Analysis of the Gene and Protein Causing Best Macular Dystrophy

BY

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Dissertation for the degree of Doctor of Philosophy (Faculty of Medicine) in Clinical Genetics presented at Uppsala University in 2003.

ABSTRACT


Best macular dystrophy (BMD) is an autosomal dominant inherited eye disease with a juvenile onset. Accumulation of the pigment lipofuscin in the retinal pigment epithelium can later cause macular degeneration and loss of vision. BMD have histopathologic similarities with age-related macular degeneration, the most common cause of blindness among elderly. BMD diagnosis is made with fundus examination and electrophysiology. The VMD2 gene, causing BMD, has previously been localized to 11q13 using linkage and recombination of a 12 generation family with BMD.

In this study the genetic region has been further narrowed using polymorphic markers in the BMD family. A human homolog for a C. elegans protein family, expressed in retina, was identified as the VMD2 gene. It has a 1755 bp open reading frame with 11 exons and encodes a 585 amino acid protein called bestrophin. Mutation analysis of the VMD2 gene in BMD families from Sweden, Denmark and Netherlands revealed 15 missense mutations, altering single amino acids in bestrophin, accumulating in the N-terminal half of the protein. VMD2 expression analysis with in situ hybridization revealed specific localization in the retinal pigment epithelium and Northern blot showed expression in retina and brain. Clinical and genetic analysis of a BMD family with generally late onset revealed a novel bestrophin mutation.

Analysis of mouse Vmd2 and bestrophin during development showed presence of mouse bestrophin in retinal pigment epithelium at postnatal day 10 and in photoreceptor outer segments during the entire postnatal period. Vmd2 expression levels were highest around birth.

Keywords: VMD2, bestrophin, macular degeneration, mutation analysis

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ISSN 0282-7476
ISBN 91-554-5494-1

Printed in Sweden by Uppsala University, Tryck och Medier, Uppsala 2003
“Our knowledge is a little island in a great ocean of nonknowledge”

*Isaac Bashevis Singer*

To my family
PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


IV Bakall B, Marmorstein LY, Hoppe G, Wadelius C, Marmorstein AD. Mouse Vmd2 expression and bestrophin localization during normal development. (Manuscript)

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## Abbreviations

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<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
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<td>ARM</td>
<td>age-related maculopathy</td>
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<td>ARMS</td>
<td>amplification refractory mutation system</td>
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<td>BMD</td>
<td>Best macular dystrophy</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>DC-ERG</td>
<td>direct current electro retinogram</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>electro-oculogram</td>
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<td>ERG</td>
<td>electroretinogram</td>
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<td>EST</td>
<td>expressed sequence tag</td>
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<td>GFP</td>
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<td>HGP</td>
<td>human genome project</td>
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<td>HUGO</td>
<td>human genome organization</td>
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<tr>
<td>Kb</td>
<td>kilo base pair</td>
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<td>OMIM</td>
<td>online mendelian inheritance in man</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROS</td>
<td>rod outer segment</td>
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<td>RPE</td>
<td>retinal pigment epithelium</td>
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<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
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<td>SFD</td>
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1. Introduction

Visual dysfunction is a very severe handicap. Diseases affecting the retina are the major cause of visual impairment in the western world. Age-related macular degeneration (AMD) is the main cause of blindness among the elderly population. Since there is no cure for the vast majority of AMD cases, much research is focused on this problem. Inherited macular degenerations with histopathologic similarities may be used as model diseases to solve general macular degeneration issues. Best macular dystrophy is an inherited disease with an onset usually early in life, leading to degeneration of the macula and loss of central vision. The gene causing this eye disease was, prior to this study, localized to the chromosomal position 11q13.

In this thesis the gene localization is studied in more detail and the gene causing Best macular dystrophy is identified. VMD2 is the name of the gene and bestrophin is the encoded protein. Disease causing mutations are revealed in patients from different families with the disease. The distribution of mutations in the gene and functional aspects of bestrophin are analyzed. One family with Best macular dystrophy is studied in greater detail from a clinical perspective and the combination of clinical and molecular diagnosis is emphasized. Finally, the $VMD2$ gene and the bestrophin protein are studied during mouse eye development in order to facilitate the analysis of future mouse models with Best macular dystrophy.

Identification of the genes causing inherited diseases is very important for gaining knowledge of the molecular defects causing the disease. By analysis of the causative gene and protein, new ways of diagnosis and treatment may be developed for previously untreatable conditions.

1.1 Basic molecular biology

The DNA, which is located in the nucleus of each cell in the body, contains the coded information for the proteins. The structure of the DNA molecule was first elucidated in 1953 by Watson and Crick. They were the first to propose that the DNA molecule has the shape of a double helix.\textsuperscript{1} This molecule consists of nitrogenous bases, attached to a ribose and phosphate backbone. The bases are either adenine (A), cytosine (C), guanine (G) or
thymine (T). The order of these bases is the key to the genetic information in the DNA. Each triplet of bases has the potential to either code for one amino acid, the basic component in proteins, or code for a stop in the protein synthesis. A DNA segment with the ability to encode a protein is called a gene and it is made up of different parts. The segments that encode messenger RNA are called exons. These are often separated by non-coding sequences, which are the introns. Before the first exon, there is a region called promoter, regulating the gene expression by the binding of transcription factors.

The process of constructing a protein from DNA involves several steps. The first step is the transcription in the nucleus of the cell, in which DNA is the template for the synthesis of RNA. This RNA is then processed so that the non-coding parts, introns, are spliced away and the coding parts, exons, are joined together. The remaining molecule is called messenger RNA (mRNA), which is first modified and then transported out from the cell nucleus to the cytoplasm. A protein is produced in a process called translation, using the mRNA as template. This protein is often modified in different ways and then transported to a part inside or outside the cell to the final location where it fulfills its designated task.

The DNA is located in the cell nucleus and can be densely packed in the structure of chromosomes. In all human cells, except for the gametes (sperm and egg cells), the number of chromosomes is 46. There are 22 homologous pairs of autosomes and two sex chromosomes, which determine the sex of the individual. XX results in a female and XY results in a male.

1.2 The human genome project

Recently two projects, one publicly and one privately financed, were able to publish the entire sequence of the human genome. This was a landmark in the history of science. The process of identification of genes causing human diseases has now been provided with a very powerful tool. The number of genes is still not definite, but the estimation is that there are between 30,000 and 40,000 protein-coding genes in the human genome. There is a great addition to these numbers if all splice variants are included. Splice variants are different proteins, which are encoded by the same gene, resulting from the usage of different sets of splice junction sequences.

An interesting feature in the genome, which can be studied, is SNPs (single nucleotide polymorphisms) and how these SNPs are linked together in the same chromosome forming a haplotype. It was proposed by Risch and Merikangas, that whole-genome association could be performed using SNPs. Later it was established that these variations in single bases can be chromosomally mapped and ordered into blocks as unique haplotypes.
Following the Human Genome Project, two other genome research projects have been initiated. The SNP consortium in combination with the Human Genome Sequencing Consortium completed the identification of over 1.4 million SNPs (single nucleotide polymorphism) in the human genome. The other project is the SNP Consortium Allele Frequency Project, which is aimed at constructing a human genome map of haplotypes based on SNPs that are commonly inherited in blocks, in a large number of individuals. The provided information from these projects will contribute to the localization of human disease genes and the understanding of gene regulation.

