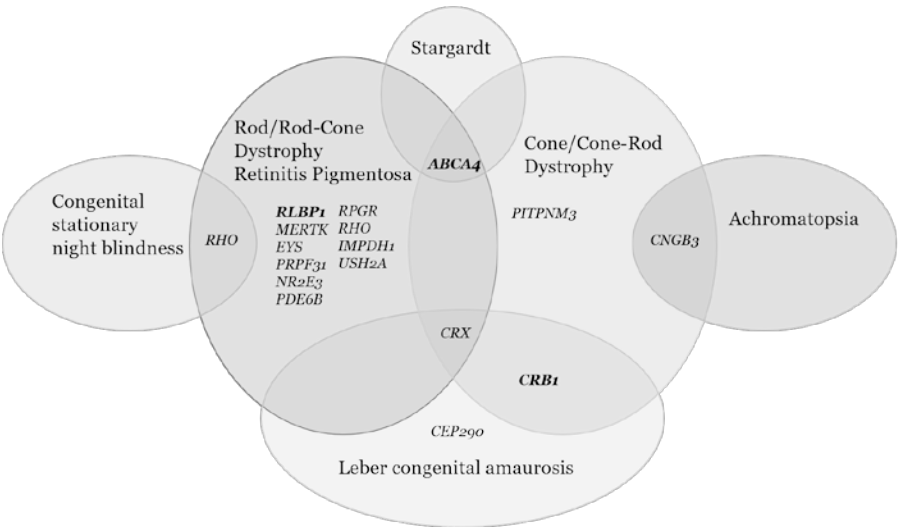


Figure 2. An illustration of retinal dystrophies and the impact of different genes. Only the genes with mutations present in the population from northern Sweden are shown. The bold indicates genes studied in this thesis.

Rod and rod-cone dystrophies

In this group of dystrophies, the rods are affected first. The diseases in this group can be progressive or stationary. The progressive form and the most common clinical manifestation of IRDs is **Retinitis Pigmentosa** (RP) (Nash, et al., 2015). RP is clinically and genetically heterogeneous with variable expression and onset. The symptoms include difficulties seeing in dim light, night blindness, and loss of peripheral vision progressing to the centre of the visual field, resulting in tunnel vision and finally complete blindness (Zhang, 2016). RP is one of the leading causes of legal blindness in the working age population in industrial countries (Buch, et al., 2004) and affects 1:4000 in Sweden. RP is also present in syndromes such as Bardet-Biedl and Usher (Koenig, 2003). The stationary form of RP is called **congenital stationary night blindness** (CSNB), also known as night blindness or nyctalopia. Mutations in 17 genes have been reported to cause CSNB (Zeitz, et al., 2015).

Cone and cone-rod dystrophies

Dystrophies affecting the cones (**COD**) or cone-rods (**CORD**) are often quite severe but fortunately less common. Affected individuals with COD usually have lost colour perception and central vision. CORD patients with additional rod involvement and increased severity are usually completely blind by the age of 40 (Thiadens, et al., 2012). **Achromatopsia**, the stationary cone dystrophy, exists in two forms: with complete absence of all colour vision or with the perception of only a specific colour (Nash, et al., 2015).

In **Stargardt** disease, the photoreceptors in the macula are affected, resulting in loss of central vision. Stargardt disease, which usually does not affect peripheral vision, is the most common cause of central vision loss in young people. The majority of cases are recessively inherited and associated with mutations in the *ABCA4* gene (Dalkara, et al., 2016).

Generalised retinal dystrophies

Retinal dystrophies with simultaneous degeneration of both rods and cones are called generalised retinal dystrophies. The most common disease in this group is **Leber congenital amaurosis** (LCA). LCA is characterised by an onset of disease in early childhood, poor vision, photophobia, nystagmus, and severe retinal dysfunction.

A rare dystrophy in this group is **choroideremia**, which is a progressive degeneration of the retina, the RPE, and the choroid. The disease progresses relatively slowly, so patients may retain good central visual function until 50-70 years of age (Zinkernagel and MacLaren, 2015).

Providing a patient with a definitive clinical diagnosis can be a challenge for an ophthalmologist due to overlap of both clinical symptoms and genetic causes, complexity of the results of ophthalmic examinations, and unpredictable disease course (Figure 3). It may take a long time for the full disease spectrum to manifest. Moreover, familiar variability in IRDs is not a rare event as different families and family members with the same disease-causing mutation(s) can manifest different phenotypes (Chiang, et al., 2015).

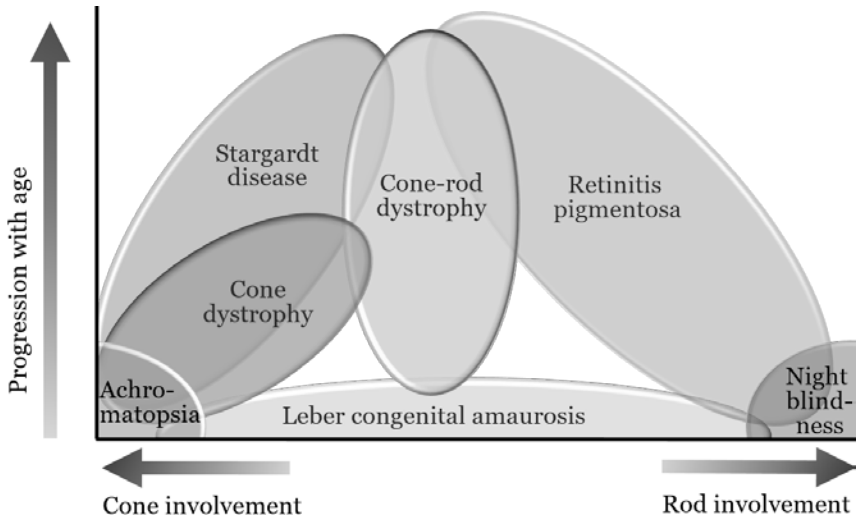


Figure 3. Phenotypic overlap among autosomal recessive retinal dystrophies. Clinical complexity and possible course of recessive retinal dystrophies is shown. Stargardt patients resemble features of cone, cone-rod dystrophy and LCA patients have features of RP, cone, or cone-rod dystrophies (den Hollander, et al., 2010). *Modified and printed with permission.*

Examinations of the eye

In both corneal and retinal diseases, visual acuity testing is important and should be examined first. Description of phenotypes is mainly done by examinations of the eyes (Bowling B, 2016; Kugelberg M, 2010).

Visual acuity

Visual acuity is the quantitative measurement of the eye's ability to see an in-focus image at a certain distance. Visual acuity is often measured using the Snellen chart, an eye chart with lines and blocks of letters decreasing in size.

