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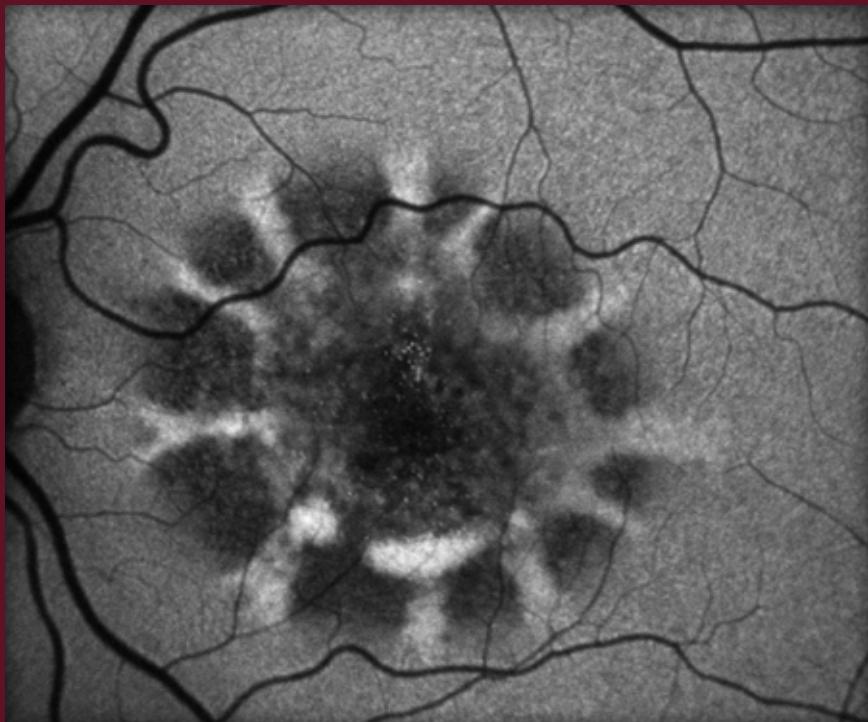
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Hereditary retinal disease

Clinical and genetic studies on the role of the
peripherin/RDS gene, the *BEST1* gene, and the *CFH* gene



Camiel J.F. Boon

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Cover image: Fundus autofluorescence image of a peculiar macular dystrophy, showing an odd radial, stellate pattern of autofluorescence changes. The small pseudohypopyon in the inferior part of the lesion gives a hint of the correct diagnosis, Best vitelliiform macular dystrophy. After many years of uncertainty about the diagnosis, molecular genetic analysis identified a mutation in the *BEST1* gene, establishing the definitive diagnosis of Best vitelliiform macular dystrophy.

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Clinical and genetic studies on the role of the
peripherin/RDS gene, the *BEST1* gene, and the *CFH* gene

Een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

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volgens besluit van het College van Decanen
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*To my parents
To Karlijn*

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List of abbreviations

A2E	N-retinylidene-N-retinylethanolamine
ABCA4	ATP-binding cassette transporter A4 (gene)
ABCR	ATP-binding cassette transporter
AD	autosomal dominant
ADVIRC	autosomal dominant vitreoretinochoroidopathy
AFVD	adult-onset foveomacular vitelliform dystrophy
aHUS	atypical hemolytic uremic syndrome
AMD	age-related macular degeneration
AP	alternative pathway
APOE	apolipoprotein E (gene)
ARMS2	age-related maculopathy susceptibility 2 (gene)
ARB	autosomal recessive bestrophinopathy
atRAL	all-trans-retinal
BEST1	bestrophin-1 (gene)
BLD	basal laminar drusen
BVMD	Best vitelliform macular dystrophy
CACD	central areolar choroidal dystrophy
Cav	voltage-dependent calcium
CCP	complement control protein
CERES	composite exonic regulatory element of splicing
CFB	complement factor B
CFH	complement factor H
CFI	complement factor I
CFHR	complement factor H-related
CNV	choroidal neovascularization
CR1	complement receptor 1
CRP	C-reactive protein
cSLO	confocal scanning laser ophthalmoscope
DAF	decay accelerating factor
DGGE	denaturing gradient gel electrophoresis
DHPLC	denaturing high-performance liquid chromatography
DNA	deoxyribonucleic acid
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1 (gene)
ELOVL4	elongation of very long chain fatty acids-like 4 (gene)
EOG	electro-oculogram
ERG	electroretinogram
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FAF	fundus autofluorescence
FFA	fluorescein angiography