In the initial effort to sequence the entire human genome, only a few individuals were analyzed. Now Venter and colleagues have a new project which plans to sequence the gene-coding regions of 1000 individuals. This will enhance the knowledge of the variation between humans, which might explain why some people are affected to a different degree by the same disease.

![Fig. 1. The human eye. Modified from National Eye Institute, National Institutes of Health.](image-url)
1.3 The retina and the RPE

The retina is the inner layer of the eye globe (Fig. 1). It consists of several cell types which can be divided into two parts. The inner part is the neural retina containing the photosensitive cells, which are rod and cone photoreceptors. The rod photoreceptors are sensitive to moderate light and are distributed all over the retina. The rods have two parts, the inner and the outer segments. The inner segments contain the most of all the organelles and have synapses to bipolar cells which transmit the light impulses. The outer segments are packed with disks containing the photo pigment rhodopsin.

The cone photoreceptors detect color and are responsible for the high resolution vision. The cones are concentrated in the macula. The rods are in majority in an adult eye (100 million cells) and the cones are less frequent (5 million cells). Other cells in the neural retina are bipolar cells and ganglion cells, which mediate the photoreceptor response to light to the brain.

The outer layer of the retina contains the retinal pigment epithelium (RPE), a pigmented monolayer of hexagonal and columnar cells. The basal part of the cells has a great number of invaginations and is firmly attached to the adjacent Bruch’s membrane. Close to the invaginations are several mitochondria. The lateral outer membrane is firmly attached with tight junctions to other RPE cells. These tight junctions limit any transport across the cell layer outside the cells and the major transport of fluids, ions and molecules is performed actively and passively through the RPE cells. The apex of the cells has long microvilli and cylindrical sheets, which are loosely associated with the outer segments of the photoreceptors. The cytoplasm contains plenty of smooth endoplasmic reticulum. In the apical part of the cytoplasm and in the microvilli are numerous melanin granules, which absorb light that has passed the photoreceptors. Each morning the tips of the photoreceptors are shed and phagocytosed by the RPE. Lysosomal vesicles in the cytoplasm are responsible for degrading shed outer segments from the photoreceptors.
Selected functions of RPE:\textsuperscript{12,13}

*Physical*
- Blood-retinal barrier which protects neurosensory retina
- Attaching the neurosensory retina by pumping fluids from subretinal space.

*Optical*
- Light is absorbed by the melanin granules, which improves image resolution.

*Electrical activity as a result of light-induction*
- The c-wave and fast oscillation are responses to ionic changes.
- EOG recordings is a response to biochemical changes.

*Metabolic-Biochemical*
- Phagocytosis of photoreceptor outer segment tips.
- Extensive lysis of outer segments with cathepsin D as major lysozyme.
- Transport of metabolites to and from neuroretina and choroid circulation
- Capture and storage of Vitamin A.
- Isomerization of all-trans to the photo-active 11-cis retinal.

The RPE fulfills various physical and metabolic functions, some listed above. RPE has an important role in the phototransduction. When a light flash reaches the rhodopsin-filled photoreceptor outer segments, the active form of rhodopsin, 11-cis-retinal, is transformed into inactive all-trans retinol, which is transported into the RPE. Biochemical reactions reactivates rhodopsin, which is transported back to the photoreceptors from the RPE.

1.4 Best macular dystrophy
Best macular dystrophy (BMD) is an autosomal dominant inherited macular degeneration. The alternative name vitelliform macular dystrophy originates from the egg-yolk like lesion in the macular region that is typical for BMD. The prevalence of BMD was estimated to 2 per 10,000 in the Swedish population with a higher prevalence in the county of Västerbotten, namely 4 per 10,000.\textsuperscript{14}
Clinical characteristics of BMD: 15

Previtelliform stage
In the first BMD stage, the fundus can be normal or have an irregular shape with granular RPE. There is a great variability in the disease phenotype. Some infants have an affected macula after a few weeks whereas other individuals have an abnormal EOG, but perfect vision their entire life.

Vitelliform stage
The “egg-yolk” lesion can be seen as a distinct yellowish round subretinal lesion that rarely elevates the neural retina. Photoreceptors are usually unaffected and vision is normal.

Pseudohypopyon stage
Some patients present with a pseudohypopyon, which is a more dense yellowish material within the original lesion. When the head is tilted, the deposit moves with gravity. Focal areas are sometimes degenerated and RPE is clumped within the lesion.

Vitelliruptive (Scrambled egg) stage
With time the lesion becomes irregular. Pigment clumping occurs and yellowish material accumulates in the lesion borders. Vision is often not affected.

Atrophic stage
The yellow pigment is disappearing and there is atrophy in central RPE, which can be difficult to separate from other macular degenerations such as Stargardt and AMD. Visual acuity loss is immense, sometimes with blindness.

Subretinal neovascularization
Subretinal neovascular membranes are present and may bleed and lead to subretinal fibrosis, seen as a whitish scar. Vision decrease when hemorrhages occur, sometimes acutely.

BMD has mainly a bimodal onset with the two peaks around 3-9 years and in the twenties, but there is a great variability in onset from infants to over 50 years of age. 16 The disease is transmitted in a dominant manner, which means it is sufficient to inherit one mutated VMD2 gene from either parent.
There is variability in expression, where some patients are legally blind and others have a very moderate or no decrease in visual acuity. Some are asymptomatic when diagnosed, sometimes because examination is performed when a relative starts to get affected. The diagnosis is then made by electrophysiological examination, based on normal ERG, but abnormal EOG, (se below). Patients with symptoms are without pain, but complain of progressive loss of central vision. Sometimes color vision is altered. Peripheral vision is usually unaffected and dark adaptation is also normal. Current there is no general and efficient treatment for BMD. A treatment is not always necessary and some patients do not have an impaired vision at all. In one study, more than three-quarters of 91 BMD cases had a vision of 20/40 (0.5) or better. Many patients had only a temporary decrease in vision during the vitelliform and pseudohypopyon stages. For those patients with an atrophic macula and fibrous scars the situation is worse. Recently, a BMD patient with choroid neovascular membrane and decreased visual acuity, was successfully treated with argon laser photocoagulation. Visual acuity improved from 20/60 (0.33) to 20/30 (0.67) and serous retinal detachment was significantly reduced. This is a positive result, but this treatment can not be applied to BMD cases with atrophic lesions and fibrous scars.

1.5 Age-related macular degeneration

Age-related macular degeneration (AMD) is an eye disease affecting the macula in people usually over the age of 50. There are two variants of AMD according to the commonly used clinical classification.

Dry AMD
- Early form: Presence of soft drusen, focal hyper- or hypopigmentation of the RPE and small atrophies in macula.
- Late form: Geographical atrophy, which is hypopigmentation in a large, limited area in the RPE.

Neovascular AMD
- RPE detachment, presence of soft drusen, choroidal neovascularization sometimes with vessel leakage and bleeding. A fibrovascular scar can be seen in the end stage.