Slit lamp

The slit lamp is an important tool for routine ophthalmic examinations that is used together with a biomicroscope. It consists of a high-intensity light source. The binocular slit lamp enables structures like the conjunctiva, cornea, lens, iris, and sclera to be examined. Using a slit lamp, the retina can be examined by a second hand-held lens.

Visual field test

Visual field is used for detection of full horizontal and vertical range and sensitivity of vision. Detection of defects in the visual field could be a sign of eye disease. Moreover, the size, shape and localisation of a scotoma can offer important clues for diagnosis.

Electrophysiology

Electrophysiology measures electrical activity generated by the photoreceptors in the retina upon light stimulation. An electrode placed on the cornea captures the activity and this graphic record is called an electoretinogram, a useful tool for diagnosing disorders or documenting functional changes in the photoreceptors of the retina.

Basic molecular genetics

All living organisms consist of cells. In a human cell the genetic information is present in the form of deoxyribonucleic acid, **DNA**, bundled up into chromosomes and stored in the nuclei. In humans there are 23 pairs of chromosomes in each somatic cell, 22 pairs are autosomes and one pair determines sex, an X and a Y chromosome in males and two X chromosomes in females. DNA is composed of a chain of four nucleotides or bases: adenine (A), cytosine (C), guanine (G), and thymine (T). **Genes** represent regions of DNA that encode proteins with various functions needed by the organism. In normal conditions, one chromosome in each pair is inherited from the mother and the other one from the father, so each gene or marker is present as two copies or alleles. One can be homozygous for the same allele at a specific locus or can be heterozygous with different alleles at a specific locus. The genes contain coding (**exons**) and non-coding (**introns**) regions. To get a functional protein, exons need to be set together to serve as a template for protein production. During gene transcription, the introns are removed in a process called **splicing** and the template becomes a single stranded complementary messenger ribonucleic acid, (**mRNA**). Splicing machinery is a complex of proteins that recognises certain sequences, forms intron loops, and fuses the exons together. The resulting mRNA is used in the translation process where three bases (**a codon**) code for one amino-acid. The resulting chain of amino-acids forms a protein (Figure 4).

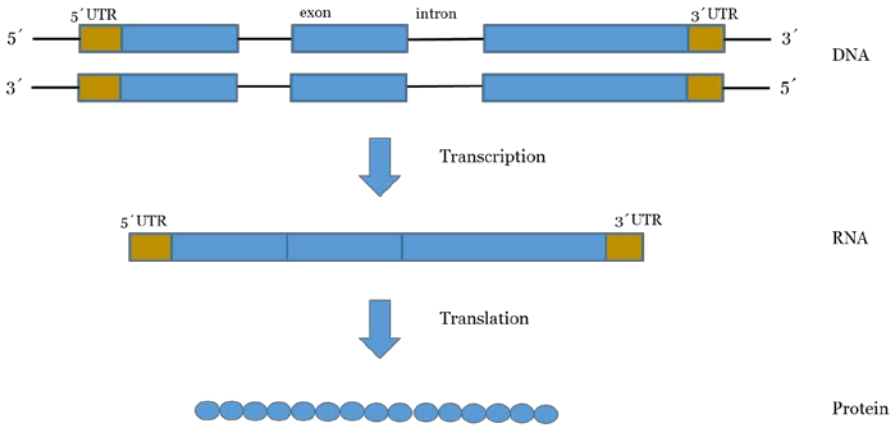


Figure 4. The central dogma: the genetic information in the DNA is transcribed into mRNA and mRNA is translated into a protein.

Sequence variations

A variation in the DNA sequence can either cause a change in the protein function or be a normal **variation** in the population without causing a disease. In this thesis, a normal variation in the genome is called a polymorphism and a disease-causing variant is referred to as a **mutation**. The most frequent variations in the DNA sequence are **single nucleotide polymorphisms** (SNPs), insertions, and deletions (Bentley, et al., 2008). SNPs, evident in more than 1% of the population, are considered non-pathogenic.

A mutation can either be a single base substitution, insertion, or deletion of nucleotides. A single base substitution can result in a **silent** or **synonymous** change, altering the codon but coding for the same amino-acid. A **missense** mutation results in an amino-acid change, while a **nonsense** mutation creates a stop codon, which terminates the translation. A single base change can interfere with the splicing processes and result in incorrect mRNA sequence, so-called **splice mutations**. A frame-shift mutation caused by an insertion or deletion in the sequence leads to a shift of the reading frame and often results in a premature stop codon.

If the insertion or deletion is large, from hundred base pairs up to several million base pairs, it is described as a **copy number variation** (CNV). **Translocations** and **inversions** represent large aberrations where a part

of one chromosome is transferred to a non-homologous chromosome or swapped in orientation, respectively.

Splice mutations

A correct splicing might be disturbed if a sequence alteration occurs in the intronic stretches (branch site) or exon-intron junctions. The splicing occurs in the spliceosomes, a complex of five small nuclear ribonucleoproteins and additional factors. The spliceosome joins a donor splice site, located at the 5´end of the intron, to the corresponding acceptor site, located at the 3´end of the intron. Within the intron, there are several important recognition sites that ensure correct splicing (Liu and Zack, 2013). The most common splicing mutations are those that induce exon skipping, form new exon/intron boundaries, or activate cryptic splice sites by alterations of the canonical donor or acceptor sites (Lewandowska, 2013; Olsson, et al., 2008). Differences in splicing have been reported between tissues as well as between cell lines (Yeo, et al., 2004).

De novo mutations

A *de novo* mutation is a new germline mutation that is not inherited from either parent. *De novo* mutations tend to be more deleterious than inherited variants because they have not undergone the same level of evolutionary selection. The incidence of *de novo* mutations has been shown to correlate to paternal age (Veltman and Brunner, 2012).

Variant evaluation

The importance of evaluation of sequence variants has been highlighted by the American College of Medical Genetics and Genomics (ACMG) (Richards, et al., 2015). In 2013, a workgroup was formed with a goal of developing recommendations for the use of standard terminology for classifying sequence variants. Two sets of criteria were presented: one for classification of pathogenic or likely pathogenic variants and another for classification of benign or likely benign variants. The information used in classification includes variant frequency, *in silico* prediction tools, reported functional testing, and segregation with disease. When the pathogenicity of a variant can be neither confirmed nor ruled out, the variant is classified “variant of uncertain significance” or VUS. The guidelines recommend a variant to be termed pathogenic, likely pathogenic, uncertain significance, likely benign, or benign per evidence evaluation (Figure 5).

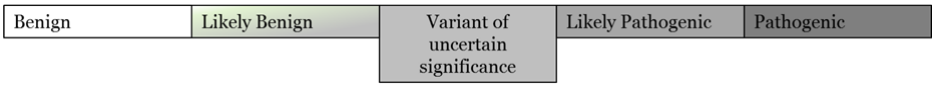


Figure 5. Classification groups according to ACMG standards and guidelines (Richards, et al., 2015).