List of abbreviations

FHL-1	factor H-like protein 1
GAG	glycosaminoglycan
GBM	glomerular basal membrane
HTRA1	HtrA serine peptidase 1 (gene)
HLA	human leukocyte antigen
HRA	Heidelberg retina angiograph
HUS	hemolytic uremic syndrome
ISCEV	International Society for Clinical Electrophysiology of Vision
LOD	logarithm of the odds
MCDR1	macular dystrophy, retinal, 1
MCP	membrane co-factor protein
MIDD	maternally inherited diabetes and deafness
MPD	multifocal pattern dystrophy simulating Stargardt disease (STGD1)/fundus flavimaculatus
MPGN	membranoproliferative glomerulonephritis
MRCS	microcornea, rod-cone dystrophy, early-onset cataract, posterior staphyloma
NIR-FAF	near-infrared fundus autofluorescence
OCT	optical coherence tomography
ORL	outer red line
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PLEKHA1	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 1 (gene)
PROM1	prominin 1 (gene)
RDS	retinal degeneration slow
RNA	ribonucleic acid
RP	retinitis pigmentosa
RPA	retinitis punctata albescens
RPE	retinal pigment epithelium
ROM1	retinal outer segment membrane protein 1 (gene)
SCR	short consensus repeat
SERPING1	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (gene)
SNP	single nucleotide polymorphism
SSCA	single-strand conformation analysis
SSCP	single-strand conformation polymorphism
STGD1	autosomal recessive Stargardt disease
TIMP-3	tissue inhibitor of metalloproteinase (gene)
TLR3	toll-like receptor 3 (gene)
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
VMD2	vitelliform macular dystrophy 2 (gene)

Figures marked with an asterisk are printed in color at the end of this thesis, in the section “Color figures”.

Preface

The patients who are described in this thesis often experience the difficult consequences of their eye disease every day. These consequences may not only include examples such as the inability to play bridge or to sew, but also the inability to recognize people on the street and to perform professional activities. Macular degeneration is not a disease that people can easily recognize from the outside. As a result, a patient suffering from macular degeneration may be judged “arrogant” behind his or her back, if he or she is unable to recognize and thus greet an acquaintance who is ignorant of the nature of the disease. More public awareness of this group of blinding diseases is therefore desired.

The patients described in my thesis were often prepared to travel to Nijmegen, sometimes from the other side of the country, to undergo the extensive examinations that have finally resulted in this thesis. Therefore, I dedicate my thesis to these patients, and I hope that our findings may contribute to a better patient information and prognosis with regard to these severe eye disorders.

Voorwoord

De patiënten die in dit proefschrift worden beschreven ervaren vaak elke dag de vervelende gevolgen van hun oogziekte. Hierbij gaat het niet alleen om zaken als niet meer kunnen bridgen en kantklossen, maar ook om het niet meer herkennen van mensen op straat en het onmogelijk worden van beroepsmatige werkzaamheden. Maculadegeneratie is geen ziekte die je aan de buitenkant aan iemand kunt zien. Dit heeft heel concreet als gevolg dat iemand met maculadegeneratie soms achter zijn of haar rug om als “arrogant” wordt versleten, als hij of zij een bekende soms niet herkent en dus niet groet. Meer bekendheid voor deze groep van oogziekten is dus hard nodig.

De in mijn proefschrift beschreven patiënten waren vaak bereid om, soms vanuit de andere kant van het land, af te reizen naar Nijmegen, en een hele ochtend of middag onderzoeken te ondergaan die uiteindelijk hebben geleid tot dit proefschrift. Mijn proefschrift draag ik dan ook mede aan hen op, in de hoop dat onze bevindingen bij kunnen dragen aan een betere informatievoorziening en prognose ten aanzien van deze ernstige oogaandoeningen.