According to the alternative classification by the International Classification and Grading System, the term age-related maculopathy, ARM, is used. ARM
is divided into an early and a late form, where the late form is equivalent to AMD and subdivided into dry and neovascular AMD, as specified above.\textsuperscript{19}

AMD is the most common reason for loss of vision in people over the age of 60 in the western world. The prevalence of AMD in Sweden is 300,000. The prevalence in the USA is 20\% of individuals over 60 and it is predicted to affect about 10 million people in 2030. According to the US Census Bureau, AMD is expected to increase by 80\% in the next 25 years, because of prolonged life span, as a function of e.g. nutritional improvements.\textsuperscript{20} The prevalence of AMD is, according to the Framingham Eye Study, 1.6\% for people 52-64 years old, 11\% for the 65-74 years interval and 27.9\% for people 75-85 years old.\textsuperscript{21,22} The prevalence for late AMD (dry and neovascular) varies between 1.2\% and 1.7\% in the whole population and increases rapidly with age, which is shown in studies from different parts of the world.\textsuperscript{21,25}

**Risk factors for AMD**

**Age** is the most important risk factor, which has been confirmed by many studies. All variants of AMD as well as the severity of the disease increase by age, especially after 75 years.\textsuperscript{21,23-25}

**Smoking** has been found to increase the risk for both dry and neovascular AMD.\textsuperscript{26}

**Certain nutrients** have a beneficial effect. The reason is that antioxidative agents such as vitamin C and E, glutathione, betacarotene, selenium and Zinc in the retina are protective against free oxygen radical accumulation, which is due to the photochemical processing of light.\textsuperscript{27-29} The high intake and high levels in blood serum of the carotenoids lutein and zeaxanthin reduces the risk for neovascular AMD.\textsuperscript{30}

**Genetics** has been established as one of the risk factors for AMD. Sibling and parent-offspring case-control studies have indicated a familial aggregation of AMD.\textsuperscript{31-35} Several small twin studies have shown a high degree of concordance for AMD.\textsuperscript{36-41} These studies were not population-based and were predominantly based on monozygotic twins. In a recent population based twin study, the inheritability of early dry AMD (ARM) was found to be 45\% for all phenotypes. A higher inheritability for soft drusen (57\%) and large, $\geq$20 $\mu$m diameter, hard drusen (81\%), was observed. The hypothesis that genes causing inherited macular dystrophies also contribute directly to some of the AMD cases has so far not been verified. The best correlation has been found for ABCA4, the gene that causes Stargardts disease, and AMD. It has been proposed that about three percent of AMD cases are associated with ABCA4,\textsuperscript{42} but this has not been confirmed by others.\textsuperscript{43,44} When the BMD causing gene, VMD2, was identified and
disease causing mutations were found in patients with BMD, the gene was screened for mutations in AMD patients. There are some indications in a study by Lotery and coworkers, that the VMD2 gene may play a minor role in AMD. A small number of AMD patient were found to harbor mutations in the VMD2 gene.45 A separate study did not detect a significantly different mutation rate in AMD cases compared to the analyzed controls.46

Histopathology in AMD
The accumulation of drusen in AMD cases is an important characteristic of the disease. The protein composition of drusen has been studied in eyes from unaffected donors and donors with AMD, by Crabb and colleagues. They found protein modifications in drusen that may have been generated by the oxidation of lipids and carbohydrates.47 These modifications may be the signal for generating a local inflammatory response, which is suggested to be a pathomechanism for AMD.48 The findings of the protein compound of drusen by Crabb et al also confirm that drusen is mainly of RPE origin. Drusen is accumulating as a result of inflammation after RPE injury.

Lipofuscin
Lipofuscin is an autofluorescent compound present in the vitelliform lesion in BMD and is associated with various manifestations in AMD.49 Lipofuscin accumulates inside and beneath the RPE cells and the reason is probably the inability of the RPE to degrade lipofuscin. This is a an age-dependent process verified by both in vivo and in vitro experiments.50,51 Lipofuscin granules are believed to contain potentially toxic compounds, which cause RPE degeneration. The most well-known compound is A2-E (N-retinylidene-N-retinylethanolamine), a Schiff base product of all-trans retinalaldehyde and ethanolamine (2:1 ratio), which has been shown to inhibit the lysosomal catalytic process in RPE.52 The knockout mouse model for Stargardts disease showed that the deficient protein, RmP, is responsible for transporting molecules that make up A2-E out from outer segment disks. In the knockout mouse there is an accumulation of A2-E in the photoreceptor outer segments, which are digested by the RPE and then cause macular degeneration by destroying the RPE.53 Recently, the protein content in lipofuscin from human AMD eyes has been studied and the results show a complex protein mixture. Some proteins originate from photoreceptors, exemplified by recoverin. Other proteins are derived from mitochondria and must come from the RPE cell itself, because the photoreceptor mitochondria are located in the inner segments, which are not digested by RPE. Normal household proteins are found in lipofuscin as well, and the reason might be
that the UV light and oxygen induced protein modifications are sufficient to inhibit proteolytic degradation, but this needs to be studied in detail.\textsuperscript{54}

**AMD treatment**

There is no treatment for dry AMD. One treatment for cases of wet AMD is targeting vessels with photocoagulation using laser, but this method results in big scars. Using the method photodynamic therapy, a photosensitive substance is intravenously injected and local endothelial cells are lysed using a weak laser. Transpupillary thermotherapy is another method affecting the vessel tissue by using a moderate increase in temperature (43\textdegree C) for an extended period. There is no pharmacological treatment for wet AMD yet, but many compounds inhibiting VEGF are being evaluated.\textsuperscript{55} Translocation of the macula is a recent surgical technique where the neural retina is moved to an area with healthy RPE.\textsuperscript{56}

1.6 Animal models for AMD

The best animal model for AMD would be a primate, the only animals having a macula. Mice do not have a distinct macula, but it is the animal model of choice for the majority human diseases. There are not many mouse models for AMD. The first was a model for Stargardt disease with the \textit{Abcr} knock-out mouse. The mice lack the rim protein, RmP, which can cause Stargardt disease when altered.\textsuperscript{57} The human disease was simulated in some aspects and there was accumulation of the major lipofuscin fluorofore A2-E in the RPE.\textsuperscript{53} Another transgenic mouse model for AMD has just been reported.\textsuperscript{58} The mice are expressing a mutated form of human cathepsin D that is enzymatically inactive. This impairs the degradation of phagocytosed ROS in RPE. The inserted gene is driven by a cytomegalovirus promoter that results in a very high expression in many different tissues. The mice presents the following histopathologic changes: pigmentary changes indicating RPE atrophy, RPE cell proliferation, photoreceptor degeneration, accumulation of lipofuscin-like substance and a significant decrease in the amplitude of the a-wave when performing ERG. It is an interesting mouse model, which shows that high accumulation of inactive cathepsin D mimics several AMD traits and it may be used for the understanding of the pathomechanism of AMD.