Patterns of inheritance

A genetic disease is any disease that is caused by an abnormality in an individual's genome. A monogenic trait can be transmitted by different inheritance modes – autosomal **dominant** (AD), autosomal **recessive** (AR), or **X-linked**. Affected individuals are heterozygous for mutation in an AD disease; they carry only one defective copy/allele of the gene (Strachan and Read, 2010). The normal allele is often referred to as the **wild type** (wt), and the disease-causing allele as the **mutant** (mt). In AD diseases, the mutation often leads to a new protein function therefore is regarded as a gain-of-function mutation. This new function causes the disease. The trait in AD diseases is often seen in every generation and in both females and males (Figure 6a). However, the condition may also result from a new (*de novo*) mutation and may occur in individuals without disease history in their family.

In AR diseases, both gene copies must be defective, and the individual must inherit a defective allele from both parents who are unaffected carriers of the disease gene. Generally in AR diseases, the mutations are found to be loss-of-function (null) mutations (Griffiths, et al., 2000). However, some loss-of-function mutations are dominant when the wt allele in a heterozygous individual can not provide the amount of the gene product needed for normal cell function. In AR diseases, the parents of an affected individual are healthy carriers of a mutant allele, typically without any symptoms of the condition. AR diseases are more common in societies with a custom of consanguineous marriages (Figure 6b) (Modell and Darr, 2002).

In consanguineous families and in families from isolated areas with low migration rate over the centuries, **homozygosity mapping** has been shown to be a powerful tool in detection of mutations causing recessive disorders (Collin, et al., 2011; den Hollander, et al., 2007; Siemiatkowska, et al., 2011). The mutation is not only homozygous but also stretches of DNA surrounding the mutation (haplotypes) are homozygous. There is a great

chance of finding cause of disease if regions with **loss of heterozygosity** (LOH) are detected in a patient with a recessive disease. Loss of heterozygosity is also a common event in cancer development (Ryland, et al., 2015).

In X-linked diseases, the mutated allele is situated on the X-chromosome, affecting males and females differently. In males, with only one X chromosome, a mutation in the only copy of the gene causes the disorder. In females, with two X chromosomes, a mutation can be either dominant-causing or recessive-causing due to X-inactivation. Typically, male carriers of an X-linked trait can not pass the disease to their sons (Figure 6c) (Lindsten and Iselius, 1987).

The inheritance of a trait can also be complex when genes in combination with environmental factors contribute to the phenotype.

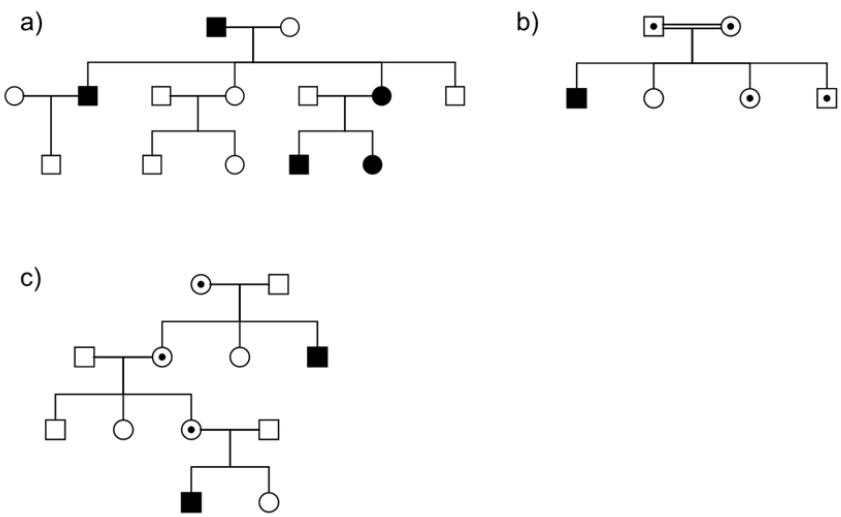


Figure 6. Pedigrees with different modes of inheritance: a) autosomal dominant; b) autosomal recessive; and c) X-linked. Squares represent males and circles females. Filled symbols represent individuals affected by disease, unfilled symbols represent individuals unaffected by disease, and symbols with a black dot represent healthy carriers. Double line represents consanguineous relationship.

Compound heterozygosity and digenic inheritance

An affected individual with a recessive disorder can be either homozygous with the same mutation on both alleles or a **compound heterozygote** with two different allelic mutations in the same gene, one from each parent. There have also been reported cases of retinal dystrophies with **digenic** inheritance (Kajiwara, et al., 1994; Liu, et al., 2016). In digenic cases, heterozygous mutations in two different genes have been shown to cause the disease. For example, 21 genes are known to cause the Bardet-Biedl syndrome with majority of patients carrying bi-allelic mutations (Suspitsin and Imyanitov, 2016).

Uniparental disomy

In some cases, a disease develops when both gene alleles are inherited from the same parent, resulting in **uniparental disomy** (UPD). Some genes are expressed differently according to parental origin or expressed from either the paternal or maternal inherited copy, which is the case for Prader-Willi and Angelman syndromes that involve the same region of chromosome 15. Loss of paternal contribution of 15q11-q13 causes Prader-Willi syndrome and loss of the maternal contribution causes Angelman syndrome articular mutation. Diseases can also result from inheriting a homologue gene with a heterozygous variant from the same parent, making the offspring homozygous for that particular mutation (Munshi and Duvvuri, 2007; Preece and Moore, 2000). This highlights the importance of establishing carrier status of parent to children harbouring homozygous mutations because presence of homozygous mutation can be the result of UPD or a deletion.

Penetrance

The **penetrance** of a disease is the probability that an individual with a disease genotype will manifest the disease phenotype. In AD diseases such as retinitis pigmentosa RP11, the penetrance might be reduced, meaning that not all mutation carriers will develop the disease (Kohn, et al., 2009). The penetrance can also differ in populations from different geographic areas despite the genotype identity (Holmgren, et al., 1994; Plante-Bordeneuve, et al., 2003).

Population genetics

Humans are genetically very similar with estimates of similarity ranging from 99.5 to 99.8% (Kidd, et al., 2004). Despite this similarity, some differences in frequencies for monogenic diseases are observed in different populations. A small number of individuals with a limited gene pool consisting of a few alleles can form the basis of a new large population. If a genetic variant appears in such an environment, the variant frequency can rapidly increase resulting in a **founder effect**. A founder mutation is more common in isolated populations; they represent a valuable source for mapping and identification of genetic causes in recessive and dominant diseases. High prevalence of disease indicates an enriched amount of risk factors or genetic mutations. The population of northern Sweden has been shown to be well suited for mapping of monogenic and complex diseases (Johansson, et al., 2005). Several Mendelian diseases are present in this population. With a history of low migration due to the river valleys, the population in northern Sweden has been quite conserved. Small populations have less genetic variants compared to larger populations and these variants might be common only in those populations.