Chapter 1

General introduction

1.1. Historical background

The anatomical and functional complex of photoreceptors, retinal pigment epithelium, Bruch's membrane, and choriocapillaris, that is essential for optimal retinal function, was first described by the renowned Dutch anatomist and botanist Frederik Ruysch (1638-1731), at the end of the 17th century.^{1,2} Half a century later, in 1744, Rüdiger F. Ovelgün reported the first case of familial night blindness.³ This case is generally considered the first documented case of probable familial retinitis pigmentosa.

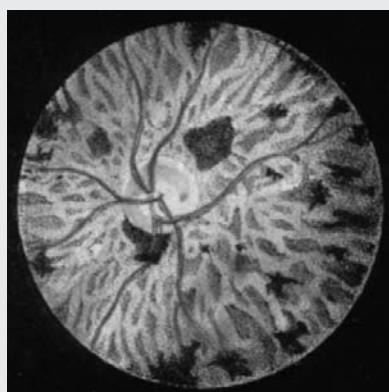


Figure 1.1.*

Original fundus image of retinitis pigmentosa, published by van Trigt in 1853.

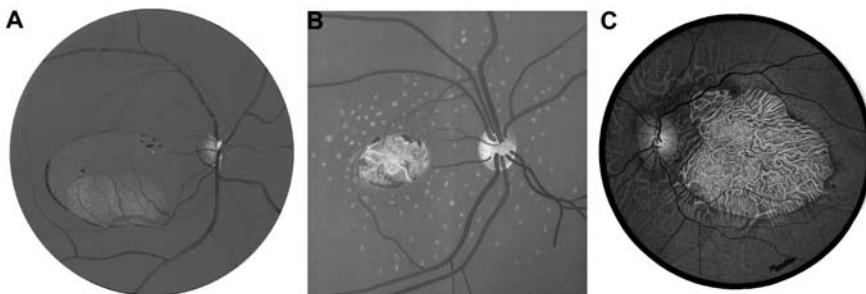
vitreous, as well as retinal and choroidal abnormalities, does not need to be explained at great length. The reported facts speak for themselves.” Drusen -the hallmark lesions of age-related macular degeneration- were first described by Franciscus C. Donders in 1854 (Fig. 1.2).⁶ The term drusen is derived from the German word “Druse”, which refers to a geode, a rock with a cavity that is internally lined by crystals. Age-related macular degeneration was reported for the first time in medical literature in 1874 by Jonathan Hutchinson (1828-1913),

A true revolution in ophthalmology started with the invention of the ophthalmoscope in 1851 by Hermann (von) Helmholtz (1821-1894), which allowed examination of the retina and the optic disc.⁴ Soon thereafter, in 1853, Adriaan C. van Trigt (1825-1864) was the first to publish an atlas with colored pictures of the normal fundus and several pathological retinal conditions, including retinitis pigmentosa (Fig. 1.1; figures in this thesis marked with an asterisk are printed in color at the end of this thesis, in the section “Color figures”).⁵ Van Trigt concludes: “The significance of the ophthalmoscope for the diagnosis of lens opacification, disorders of the



Figure 1.2.

Franciscus Cornelis Donders (1818-1889).

**Figure 1.3.***

Original figures published by Best, Stargardt, and Sorsby. A. Best vitelliform macular dystrophy, drawing published by Friedrich Best in 1905. B. Stargardt disease, drawing published by Karl Stargardt in 1909. C. Central areolar choroidal dystrophy, drawing published by Arnold Sorsby in 1939.

with the description “symmetrical central chorido-retinal disease occurring in senile persons”.⁷ In 1905, the German ophthalmologist Friedrich Best (1871-1915) reported typical macular lesions in eight affected members of a large family, a condition which we now know as Best vitelliform macular dystrophy (Fig. 1.3A).⁸ Stargardt disease (*fundus flavimaculatus*) was named after Karl B. Stargardt (1875-1927), who described the clinical features of this retinal dystrophy in 1909, in seven patients from two families (Fig. 1.3B).⁹ Clear-cut cases of central areolar choroidal dystrophy in multiple generations were first described in 1931 by Hendrik K. de Haas (1873-1953),¹⁰ followed by a more elaborate discussion by Arnold Sorsby (1900-1980) in 1939 (Fig. 1.3C).¹¹ Sorsby observed that the age at onset of a macular dystrophy could vary considerably within a family, and also remarked that so-called “macular” dystrophies were often more widespread than initially suggested by the ophthalmoscopic picture.¹² In 1949, Jules François (1907-1984) was the first to describe a family with autosomal dominant inheritance of a widely variable phenotype, ranging from Stargardt disease to retinitis pigmentosa.¹³