Sorsby's Fundus Dystrophy (SFD) is an inherited late-onset macular degeneration caused by mutations in the tissue inhibitor of metalloproteinase (TIMP-3) gene. A knock-in mouse model has been constructed by inserting mutation causing an amino acid exchange (Ser156Cys), which has been found in SFD patients. Extensive analysis of this mouse model has not contributed to a greater understanding of the disease pathology. Results
show that mutated TIMP-3 tended to aggregate in higher-molecular-weight-complexes and was found in a higher concentration compared to wildtype TIMP-3. This might be an explanation for SFD. The construction of mouse models for other inherited macular dystrophies, including BMD, are still relevant, in particular if there are specific methods available for functional analysis.
2. Aims of the present investigation

The aims for this thesis were the following:

**Paper I**
To identify the gene causing the autosomal dominant eye disease Best macular dystrophy. To study the basic expression pattern of the gene in human tissues in general and the expression in the retina in particular. To perform the initial characterization of the predicted protein, bestrophin, that is defect in BMD.

**Paper II**
To search for mutations in the *VMD2* gene in a number of families affected by BMD from different countries. To study the mutation distribution in the gene and analyze the predicted protein sequence for secondary structure and transmembrane domains with publicly available computer programs.

**Paper III**
To identify the disease causing mutation and clinically examine individuals in a BMD family with mostly late onset.

**Paper IV**
To clone the mouse *Vmd2* gene and produce an antibody for mouse bestrophin. To analyze the mouse *Vmd2* expression and study the mouse bestrophin localization in eyes during normal mouse development.
3. Methods

3.1 Family material

The first cases of BMD were described 1905 by Best as a juvenile vitelliform macula degeneration. For Papers I and II, in total 22 different families with individuals affected by BMD were analyzed. The largest and most important family is Swedish with branches in Älvdalen and Vilhelmina. This family has been traced back for 12 generations after extensive research. The oldest ancestral couple were born in the 1670-ies and lived close to Älvdalen, but which one of the spouses that was affected has not been established. An affected descendant moved to Vilhelmina and brought the disease causing mutation to the area. The relationship between the family branches was established by studying old parish records. From these records it was also possible to obtain information about progressive loss of vision by assuming that progressive inability to read was correlated to decreased visual function. Later epidemiological studies suggest that families in Minnesota, USA, carrying the BMD disease are relatives to Swedish immigrants.

From the large Swedish study over 230 individuals have been used to localize and refine the VMD2 region to the chromosomal position 11q13. For detection of BMD causing mutations in the VMD2 gene for Papers I and II, 22 different families from the following places were used: Vilhelmina and Älvdalen, Sweden (S1), Lund, Sweden (SL 1-5, 76), Umeå, Sweden (SU5, SU11), Gothenburg, Sweden (SG1), Netherlands (Nx1-4, 6, 8, 10-12), Denmark (Dx1, 2), Morocco (Maro). These families consisted of at least one affected individual, diagnosed by both fundus examination and EOG/ERG analysis. In the other family members, diagnosis were made with either ophthalmic examination or electrophysiology.

3.2 Animal tissue samples

The animals used in this thesis were handled according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal experiments in Sweden were approved by the Uppsala Animal Ethics
Committee, March 2nd 2001, C38/1. Balb/c mice for RT-PCR and TaqMan analysis (Paper IV) were obtained from M&B, Denmark. Swiss Webster and Balb/c mice were obtained from Harlan, USA, for in situ hybridization (Paper I) and immunohistochemistry (Paper IV).

3.3 Identification of disease genes

BMD is a dominant disease and the method of choice for gene identification in this investigation was positional cloning combined with candidate gene approach.

Positional cloning

In positional cloning, the chromosomal region for the disease gene is determined. The method linkage analysis uses genetic markers, mainly microsatellites, distributed along the genome. These markers vary between different chromosomes and a subset of these were analyzed in both affected and normal BMD family members to localize the region for the VMD2 gene to a 1 cM interval on 11q13.64

Crossover analysis

When the candidate region is known, it can be further narrowed down by analysis of meiotic recombinations. A more refined map over the region is constructed using a more dense collection of microsatellites. In Paper I, 14 new CA-repeat markers were identified and used for the region. Haplotypes were constructed to localize crossover events during the meiosis. By analyzing the haplotypes in family members, the region containing the VMD2 gene was further refined.65

Candidate gene selection

The minimal chromosomal region for the unknown disease gene still contained a great number of genes. The region was spanning over several PACs and BACs in a contig, which means that overlapping parts of this region was cloned into bacterial chromosomes and thus available for analysis. Nine PAC-clones were subcloned and sequenced using Sanger sequencing. Subclones containing microsatellite CA-markers were selected in order to develop new polymorphic markers. In addition, subcloned sequences were compared to databases with known expressed sequence tags, ESTs, using BLAST.66 A gene matching known EST sequences expressed in retina was selected as a candidate and resequenced. The number of exons was determined using the programs Crossmatch and AceDB, combined with 5´-RACE to obtain the 5´-cDNA sequence.
3.4 Mutation detection methods

To detect if the identified gene was causing BMD, exon flanking primers were used to PCR amplify all the 11 exons in the gene from genomic DNA from BMD patients and normal controls.

**PCR** is a method for amplifying DNA fragments limited by oligonucleotide primers. Amplified *VMD2* exons were sequenced and the gene sequence was searched for differences between affected and controls.

**Sanger sequencing** is the most commonly used method for analyzing each single base in a DNA fragment of maximum around 800 bases. When using fluorescent dye terminators, copies of the template fragments are made with the four terminators randomly incorporated into the sequence copies. When a dye terminator is inserted, the copying process stops. The result is a collection of many sequences with variation in lengths corresponding to each base in the original template. Size separation is performed with laser detection of the fluorophores and the base at each position is interpreted by a computer.

All the missense mutations causing an amino acid alteration in the protein, which were found in BMD cases but not the controls, were considered to be disease causing mutations. All nucleotide substitutions that did not cause exchange of an amino acid, were determined as non causative polymorphisms. These were also detected in the some of the controls. The identified mutations found in one BMD affected family member were screened for in other affected and unaffected family members using **restriction enzyme digestion** or **ARMS** (amplification refractory mutation system). If the mutation altered or introduced a site recognized by a restriction enzyme it was used to digest the PCR amplified exon in which the mutation was located. If no restriction site was altered, the ARMS method was used. A PCR fragment was amplified using alternative primers in one end, with the identified nucleotide exchange located in the very 3’-end of the primers and a second mismatch located 2-3 bases from the end. One primer had a 3’ end matching the wildtype nucleotide and the other primer would only amplify the alteration. The results of the mutation detection methods were visualized with agarose gels electrophoresis.

**Passport Mutation Scanning** is based on Enzymatic Mutation Detection technology by Amersham Biosciences, Uppsala, Sweden. This method was used in Paper III to scan PCR amplified *VMD2* exons in one BMD patient to detect heterozygous changes. During the PCR, a low concentration of fluorescently labeled dCTP (1:1000 of the dCTP concentration) was incorporated into the amplified fragments of *VMD2* exons. The products were first heat denatured to separate the DNA strands and the strands were then reannealed, creating mismatches at the location of heterozygous mutations. This forms a “bubble” in the sequence, which is digested with the
enzyme Endonuclease VII that recognizes mismatches. The fragments were visualized on a polyacrylamide gel in an ABI Prism 377 DNA Sequencer. Presence of novel peaks indicated a cleaved fragment and a heterozygous mutation. The mutation was identified using direct sequencing.