Aim of this thesis

The general aim of this thesis is to identify unknown genetic causes in patients with corneal and retinal dystrophies. The specific aims of each paper that forms this thesis are elucidated below.

Paper I – *COL17A1*

To identify genetic cause of disease in families with corneal dystrophy with recurrent erosions.

Paper II – *CRB1* and *ABCA4*

To identify mutations causing disease in a family with Leber congenital amaurosis and Stargardt disease.

Paper III – *ABCA4*

To perform a functional study of two intronic *ABCA4* variants identified in Stargardt patients.

Paper IV – *RLBP1* and *CAIV*

To identify the second mutation causing recessive retinitis pigmentosa of Bothnia type in *RLBP1* c.700C>T heterozygous carriers.

Methodology

A summary of the main methods is given in the section below. Informed consent was obtained from all participants and the Regional Ethical Review Board in Umeå approved the research. All studies were performed according to the principles of the Declaration of Helsinki.

Patients and control material

Clinical examinations of all patients have been performed at the Department of Ophthalmology, University Hospital of Umeå. DNA from individuals included in Papers I-IV was extracted from either peripheral blood lymphocytes using the salt method described previously (Balciuniene, et al., 1995) or buccal swabs using a Puregene ™ DNA Purification kit (Qiagen, MD USA). The control samples used in **Papers I**, **II**, and **IV** for gene frequency estimations consisted of drafted men and blood donors from Västerbotten County. The number of controls was different due to technical reasons or sample access.

In **Paper I**, one extended family with corneal dystrophy and recurrent erosions was investigated. Samples were collected from 46 individuals (35 affected, nine non-affected, and two with unknown disease status). All individuals originated from the same geographic area in northern Sweden but now live in different parts of Sweden. A control group of 23 individuals from the same restricted geographic area of northern Sweden (West Bothnia) was used for filtering of WES data.

The patients described in **Paper II** originated from Jämtland County in northern Sweden. DNA from six affected individuals and ten unaffected relatives were available for this study. A control material from a matched population was used for frequency estimation of *CRB1* mutation and *ABCA4* mutations. One of these samples was also included in **Paper III**.

In **Paper IV**, individuals (n=121) with a recessive RP were genotyped and 67 patients were shown to be homozygous for c.700C>T (R234W) mutation in *RLBP1* associated with Bothnia dystrophy (BD). Furthermore, ten individuals presented with BD phenotype were heterozygous carriers of *RLBP1* c.700C>T mutation. To identify a cause of their disease, these ten patients were further analysed.

Molecular genetic methods

Genealogy analysis (Paper I)

For genealogic studies, Swedish church records were used. The church records were initiated in the 17th century and contain information about all births, deaths, marriages, health status, and migrations of the inhabitants. All records have been digitalized and are available through the Swedish Archive Information homepage (www.svar.se). Additional information was collected from the Demographic database at Umeå University, INDIKO, and personal communications.

DNA sequencing (Papers I, II, III, IV)

Sanger sequencing was used in **all papers** for mutation screening, verification, and confirmation of certain findings. With Sanger sequencing, regular nucleotides are mixed with fluorescently-labelled termination nucleotides, resulting in fragments with different lengths and a fluorescent nucleotide at the end. The fragments are separated according to their size and results in a consensus sequence corresponding to the targeted DNA. Most primers used in the projects have an M13-tag sequence to simplify the sequencing PCR. All sequencing reactions were performed in a final reaction of 10µl using Big Dye Terminator (v.3.1 Cycle sequencing kit, Applied Biosystems, Foster City, CA). The products of the sequencing reactions were run on an ABI 3730xL (**Paper IV**) and ABI 3500xL (**Papers I**, **II**, **and III**) Dx Genetic analyser (Applied Biosystems). Sequences were aligned and evaluated using Sequencher software version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA).

PCR-restriction Fragment Length polymorphism (PCR-RFLP) (Papers II and IV)

Mutation segregation in **Paper II** and **Paper IV** was done with PCR-RFLP analysis. Restriction endonucleases cut the DNA sequence at a certain known sequence distinguishing between the mutant and the wild type nucleotide. The digested PCR products were easily separated on an agarose gel and the fragment length was visualised under UV-light by DNA staining with ethidium bromide.

Denaturing High Performance Liquid Chromatography (dHPLC) (Paper IV)

CAIV sequence variant in control samples was detected by dHPLC (Wave Nucleic Acid Fragment Analysis System, Transgenomic, Omaha NE, USA) (**Paper IV**). The data were analysed with Navigator Software v.2.1 (Transgenomic) (Keller, et al., 2001). The manufacturer's software (Wavemaker; Transgenomic) was used to predict melting characteristics of an amplicon encompassing exon 1 in the *CAIV* gene. During dHPLC, DNA fragments were separated according to their binding affinity and size. An additional denaturing step with a slow cooling to room temperature was performed at the end of the PCR to allow fragments to form heteroduplexes if a variation in the amplicon existed (Figure 7).

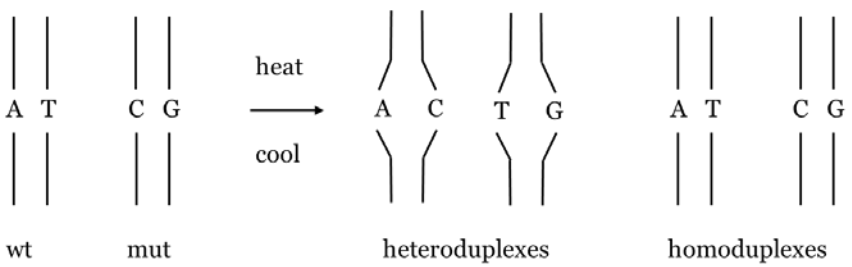


Figure 7. Heating and slow cooling at the final step of PCR theoretically yields four types of fragments in heterozygous amplicons: a mutant and a wild-type homoduplex, and two heteroduplexes.

Arrayed Primer Extension (APEX) Analysis (Papers I, II, and IV)

The APEX technology uses hybridization between DNA samples to a chip with oligos extended by fluorescently-labelled terminator nucleotides. An array contains thousands of oligonucleotides that hybridises to the surfase, and PCR-amplified fragments anneal to the complementary oligonucleotides on the microarray extended by fluorescently-labelled terminator nucleotides. The emitted light is detected with lasers and detection of many sequence variants is performed at the same time (Kurg, et al., 2000). APEX analysis was used by Asper Biotech to screen for known mutations in **Paper I**, **II**, **and IV**.