3.5 Electrophysiology

The classical way of diagnosing BMD has been fundus examination and electrophysiology. The eye is a dipole with the cornea as the positive pole and the potentials can be clinically measured to obtain Electoretinogram (ERG) and Electro-oculogram (EOG).

**ERG** is a recorded response to light, by the retinal action potentials. The components of the ERG are the a-wave, originating from the photoreceptors, the b-wave, originating from the bipolar cells and slow PIII from Müller cells. The defect in BMD does not affect the ERG.

**Multi-focal ERG** is a variant of ERG, in which the neural retinal function is simultaneously measured in many small areas of the retina. It is useful for studying neural retinal diseases restricted to the macular area, but is not a standard method for diagnosing BMD.

**EOG** is the recordings of the light response by the RPE. The RPE is a bipolar layer of cells, which has a constant electric potential. Electrodes are placed on each canthus of the eye, i.e. on the skin in the two corners of an eye. The eye is then moved back and forth to each side in order to record the electric potentials. During this procedure, the eye is first dark adapted for 15 minutes to generate the Dark trough (Dt). Then standard light is turned on for 15 min to generate a Light peak (Lp). The Arden Ratio is the ratio for Lp/Dt and should be over 1.5 in normal eyes. BMD patients have an Arden Ratio below 1.5, which can be detected by EOG recording even before any macular abnormalities can be observed by fundus examination. A few individuals with a low Arden ratio never develop BMD symptoms because of variable expression of the disease.

3.6 Gene expression and protein localization

Gene expression studies are important tools for obtaining additional information about disease genes. The study of transcribed mRNA of a gene is more sensitive than analyzing the translated protein and requires less tissue samples than protein studies. The level of gene expression is usually correlated to the amount of protein in the cell.

**RT-PCR** is used for analyzing gene expression. RNA molecules are first isolated from a tissue and then used as templates for construction of cDNA copies (complementary DNA) by a reverse transcriptase enzyme, followed
by DNA amplification with PCR. The PCR product is visualized on an agarose gel.

**Northern blot** is a method for detecting specific gene transcripts in RNA from a tissue, by first electrophoresis separation of the transcripts on an agarose gel, which is blotted onto a membrane. A gene specific, radioactively labeled, DNA probe is hybridized to the membrane to bind the mRNA of interest. Detection is performed with a radioactivity sensitive film. The results show the size of the gene transcripts only in the tissues where the gene is expressed. In Paper I, Northern blot was performed on several human tissues using a VMD2 probe.

**In situ hybridization** is a method for mRNA detection in a tissue section using a labeled cDNA sequence as a probe. A mouse Vmd 2 probe was used in Paper I to sublocalize the cellular expression in a mouse retina section.

**Real-time quantitative PCR** using TaqMan technique is a method based on the same principles as RT-PCR. The main difference is that during the PCR reaction a TaqMan probe is added, which is complementary to one of the DNA strands and is fluorescently labeled with 6-FAM reporter in one end and a TAMRA quencher in the other. The probe binds to a DNA strand during the thermal cycling process and is digested by the exonuclease function of the DNA polymerase when a new DNA strand is formed. The digestion of the probe releases the FAM molecule from the quencher and a fluorescent signal can be detected in real time after each thermal cycle, e.g. by using a Model 7700 Sequence Detector by Applied Biosystems. The mouse Vmd2 expression during development was analyzed and mouse Gapdh was used as normalizing control.

**Immunohistochemistry** is a procedure for determining localization of proteins within a tissue section. It is a sensitive method that can localize a protein to a specific cell type in a tissue and in some cases even sublocalize it to a certain part of the cell. Functional aspects of the protein can be elucidated by the localization, especially if a mutated form of the protein has a different localization compared to the wildtype variant. In immunohistochemistry an antibody, binding to a specific protein, is applied to a tissue section and detected with another antibody labeled with a fluorescent dye or an enzyme that can react with a chromogen. In Paper IV a polyclonal mouse bestrophin antibody raised against a C-terminal peptide was applied to 8 µm sections of mouse eyes from different days of the postnatal development. The eyes had been fixed in paraformaldehyde and embedded in paraffin before sectioning. A biotinylated antibody detected the bestrophin antibody and avidin-enzyme complex was used to amplify a visible signal.

**Immunofluorescence** was used test the specificity of the produced antibodies to mouse bestrophin. Mouse Vmd2 cDNA was amplified with
PCR from a mouse testis cDNA library. It was cloned into two expression vectors. One produced mouse bestrophin alone and the other produced a fusion of mouse bestrophin and GFP (green fluorescent protein). The cell line RPE-J was transfected with the vectors and mouse bestrophin was detected with the antibodies.

3.7  Computer based transmembrane prediction

Publicly available computer programs designed to predict topology and subcellular localization based on the amino acid composition were used. Since this is prediction, several different computer programs should be used and the consensus of these defines the best estimation of predicted transmembrane domains. In paper 1 and 2 hydropathy profile and transmembrane prediction was performed using computer programs Tmpred (www.ch.embnet.org/software/TMPRED_form.html), PSORT (psort.ims.u-tokyo.ac.jp) and PredictProtein (cubic.bioc.columbia.edu/predictprotein). In Paper IV, the following transmembrane prediction programs were used: HMTOP (www.enzim.hu/hmmtop), TMHMM 2.0 (www.cbs.dtu.dk/services/TMHMM-2.0), SOSUI (sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), DAS (www.sbc.su.se/~miklos/DAS) and TOPPRED (bioweb.pasteur.fr/seqanal/interfaces/toppred.html).
4. Results and Discussion

4.1 Papers I and II

The gene and protein causing Best macular dystrophy were identified by positional cloning. This achievement was performed in several steps. The BMD gene had been located to chromosome 11q13 to the VMD2 locus.\textsuperscript{44,73} Later the minimum interval for the gene had been further refined to a 1 cM area using DNA from a 12 generation Swedish family,\textsuperscript{65} comprising 9 overlapping PAC clones with several genes.\textsuperscript{74} In Paper I, novel CA-repeat microsatellites were discovered and used in cross-over analysis to limit the region further to an 800 kbp region flanked by the markers BM14 and BM2. In order to identify candidate genes, direct sequencing was performed on shotgun libraries. BLAST computer analysis revealed a retina-specific human EST as well as five other ESTs in the close proximity. The ESTs were homologous to the large RFP family of \textit{Caenorhabditis elegans} proteins sharing a common conserved motif containing the amino acids arginine (R), phenylalanine (F) and proline (P), see below. The cDNA of the human gene, \textit{VMD2}, was cloned from human retina and the gene was characterized. The \textit{VMD2} cDNA is a 2.2 kbp sequence with a 1,755 bp open reading frame. RT-PCR revealed an alternative splice variant that included an in-frame Alu-repeat into exon 7, and is therefore not believed to be a functional variant.

The \textit{VMD2} gene was scanned in BMD patients for sequence variants. Mutations in the \textit{VMD2} gene that are changing amino acids in the protein bestrophin were identified. Other genes in the same region, including FTH1, were also scanned for sequence variants, but were excluded as not causative for BMD.