Immunohistochemistry (Paper I)

In **Paper I**, immunohistochemistry (IHC) was performed on sections of corneas obtained from deceased donors without any known corneal disease to study the expression of collagen type XVII (COL17A1). Corneal cross-sections, 5- to 7-µm thick, were used for IHC with polyclonal antibodies against COL17A1 and laminin β3 (Table 2). Laminin β3 chain is a known constituent of the corneal epithelial basement membrane (Bystrom, et al., 2007) and was used as a basement membrane marker. Normal swine serum was used for both COL17A1 and laminin β3 to block any potential unspecific binding. The slides were mounted in the media containing DAPI (Vector Laboratories, Burlingame, CA) to stain the nuclei of the cells. In negative controls, the primary antibody was replaced with PBS.

Table 2. List of primary and secondary antibodies for IHC.

Primary antibodies	**Source**	**Code**	**Epitop**
COL17A1 (rabbit)	Abcam, Cambridge, UK	ab28440	507–548
Laminin β3 (rabbit)	Thermo Scientific, IL, USA	PA5-21514	~644- 960
Secondary antibodies	**Source**	**Code**	
FITC Swine anti-rabbit	Dako, Copenhagen, Denmark	F0205	
TRITC Swine anti-rabbit	Dako, Copenhagen, Denmark	R016/Z02239	
Normal sera	**Source**	**Code**	
Swine	Jackson I.R, west Grove, PA, USA	014-000-121	

Allelic Discrimination Assay Taqman (Paper IV)

The genotyping in **Paper IV** was performed using endpoint analysis with specific minor groove binder probes (MGB). TaqMan assay designed by Applied Biosystems is a mix containing two primers, one forward and one reverse, for the amplification of the sequence of interest, and two MGB probes for the allele discrimination, one wt and one mutant. Each probe contains a fluorescence reporter in the 5´-end and a non-fluorescence quencher in the 3´-end. When the probe is intact, the quencher absorbs the reporter's fluorescence. The reporters used in our assay were VIC and FAM. When the amplification occurs, the polymerase cleaves the probes that are hybridised to the target, separating the reporter and quencher, producing a detectable fluorescence (Yao, et al., 2006). The PCR and MGB analysis were performed on an Applied Biosystems 7000.

Real-time PCR expression analysis with digital droplet PCR (Paper I)

In real-time PCR analysis, the amplification of a target is monitored in real-time and not just as it ends, as in conventional PCR. This method is used to detect small amounts of specific targets and is often used in diagnostics of haematological diseases and treatment follow-ups. The gene expression is estimated by measuring the number of RNA transcripts in a sample. Fluorescent probes, MGB probes, are used in the same way as in allelic discrimination assay. In traditional real-time PCR, the amount of target is calculated using an external reference gene with a known value (Raeymaekers, 2000). In ddPCR, mineral oil is added to PCR mix to generate thousands of oil drops. PCR reaction using one sample, in fact, represents thousands of reactions, a number equal to the number of oil drops. The amount of template is calculated according to Poisson statistics as the number of drops with a positive amplification in a total number of analysed drops (Hudecova, 2015). In **Paper I**, mRNA expression of *COL17A1* was studied in a library consisting of RNA from 20 different human tissues (Ambion, Life Technologies) and RNA from Corneal Epithelial cells (ScienCell, Carlsbad, CA).

Single Nucleotide Polymorphism Array (Papers I, II)

Single nucleotide polymorphism array or SNP array is a chip-based technique. The arrays used (The HumanOmniExpress-24 and Human610-Quad BeadChip, Illumina) consist of a grid with BeadChips covered with locus-specific oligos or 50-mers for detection of 715 000 different SNPs and markers scattered over the whole genome (LaFramboise, 2009). DNA is amplified and fragmented before hybridization to the BeadChip. The HiScan reader detects fluorescence, and software (GenomeStudio, Illumina) performs genotyping calling. The assay delivers two principal types of data – genotype and intensity – for identification and breakpoint determination of copy number variants (CNV) for every SNP. The GenomeStudio and cnvPartition 3.2.0 detects CNVs by retrieving Log R Ratio (LRR) and the B Allele Frequency (BAF). LRR is the ratio between the observed and the expected probe intensity. A region without evidence of CNV shows a LRR of around zero and a BAF of 0, 0.5, or 1 for the genotypes AA, AB, and BB, respectively.

The LRR and BAF can determine deletions and duplications, while BAF is helpful in detection of loss of heterozygosity (LOH) and UPD. In **Paper II**, regions of homozygosity (ROH) were detected by SNP array. SNP array data were used for haplotype analysis in **Paper I**. When searching for disease causing mutations, a common haplotype shared by affected individuals can

often be found as a result of genome-wide genotyping by SNP array. The disease gene is usually located within such a haplotype representing a group of genes that are inherited together from a single parent.

In vitro splice assay (Papers I, III)

In vitro splice assay was used in both **Paper I** and **Paper III**. In **Paper I**, the α-globin-fibronectin-extra domain B minigene (Baralle, et al., 2003) was expressed in HEK293 cells to investigate the synonymous variant c.3156C>T in the *COL17A1* gene. In **Paper III**, the splicing vector pSPL3 (Church, et al., 1994) was expressed in both HEK293T cells and ARPE-19 cells. In both papers, the fragments were cloned into pGEM-T Easy Vector System (Promega) and then subcloned into the expression vector. Mutations were introduced into wildtype constructs by site-directed mutagenesis kit: **Paper I** with Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Hitchin, UK) and with QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) in **Paper III**. The cells were transfected with lipofectamine and RNA was extracted and reverse transcribed into cDNA. Spliced products were visualised using vector specific primers on agarose gel. Sanger sequencing with the same primers was used for sequence determination.

Whole Exome Sequencing (WES) (Paper I)

WES was performed in seven individuals from families with corneal dystrophy using exome enrichment kit (NimbleGen SeqCap EZ Exome Library SR) and the HiSeq2000 sequencer (Illumina) using Genomic Services at Ambry Genetics (San Diego, CA, USA). Generated paired-end reads were aligned to the human reference genome UCSC hg19 using the Illumina CASAVA 1.8.2 software. RTA 1.12.4 (HiSeq Control Software 1.4.5) was used for initial data processing and base calling. Sequence quality filtering script was executed in the Illumina CASAVA software (version1.8.2; Illumina, Hayward, CA). A single bam alignment file was used in SoftGenetics' NextGEN ev 2.16 for SNP and indel analysis (Liu, et al., 2011).