In Paper I, 10 different \textit{VMD2} mutations were found in 11 different families and Paper II added to the list by identifying six more mutations in six families. For mutations identified in Papers I and II, screening analysis was designed based on restriction enzyme digestion or ARMS. The initial mutation identification had been performed on DNA from one BMD case in each family. DNA samples available from other members were screened and the mutation was found in all BMD cases and excluded in the unaffected
individuals. All the mutations were excluded in 100-200 controls of Swedish and North American descent. In total we identified 15 unique missense mutations in the \textit{VMD2} gene in 19 different BMD families. Most of these mutations affect amino acids that are highly conserved in all the proteins in the \textit{C. elegans} RFP family.

\textbf{Table 1 Bestrophin mutations in the family material}

<table>
<thead>
<tr>
<th>Amino acid exchange</th>
<th>VMD2 Mutation</th>
<th>VMD2 Exon</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>T6P ACA→CCA</td>
<td>exon 2</td>
<td>Nx2, Nx6, Nx8, Nx11</td>
<td></td>
</tr>
<tr>
<td>V9A GTG→GCG</td>
<td>exon 2</td>
<td>SL3</td>
<td></td>
</tr>
<tr>
<td>A10V GCA→GTA</td>
<td>exon 2</td>
<td>Nx3</td>
<td></td>
</tr>
<tr>
<td>L82V CTG→GTG</td>
<td>exon 3</td>
<td>Dx2</td>
<td></td>
</tr>
<tr>
<td>Y85H TAC→CAC</td>
<td>exon 4</td>
<td>SL76</td>
<td></td>
</tr>
<tr>
<td>R92C CGC→TGC</td>
<td>exon 4</td>
<td>SL5</td>
<td></td>
</tr>
<tr>
<td>W93C TGG→TGC</td>
<td>exon 4</td>
<td>S1</td>
<td></td>
</tr>
<tr>
<td>Q96H CAG→CAC</td>
<td>exon 4</td>
<td>Nx10</td>
<td></td>
</tr>
<tr>
<td>D104E GAC→GAA</td>
<td>exon 4</td>
<td>SL2</td>
<td></td>
</tr>
<tr>
<td>G135S GGC→AGC</td>
<td>exon 4</td>
<td>SL4</td>
<td></td>
</tr>
<tr>
<td>R218C CGT→TGT</td>
<td>exon 6</td>
<td>Dx1</td>
<td></td>
</tr>
<tr>
<td>R218S CGT→AGT</td>
<td>exon 6</td>
<td>SU5</td>
<td></td>
</tr>
<tr>
<td>Y227N TAC→AAC</td>
<td>exon 6</td>
<td>Nx1, Nx12</td>
<td></td>
</tr>
<tr>
<td>Q293K CAG→AAG</td>
<td>exon 8</td>
<td>Nx4</td>
<td></td>
</tr>
<tr>
<td>G299E GGA→GAA</td>
<td>exon 8</td>
<td>SG1</td>
<td></td>
</tr>
<tr>
<td>D302A GAT→GCT</td>
<td>exon 8</td>
<td>Maro</td>
<td></td>
</tr>
</tbody>
</table>

\textit{Mutations identified in BMD families in Papers I and II}

In Paper I, two mutations were misinterpreted as silent, non amino acid changing polymorphisms, but in Paper II this was corrected. In Paper II, \textit{VMD2} mutations were not found in the three BMD families Maro, SL1 and SU11. After Paper II was published, additional Passport mutation screening and direct sequencing were performed and a novel \textit{VMD2} mutation was found in the Moroccan family. It was located in exon 8, altering bestrophin D302A (unpublished data). The reason for not finding pathogenic mutations in the two remaining families may be due to location of mutations in the promoter region or unsequenced parts of the introns. For a list of all mutations identified in BMD families analyzed in Papers I and II, see table 1. A more comprehensive list of all reported mutations in the \textit{VMD2} gene is summarized in the VMD2 mutation database, last updated Sep 2001. (www.uniwuerzburg.de/humangenetics/vmd2.html). Additional mutations
have also been identified by us (unpublished data) and others.\textsuperscript{75,76} For a summary of all bestrophin mutations in BMD patients, see Fig. 2. Up to date, 87 BMD causing mutations have been found in $VMD2$. Strikingly, 82 of these are missense mutations altering a single amino acid in 59 different positions. Three mutations are single amino acid deletions caused by deletions of single $VMD2$ codons. Only one frameshift mutation has been reported in an isolated case. It is located in exon 10 (1574delCA) and the frameshift starts at codon 490. A stop codon terminates the product after 24 amino acids so the product has 71 amino acids less in the C-terminal compared to normal bestrophin.\textsuperscript{77}

**Fig. 2**
Secondary structure of bestrophin by computer-based prediction with four transmembrane domains. N- and C-terminal parts are indicated. Amino acids altered to another amino acid in BMD patients (red). Amino acids altered to at least two other amino acids in different BMD families (black). A frame-shift mutation in a single BMD case (blue).
Some interesting features in the mutation spectrum can be noted:

- The absolute majority of the causative mutations alter a single amino acid.
- Almost all mutations aggregate in the N-terminal half of bestrophin.
- There is a tendency for mutations to accumulate within or close to the four predicted transmembrane domains.
- Some positions are important for bestrophin function since those have been mutated to different amino acids in different BMD families.

Bestrophin is a 585 amino acid protein with a mass of 68 kDa. Computer predictions showed that there are four transmembrane domain located in the N-terminal half of the protein. The only protein domain similar to other proteins is a conserved stretch of 300-400 amino acids including 25 highly conserved residues. This domain, with unknown function, has been called RFP, because of the amino acids amino acids arginine (R), phenylalanine (F) and proline (P), invariant among members. According to Pfam database the domain previously had the name Worm family 8, but it is changed to DUF289 (www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01062).

RT-PCR on a number of human tissues showed specific \( VMD2 \) expression only in retina and brain. Northern blot analysis showed a strong \( VMD2 \) expression with a 2.2 kB and a 4.3 kB band in retina. A weaker expression of \( VMD2 \) in brain and testis could also be detected. The 2.2 kB band corresponds well to the predicted size of \( VMD2 \) cDNA, but the nature of the larger band is unclear and currently under analysis. In situ hybridization of \( VMD2 \) mRNA in a section of mouse retina revealed specific staining of the RPE layer. The mutation analysis in combination with the expression studies verified the fact that the newly discovered \( VMD2 \), and the product bestrophin, were the main cause of BMD.

The W93C mutation has been found in an American family by Caldwell and coworkers. Since this is the same mutation found in the large Swedish BMD family and there are epidemiological indications that immigrants from the family have moved to Minnesota in the USA, there is a possibility that American family members carrying the W93C alteration are descendants of Swedish immigrants that emigrated during the great famine in Sweden in the 1900th century.
Polymorphisms
Several nucleotide exchanges have been identified in Papers I and II as well as by others.45,46,77-80 These variations are silent or noncoding substitutions or deletions that do not alter any amino acids. The majority of the polymorphisms are located in the 3’-half of the gene, which is less evolutionary conserved compared to the 5’-half. The polymorphisms are not believed to be the cause of BMD, since they have been found in several controls. They might have an effect on the expression rate of VMD2, but this has not been studied.

VMD2 mutations in AMD and other non-BMD diseases
A genetic component in AMD has been reported,32,33 and as soon as a new gene for an inherited macular degeneration has been identified it is interesting to screen that gene for alterations in AMD patients. Analysis of the VMD2 gene in AMD cases has been performed in a number of studies.45,46,79,81 A few amino acid changing mutations in bestrophin have been identified in AMD cases and were excluded in controls: R105C, E119Q, K149X, T216I, V275I and L567F.45,46,81 These mutations have not been found in BMD cases and represent the correlation between the bestrophin and AMD. For “Bulls eye” maculopathy, two different patients had an E119Q bestrophin alteration.46,81 For patients with Adult vitelliform macular dystrophy, the five bestrophin mutations, T6P, R47H, A146K, A243V and D312N have been found.46,79,81 Of these, only T6P is present in BMD cases, reported in Papers I and II. The general conclusion from these studies is that mutations in VMD2 do not play a major role in the development of AMD, but mutations in a few specific positions may result in AMD or macular degenerations with clinical similarities to BMD with a late onset.

4.2 Paper III
A family with several late-onset BMD cases was studied with electrophysiology, fundus examination and mutation detection (Fig. 3). Nine family members (9-78 years old) were included in the study. Six members presented with an EOG Arden ratio lower than 1.5, indicating BMD. Four had manifest visual problems and three out of four had a late onset with the first symptoms at the age of 40-50 years. BMD usually occurs in the first two decades of life, and BMD cases with later onset have previously only been reported occasionally.82-84
PCR amplified VMD2 exons were scanned for heterozygous mutations in one family member, III-I, using the Passport Mutation Scanning Kit. An alteration was found in exon four and direct sequencing revealed a novel VMD2 mutation resulting in the change from valine to alanine in amino acid position of 89 of bestrophin. The mutation removed a recognition site for the restriction enzyme PshAI, which was used to screen the VMD2 gene for this mutation in the other family members. It was present in five of the six individuals with an abnormal EOG. The late BMD onset may be explained either by the fact that the V89A mutation is a relative mild exchange of nonpolar amino acids. As mentioned, other bestrophin alterations can result in a late onset macular degeneration with similarities to BMD.

In a previous article, three Swedish BMD families with VMD2 mutations identified in Papers I and II were clinically characterized. The mutations in bestrophin were V9A, Y85K and D104E. The clinical phenotype showed a variable degree of visual acuity reduction and marked intrafamilial variability in macular pathology. Notable is that one patient...
with the Y85K mutation improved the visual acuity from 20/100 at age 7 to 20/20 at age 24.

A variable expression of BMD has been reported by Caldwell and coworkers. Two children with BMD from different families had the same bestrophin mutation, E300D. The parents contributing with the mutation were unaffected by the disease.

To evaluate the function of different missense mutations in bestrophin, an in vivo assay should be developed. Possibly different mutated constructs could be injected to the RPE layer of laboratory animals and the changes in transepithelial potential measured. This would be a good way of correlating different mutations to bestrophin function and BMD prognosis. Changes in other genes affecting the function of bestrophin may affect the onset and severity of BMD, but this has not been studied.

A young boy, IV-2 (Fig. 3), that presented with an EOG Arden ratio of 1.3 did not have the Val89Ala mutation. After re-examination his Arden ratios were 1.3-1.35. To exclude a potential mistake of sample mix-up, a second blood sample was collected for DNA extraction, and the restriction based method and direct sequencing confirmed that the boy did not have the mutation present in other family members. A conclusion is that caution should be taken when only using EOG Arden ratios for diagnosis. We suggest that a combination of electrophysiology, fundus examination and genetic analysis of the VMD2 gene should be performed to define disease status.

4.3 Paper IV

By using animal models for human diseases, new knowledge about the disease pathology can be gained. The mouse is the most commonly used animal model for human diseases because of the short pregnancy (about 20 days), the efficient protocols of generating genetically modified mice, the moderate cost and the relatively high resemblance to humans. One drawback by using mice as models for macular degeneration, is that rodents do not have an anatomical macula. Only primates have a macula with a high concentration of cone photoreceptors, but it is more costly, very time consuming and difficult to ethically motivate the use of primates as animal models for macular degenerations. BMD is an inherited disease with a mainly juvenile onset. In order to facilitate the generation of a future transgenic BMD mouse, the normal mouse eye phenotype during development must be known.

The mouse Vmd2 is located on mouse chromosome 19, which is the syntenic region of human 11q13 where VMD2 is located. An alignment of mouse and human bestrophin sequences shows that the N-terminal half,
where the BMD causing mutations have been found, is highly conserved between the two species. The C-terminal half is less conserved, which has been noted in other species as well (Papers I and II).

The sequence of the mouse Vmd2 cDNA was retrieved from the Celera database and a cDNA was successfully amplified with 5'- and 3'-end primers of the cDNA, yielding a 1653 bp fragment. This was cloned into two different expression vectors, one of them containing an in frame cDNA for GFP (green fluorescent protein). Polyclonal mouse bestrophin antibodies, called Pab-003, were generated by immunizing rabbits with a C-terminal peptide. The expression vectors were successfully used to express mouse bestrophin in a rat pigment epithelial cell line, RPE-J. Pab-003 was used for detection in combination with a fluorescently labeled secondary antibody. The construct that produced mouse bestrophin fused to GFP resulted in an expression pattern with overlapping GFP and mouse bestrophin. Because the transfected cell line was not confluent, the expression pattern did not exhibit the basolateral localization that was reported previously.86 Mouse bestrophin has 551 amino acids and the molecular mass is predicted to be 64 kDa. Western blot analysis using Pab-003 was performed to confirm predicted protein size and that the RPE-J cell line after transfection really produced mouse bestrophin. When using a GFP antibody, GFP was detected as a component of a GFP-mouse bestrophin complex. When Pab-003 was inhibited with the same peptide used to raise the antibody, no band was visualized on the Western blot, as predicted.

The first expression experiment of mouse Vmd2 was performed on mRNA isolated from whole eyes from different postnatal development stages. The results indicated a relatively high Vmd2 expression in early development with a decreasing level through out development with a low level in adult mice eyes. To verify these results and make a more detailed analysis, we performed real-time quantitative PCR using TaqMan analysis. RNA extraction from two embryonal stages was then included. The quantitative PCR confirmed the RT-PCR results. The highest Vmd2 expression was detected around the late embryonal period and early postnatal period. This can be developmentally correlated with the maturation of photoreceptors. The maturation peaks for cones and rods are at embryonal days 15 and 18, respectively.87

Immunohistochemistry was performed using Pab-003 on 8 µm sections of mouse eyes from different postnatal days. The findings were very interesting. Mouse bestrophin was detected in RPE cells with a basolateral pattern as previously reported.86 The first time staining in RPE could be detected was postnatal day 10. Electroretinogram recordings from dark-adapted mice from different stages during development showed no response until postnatal day 10, when a-wave, b-wave and slow PIII could be detected.
for the first time. These events coincides with the opening of the eyelids, which is around postnatal day 10 and is an important signal for several processes. According to studies in rat, which have approximately the same gestation period as mice, it has been reported that in normal development in situ, RPE phagocytic activity was shown to begin on the tenth day of age.\textsuperscript{88-90} This is also the signal for the adaptation of phagocytosis to the circadian rhythm, in which shed photoreceptor outer segments are digested every morning just after the opening of the eyes.\textsuperscript{91}

Surprisingly, bestrophin was also detected in photoreceptor inner segments as early as the first postnatal day. The inner segment localization persists during developments, but fades gradually and is present, but faint, during adult phase. All the detected mouse bestrophin staining is specific, because when Pab-003 was inhibited with the antibody-specific peptide, the staining was no longer present. This is a novel finding and inner segment localization was not seen in adult human eyes using a monoclonal human antibody.\textsuperscript{86} The expression in neural retina and brain (Paper I) implies that bestrophin plays a role in neural tissue, which needs to be studied further.