Bioinformatics and Web Resources

Primer design was performed using a program available on http://simgene.com/Primer3. Genomic sequences of the genes studied are available on http://www.ensembl.org. All identified variants were denoted as recommended by Human Genome Variation Society (HGVS). To predict the impact of sequence variants, missense mutations were analysed by Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org) and Polymorphism Phenotyping (PolyPhen; http://genetics.bwh.harvard.edu/pph). Variants

detected in intronic sequences were analysed with the splice site prediction programs GeneSplicer (http://www.cbcb.umd.edu/software/GeneSplicer) and Human Splicing (v.2.4.1) (http://www.umd.be/HSF/). The frequencies used were from the NHLBI Exome sequencing project (ESP; http://evs.gs.washington.edu/EVS/), the 1000 genome projects (http://www.internationalgenome.org/), Swegen variant frequency browser (https://swegen-exac.nbis.se/) (Ameur, et al., 2016) (dataset released 20161019), and the exome aggregation consortium (ExAC; http://exac.broadinstitute.org/) (Lek, et al., 2016). All bioinformatics tools were available via the Alamut software version 2.0 (Interactive Biosoftware, Rouen, France). The Swedish archive information is available at http://www.svar.ra.se. The information on testing for mutations in cornea associated genes by Asper Biotech and Tartu can be found at http://www.asperbio.com/asper-ophthalmics. The Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/ac/index.php) was used to access mutations in analysed genes. Data services and software for identifying genes and mutations causing retinal degenerations are available online: http://www.sph.uth.tmc.edu/RetNet/ [updated Oct 04, 2016; cited Aug 24, 2016].

Results and discussion

Paper I, Autosomal Dominant Corneal Dystrophy

In **Paper I**, we studied several families experiencing similar corneal problems. A history of corneal erosions with an early onset resulting in subepithelial opacifications and vision loss were the main clinical characteristics of the affected individuals. Initially, DNA samples from four affected individuals were tested by an APEX (Asper Biotech) panel for corneal dystrophies; however, no known mutations in 13 corneal genes were found. Seven samples (six affected and one non-affected sibling from four families) were then analysed by WES. The WES results were filtered so that only heterozygous non-synonymous variants affecting protein structure shared by all six patients remained. The filtering yielded three variants all residing on chromosome 10. Only one variant was novel while the other two had been seen previously and were identified as rather common in either the ESP database or in matched Swedish controls.

The only variant segregating with the disease was a novel variant in the *COL17A1* (Collagen Type XVII, Alpha 1) gene, c.2816C>T, p.Thr939Ile (NM_000494.3). COL17A1 is a transmembrane protein with 15 collagen and 16 non-collagen subdomains. It forms a homotrimer of three alpha 1 chains, functioning as a cell surface receptor and as a matrix component (Franzke, et al., 2002; Franzke, et al., 2003). COL17A1 is a component of the hemidesmosomes, which is a complex attaching basal epithelial cells to underlying basement membrane (Powell, et al., 2005; Van den Bergh and Giudice, 2003). Mutations in the *COL17A1* gene have previously been associated with a recessive junctional epidermolysis bullosa with corneal changes described in some cases (Fine, et al., 2004).

COL17A1 expression in the cornea was demonstrated by immunohistochemistry. As expected, the staining showed COL17A1 expression in the basement membrane underneath the epithelial cells. We also showed that the highest level of *COL17A1* mRNA expression was found in placenta; however, in human corneal epithelial cells from healthy cornea donors and from commercially available corneal epithelial cells, the mRNA expression was eight and two times higher, respectively, than in placenta. This confirmed the immunohistochemistry results that COL17A1 is expressed in the cornea.

Segregation analysis performed by sequencing confirmed that the novel heterozygous missense variant, c.2816C>T in *COL17A1*, segregates with the

disease. The variant was present in all 35 affected individuals and absent in all nine unaffected individuals. All families in this project could be linked to a couple born in late 1700´s, confirming a founder mutation in the population of northern Sweden. A couple of families referred the disorder as "Theodors eyes", without knowing who he was; we discovered that one of their ancestors was called Theodor.

Previously, *COL17A1* variant c.3156 C>T, p.Gly1052Gly completely segregating with the disease was excluded in a family with Thiel-Behnke dystrophy mapped to chromosome 10 (Sullivan, et al., 2003; Yee, et al., 1997). The affected individuals in this family also experienced erosions and scarring of the cornea. We undertook *in-silico* analysis of this variant and several splicing tools – i.e., Human Splicing Finder (HSF) (Desmet, et al., 2009), NNSPLICE (Reese, et al., 1997), and NetGene2 (Hebsgaard, et al., 1996) – all predicted this variant would create a new donor site. *In vitro* splice assay confirmed that the c.3156 C>T creates a splice site resulting in an insertion of a single amino acid, p.Gly1052Ala, followed by an in-frame deletion of 17 amino-acids, p.Gly1053_1070del.

In 2016, Lin et al. (Lin, et al., 2016) confirmed that the c.3156 C>T change in *COL17A1* causes the disease in the family investigated by Yee (1997) and Sullivan (2003). Furthermore, Oliver et al. (2016) reported that the same c.3156 C>T mutation was present in affected individuals from New Zeeland, the United Kingdom, and Australia, all with recurrent corneal erosions (Oliver, et al., 2016). A Finnish family with ERED symptoms also harbours the c.3156C>T variant in *COL17A1* gene (personal communication, Prof. Tero Kivelä and Dr. Ilpo Tuisku). Whether the mutation segregates in this family is under investigation. So far, two *COL17A1* mutations causing ERED have been reported. Both mutations are in non-collagenous domains of the COL17A1 protein (Figure 8).

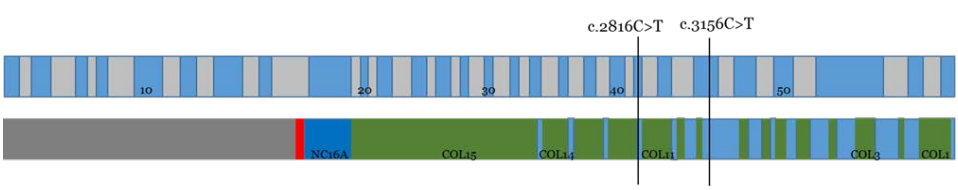


Figure 8. Figure shows the *COL17A1* gene on top and the protein below. The gene shows all the transcribed exons shown in light gray and blue. The two reported mutations causing ERED are marked with a line through the gene and protein. Intracellular domain is in dark gray, the transmembrane domain is in red, the collagenous domains are in green, and the non-collagenous domains are in blue.

The mutations found in recessive form of severe epidermolysis bullosa are presumed to be loss-of-function variants often causing premature termination codons. Missense mutations resulting in preserved COL17A1 expression are typically associated with milder phenotypes. The two mutations found in ERED are milder variants, so a dominant negative effect is possible. Furthermore, both *COL17A1* mutations found in ERED patients are in the non-collagenous domains, which might explain the relatively mild symptoms compared to epidermolysis bullosa phenotype.

Three entities in the Epithelial Recurrent Erosion Dystrophies (ERED) group – i.e., Franceschetti CD, Dystrophia Smolandiensis, and Helsinglandica – are included in the IC3D classification. Dystrophia Smolandiensis and Helsinglandica were described in Swedish families with clinical symptoms similar to our family. It would be important to analyse the *COL17A1* gene in affected individuals from these families to confirm or exclude *COL17A1* mutations as a cause of the disease.

Paper II, Retinitis Pigmentosa and Stargardt disease in the same family

In a large family from northern Sweden, individuals with both LCA and Stargardt disease were identified. One of the LCA patients analysed with a panel of 641 known variants in 13 LCA associated genes was a carrier of four heterozygous variants, all predicted to be benign. One of the STGD patients was analysed with a panel of 594 known variants in 19 RP associated genes. In this patient, only one heterozygous variant c.5461-10T>C (NM_000350.2) in the *ABCA4* (ATP binding cassette subfamily A member 4) gene was found and was predicted to affect weak splicing defect. SNP array was performed to identify potential homozygous regions.

Eight regions of homozygosity (ROH) were found in the LCA patient but only two of them harboured retinal genes. Two of these genes, *CRX* (Cone-Rod Homeobox) and *CRB1* (Crumbs 1, Cell Polarity Complex Component, former known as Crumbs Homolog 1), have been reported as a cause of LCA. Screening of these two genes resulted in identification of a novel homozygous nonsense mutation in the *CRB1* gene, c.2557C>T, p.Gln853Stop (NM_201253.2). It is known that CRB1 is expressed in Muller glia cells, cone and rod photoreceptors (Slavotinek, 2016). Segregation analysis performed by PCR-RFLP and sequencing revealed complete segregation for the *CRB1* mutation and the disease; all four affected individuals were homozygous for c.2557C>T.

In the STGD patient, only one ROH was detected with no known retinal genes included. With no other indications of disease causative genes, all exons and flanking introns of the *ABCA4* gene were sequenced and several variants were found (Table 3). One exonic variant, c.5603A>T, p.Asn1868IIe, was possibly damaging according to bioinformatics tools and is listed in The Human Gene Mutation Database (HGMD) as a “disease-associated polymorphism with supporting functional evidence”. Frequency in matched controls (n=115) was estimated to be over 13%, indicating that the c.5603A>T variant is a common polymorphism in the northern Sweden population.

Table 3. *ABCA4* sequence variants found in STGD patient.

Position	Nucleotide change	Amino-acid change	RefSNP	SIFT	PolyPhen	Splice site effect	Frequency [a]/[b]/[c]/[d]
Exon 10	c.1268A>G	p.H423R	rs3112831	Tolerated	Bening	No effect	C = 0.25/0.30/0.28/NA
exon 28	c.4203C>A	p.P1401P	rs1801666	Not predictable	Not predictable	No effect	T = 0.024/0.035/0.05/NA
Exon 40	c.5603A>T	p.N1868I	rs1801466	Deleterious	Possible damaging	No effect	A = 0.045/0.065/0.077/0.139
Exon 40	c.5682G>C	p.L1894L	rs1801574	Not predictable	Not predictable	No effect	G = 0.22/0.25/0.24/NA
Intron 3	c.302+26A>G	-	rs2297634	-	-	No effect	T = 0.52/0.48/0.48/NA
Intron 7	c.769-32T>C	-	rs526016	-	-	No effect	G = 0.246/0.30/0.287/NA
Intron 9	c.1240-14C>T	-	rs4147830	-	-	No effect	G = 0.48/0.46/0.46/NA
Intron 12	c.1761-54G>A	-	rs4147833	-	-	Cryptic site change	T = NA/NA/0.25/NA
Intron 33	c.4773+3A>G	-	rs759672616	-	-	Prediction: -46.5	G = 0.011/0.018/NA/0.009
Intron 38	c.5461-10T>C	-	rs1800728	-	-	Prediction: -4.3	C = 0.02/0.04/0.0005/0.0000
Intron 38	c.5461-51delA	-	rs4147899	-	-	No effect	= 0.21/0.25/0.245/NA

[a] ExAc total allele frequency, [b] allele frequency in European (non-Finnish) population from ExAc, [c] SweGen frequency, [d] Frequency estimated in this study. NA – Not avaliable

A novel intronic variant c.4773+3A>G with a predicted splice site effect as well as the disputed variant c.5461-10T>C were found. Segregation analysis on parents and siblings showed these two variants were allelic. One STGD patient was homozygous for the c.5461-10T>C variant and the other STGD patient was compound heterozygous for c.5461-10T>C and c.4773+3A>G.

The genomic region covering exon 33 and intron 33 seems to be a hot spot when it comes to mutations causing retinal dystrophies. Variants in c.4773+1, +2, and +5 have been reported to cause STGD, AD RP, and age-related macula degeneration (Birch, et al., 2001; Duno, et al., 2012; Jaakson, et al., 2003; Pang and Lam, 2002).

The variant c.5461-10T>C was first observed in 1999 (Maugeri, et al., 1999). Since then, the variant has been seen in several studies on identification of the genetic cause in STGD patients (Duno, et al., 2012; Rivera, et al., 2000; Zernant, et al., 2011). In 2000, Rivera failed to demonstrate a presumed effect on mRNA splicing, leading to the assumption that the c.5461-10T>C is in linkage disequilibrium with a yet unknown mutation and consequently represents a risk allele (Rivera, et al., 2000).

Mutations in the *ABCA4* gene have been seen in STGD disease, AR cone-rod dystrophy, and RP (Figure 2). Most of the identified *ABCA4* sequence variants so far are missense mutations that are rare in both patients and control populations. At present, over 900 disease-associated *ABCA4* variants have been reported (Tanna, et al., 2016). The transporter protein ABCA4 (ABCR) is localized to the disc membranes of cones and rods photoreceptors where it participates in the retinoid cycle by transporting retinoids from the lumen of the disc to the photoreceptor cytoplasm (Tsybovsky, et al., 2010). One challenge is to determine the degree to which each pathogenic variant impairs the ABCA4 protein function (Sun, et al., 2000), so the evaluation of mutations in the *ABCA4* gene will continue to be complex.

In the reported family, we identified two retinal diseases caused by mutations in two different genes. This finding highlights possible difficulties in diagnosing IRDs when multiple diseases are present in the same family, but it also highlights the importance of evaluating variant severity in a gene with many variants reported.