It will be very interesting to see if the findings in normal mice are persistent or altered in transgenic mouse models expressing mutated bestrophin.
5. Conclusions and future perspectives

The work presented in this thesis resulted in the identification of the gene causing Best macular dystrophy (BMD). The \( VMD2 \) gene encodes a protein called bestrophin.

**Conclusions from the work done performed in this thesis:**

- The majority of BMD causing mutations in \( VMD2 \) are missense mutations, altering a single amino acid.
- The majority of the mutations are accumulated in the N-terminal half of bestrophin, within or close to the four predicted transmembrane domains.
- The \( VMD2 \) gene is expressed in the retinal pigment epithelium, brain, spinal chord and testis.
- A BMD family with mainly late onset had a novel bestrophin mutation and a conclusion is that BMD diagnosis should be made as a combination of fundus examination, EOG and genetic analysis.
- Bestrophin in mouse eyes showed expression in photoreceptor outer during the postnatal period. This indicates a neuronal function for bestrophin.
- Bestrophin expression in mouse RPE had an onset at postnatal day 10, the same day as ERG could be recorded for the first time. This indicates that bestrophin expression in RPE is closely correlated to photoreceptor development.

The most important aim for the future of BMD research is to elucidate the function of bestrophin, the protein mutated in BMD cases. So far bestrophin has been localized to the basolateral outer membrane of the RPE\(^{36} \) and has been shown to physically interact with and be dephosphorylated by PP2A.\(^{92} \)

The electrophysiological hallmark in BMD pathology is a diminished light peak in EOG, resulting in an abnormal Arden ratio, below 1.5. This can be observed even before any BMD symptoms occur. The proposed theory for the pathway initiating the light peak is the following: A secreted paracrine or endocrine “light-peak substance” from the neurosensory retina
is stimulating the apical RPE membrane. An unknown pathway mediates a signal via the RPE to the basolateral membrane. An unknown secondary messenger, alternatively intracellular changes in \( \text{Ca}^{2+} \) or pH levels initiates the light peak, which is the result of an increase in basolateral RPE membrane Cl\(^{-} \) conductance.\(^{93} \) Bestrophin must be affecting the initiation of the light peak in some way, since mutated bestrophin diminishes the light peak. Recently, evidence has been provided to show that bestrophin is a \( \text{Ca}^{2+} \)-sensitive chloride channel.\(^{94} \) If this is correct, then bestrophin is directly responsible for activation of the light peak.

**How can mutated bestrophin be studied in future research?**

An mouse model for BMD with mutated bestrophin could contribute with increased knowledge of disease pathology. Relevant methods are needed for evaluating the symptoms. DC-ERG (direct current-electro retinogram) is method that is used for studying the transepithelial function in animals and corresponds well to the diagnostic ability of EOG for BMD in humans. DC-ERG has in the past only been used on relatively large animals such as cats and rabbits,\(^{95,96} \) but now a method has been developed for DC-ERG on rats,\(^{97} \) which can also be applied on mice.\(^{98} \)

The ultimate aim for the research on VMD2 and bestrophin is the development of new therapies primarily for BMD, which can hopefully also be applied to AMD. Using a combination of clinical fundus examination, EOG and molecular diagnosis, BMD can be diagnosed at an early age and potential therapy can reverse or halt the progression of decreased visual acuity. This can preserve the vision in a great number of people, whom otherwise would suffer from loss of vision.
6. Acknowledgements

The work in this thesis has mainly been performed at the department of Genetics and Pathology, Rudbeck laboratory, Uppsala University, Sweden and in part at the Cole Eye Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA.

I would like to express my sincere gratitude to the following:

Professor Claes Wadelius, my supervisor, for providing a good scientific environment and for believing in my abilities from the very beginning. Also for giving opportunities to perform important research, both in Sweden and the USA.

Dr. Alan Marmorstein, for welcoming me to his lab and also a stimulating collaboration.

Lore Zech, Karl-Henrik Gustavson and Göran Annerén for encouragement and showing interest in my work.

Ulf Petterson, director of the Rudbeck laboratory, who has provided an excellent atmosphere and has supported the issues of the PhD students in the department board.

All the present and former members of the research group of ophthalmic genetics: Caroline Graff (who has done excellent work on BMD), Towa Marknell, Sofie Ingvast, Petra Sandell, Mattias “Foppa” Jansson (according to Canadians in Florida), Fredrik Aspgren (who knows how to win a costume contest), Ola Wallerman, Álvaro Rada and Mehdi Motallebiipour. Also all the undergraduate students that participated during the past couple of years.

Present and past members of Marmorstein lab: George Hoppe, Lihua Marmorstein, Bret Stanton and John C.

The group of clinical molecular genetics, including professor Niklas Dahl, Peter Gustavsson, Maritta Pigg, Dina Tentler, Hans Mattson, Joakim Klar, Larry Mansouri, Miriam Entesarian, Birgit Carlsson, Ed Dawey and Lena Marklund.

Gerth Hedow, for interesting discussions.

I would also like to thank Mats Gullberg, Lina Emilsson and Anja Castensson for help with TaqMan. Bo Johannesson for showing how stressful it can be to write a thesis.

Jeanette Backman, Elisabeth Sandberg, Margaretha Uvhagen Antoni, Ulla Hedvall and Mia Hedefalk for administrative help. William Schannong and Viktor Persson for all help with computers.

The people at Clinical Genetics.

All the nice people at dep. of genetics and pathology, who makes this a fun place to work. Some of them are: Veronica Magnusson, Anna Beskow, Patrik Magnusson, Martin Moberg, Anna Maria Divine, Hanna Andreasson, Malin Engelmark, Lina Dimberg, Per-Ivan Wyömi, Inger Jonasson, Anh-Nhi Tran, Britt-Marie Carlberg, Johan Banér, Simon Fredriksson,
Ann-Sofi Strand, Jonas Jarvius, Jenny Jonsson and Kicki Lagerstedt (it was really fun in Italy, except for the weather).

The patients with Best macular dystrophy and all collaborators who made this work possible.

Dr. Anders Wall for believing in and encouraging young, talented and ambitious people.

My family, including my parents, Aleksander and Julia Bakall, my sister Susanna and my brother David with family, Liz and little Samuel.

The most important people in my life: Maja, for love, support and making me realize that there are other important things in life than work.
My children Jakob and Emma!

This thesis was supported by grants and stipends from the following:

- Anders Walls foundation
- Synfrämjandet
- Svenska RP-föreningen
- The Swedish Research Council
- Swedish medical society (SVLS)
- Swedish society for medical research (SSMF)
- Merck
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