Paper III, Functional impact of *ABCA4* splice mutations

In **Paper III**, we performed *in vitro* splice assay of the two potential splice mutations found in the *ABCA4* gene in **Paper II**. The two *ABCA4* intronic variants were c.4773+3A>G and c.5461-10T>C. DNA from the compound heterozygous STGD patient with both mutations was used. PCR amplicons with the c.4773+3A>G contained exon 33 and 34 with flanking intronic regions. For the c.5461-10T>C variant, two constructs were designed, one with only exon 39 (R1) and one with both exons 39 and 40 (R2) with flanking intronic regions. Mutant variant c.5461-10C was introduced into wild type constructs by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) in accordance with the manufacture's protocol.

All amplicons were, first cloned into pGEM-T Easy Vector System (Promega) and then sub-cloned into the pSPL3 splicing vector (a kind gift from Prof. Monica Holmberg, Dept. of Medical Biosciences). Minigene constructs for wild type and mutant variants together with an empty vector were transfected into two cell lines, HEK293T (embryonic kidney cells) and ARPE-19 (retinal pigment epithelium cells), using Lipofectamine® 3000 Transfection kit (ThermoFisher, USA). After 46-48h, the cells were harvested. RNA was extracted and approximately 1µg of RNA was reverse transcribed.

RT-PCR was performed using pSPL3 vector specific primers and run on an agarose gel to detect splicing products. Different splicing products were present in HEK293T and ARPE-19 cells transfected with either wildtype or mutant c.4773+3A>G minigene. Amplified products were gel extracted and Sanger sequenced using the same vector specific primers. Sequencing of RT-PCR products from HEK293T transfected with wild type minigene revealed correct spliced product with exon 33 and 34 present as well as the absence of exon 34. RT-PCR on mRNA from HEK293T and ARPE-19 cells transfected with mutant minigene showed transcript with loss of exon 33 or both exons. Thus, c.4773+3A>G variant causes exon skipping that would result in protein truncation.

RT-PCR on mRNA from HEK293T cells transfected with wild type minigene c.5461-10T R1, presented a correct splicing product of exon 39 while transfection with the mutant minigene resulted in exon 39 skipping. Transfection with c.5461-10T R2 wild type minigene resulted in one splicing product containing exon 39 and 40. However, transfection with mutant type c.5461-10C R2 revealed two transcripts, one with skipped exon 39 and the other one with skipping of exons, 39 and 40. Unfortunately, the transfection

of the ARPE-19 cells with all constructs did not produce any transcripts. In conclusion, our results provide experimental evidence of pathogenicity of one common and one rare intronic variant in Stargardt disease.

Paper IV, Bothnia dystrophy (BD)

In **Paper IV**, we had a group of 10 affected individuals with a phenotype resembling RP of Bothnia type, all heterozygous for mutation c.700C>T, p.Arg234Trp (NM_000326.4), in the *RLBP1* (retinaldehyde binding protein 1) gene. The *RLBP1* gene encodes the CRALBP protein - carrier for 11-*cis* retinol and 11-*cis* retinal in the vision cycle (Golovleva, et al., 2003). DNA from two of these patients was analysed with APEX panel for autosomal recessive RP, and one patient was also analysed with APEX panel for autosomal dominant RP.

With the recessive panel, an additional mutation in the *RLBP1* gene was identified, c.677T>A, p.Met226Lys. All ten affected were carriers of c.677T>A; the mutation was tested by PCR-RFLP. Segregation analysis was performed by analysing parents of six index cases and in all tested families the c.700C>T and c.677T>A were allelic. In a matched control population (n=233), only one carrier of the c.677T>A was detected. Analysis of our RP population revealed two homozygous individuals for the c.677T>A mutation.

The APEX panel for autosomal dominant RP revealed an interesting finding. One of the patients were compound heterozygous for the two *RLBP1* mutations but also a carrier of the c.40C>T, p.Arg14Trp (NM_000717.4), variant in the *CAIV* (carbonic anhydrase 4), known to cause a dominant RP in South African families of European ancestry (RP17) (Rebello, et al., 2004; Yang, et al., 2005). The non-affected mother of our patient without clinical changes in retina at 61 years of age carried both variants, the c.700C>T in the *RLBP1* and the c.40C>T in the *CAIV* gene.

Further analysis of matched control population demonstrated a c.40C>T frequency over 4% in our region. This indicates that c.40C>T mutation in *CAIV* is a common polymorphism in northern Sweden. We have reported two mutations, c.677T>A and c.700C>T, in the *RLBP1* gene that cause RP of Bothnia type in homozygous or compound heterozygous patients. The c.700C>T variant seems to be a founder mutation in northern Sweden. Overall frequency in the Swedish population is 0.2% (4/2000 alleles) according to SweGen.

Conclusions

The general aim of this thesis was to identify the genetic cause of disease in families with corneal or retinal dystrophies and to gain an understanding of disease mechanisms. The main conclusions are discussed below.

Paper I

A novel mutation, c.2816C>T, p.Thr939Ile, in the *COL17A1* gene was proven to cause epithelial recurrent erosion dystrophy in a large family from northern Sweden. A previously excluded variant, c.3156C>T, p.Gly1052Gly, in the same gene was also proven to effect splicing and cause the disease.

Paper II

A novel homozygous mutation, c.2557C>T, p.Gln853Stop, in the *CRB1* gene was identified as a cause of Leber congenital amaurosis in a family from Jämtland. In the same family, two individuals with Stargardt disease were present. The genetic cause of the disease in those patients were either homozygous *ABCA4* c.5461-10T>C mutation or allelic *ABCA4* mutations, c.5461-10T>C and novel c.4773+3A>G mutation.

Paper III

The two *ABCA4* splice mutations found in Paper II were evaluated in an *in vitro* splice assay and splice alterations were shown.

Paper IV

Bothnia dystrophy is caused not only by the homozygous *RLBP1* c.700C>T, p.Arg234Trp, mutation but also by homozygous c.677T>A, p.Met226Lys or a combination of these two mutations, c.[700C>T];[677T>A]. The c.40C>T, p.Arg14Trp variant in *CAIV* gene is a benign polymorphism in northern Sweden.

Overall conclusions

Corneal and retinal dystrophies are two clinically and genetically heterogeneous groups of diseases. Many genes are involved in the pathogenesis of these diseases although genotype-phenotype correlations are not always well defined. Variant assessment can change over time, making variant evaluation difficult, demanding, and time consuming. In simple cases with no family history, the search for genetic cause of disease is a true challenge. Better understanding of rare variants can be done by vigorously genotyping large cohorts of patients and healthy populations. This strategy together with functional testing of suspected disease variants can facilitate more powerful variant characterization. A better understanding of the overall genetic causes of eye diseases will result in better genetic counselling and treatment.

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