The remarkable clinical variations of macular dystrophies were already noted by Stargardt in 1917. He proposed to separate these clinical entities into different groups, based on the clinical presentation and ophthalmoscopic appearance.¹⁴ Nevertheless, the “monistic” view that all macular dystrophies represent an expression of a single clinical entity, advocated by for instance Leber,¹⁵ Sorsby,¹² and François,¹⁶ dominated until the late 1960s.¹⁷ In 1967, August F. Deutman proposed a classification that differentiated the hereditary dystrophies of the posterior pole of the eye according to their primary localization within the retinal and/or choroidal layers, as evidenced by clinical, functional, and histopathological studies.¹⁸ In addition, he underscored the likelihood that these different clinical entities were caused by different genes.

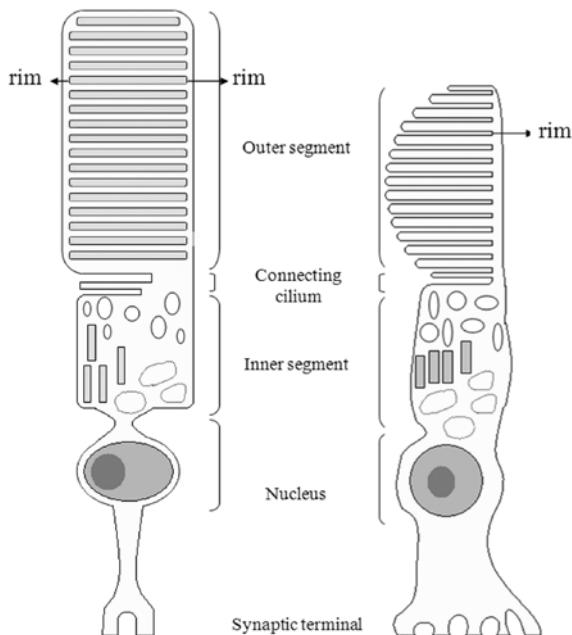
Sorsby was among the first to thoroughly explore and describe the role of genetics and heredity in ophthalmology, which resulted in his handbook “Genetics in Ophthalmology” (1951).¹⁹ The era of molecular genetics in ophthalmology was announced in 1984 by Bhattacharya and colleagues, when they mapped a gene involved in X-linked retinitis pigmentosa to a specific area on the X chromosome.²⁰ The first autosomal dominant retinitis pigmentosa gene was subsequently identified by Dryja and co-workers in 1990,²¹ whereas Cremers and colleagues identified the X-linked choroideremia gene in the same year.²² One year later, the *peripherin/RDS* gene was identified as a cause of autosomal dominant retinitis pigmentosa.^{23,24} Soon thereafter, its involvement in various macular dystrophies would come to light,²⁵ including its role as a cause of central areolar choroidal dystrophy, which was shown by Hoyng and colleagues (1996).²⁶ The genetic background of Best vitelliform macular dystrophy was elucidated in 1998, with the identification of the *BEST1* gene (formerly known as *VMD2*) by Petrukhin and co-workers.²⁷ The CFH protein was already identified in 1965 by Nilsson and Müller-Eberhard,²⁸ followed by the identification of the gene cluster harboring the *CFH* gene in 1985, by Rodríguez de Córdoba.²⁹ However, it would take until the year 2000 before its complex genomic structure would be unraveled by Male and co-workers.³⁰

1.2. The retina and choroid

Light is converted to a neuronal signal in the retina, which is a layer of highly specialized neural tissue covering the posterior inner eye. The retina is as thin as 0.4 mm, and finds its embryologic origin in the developing forebrain. Therefore, it may be considered a part of the brain. The retina can be divided into the neurosensory retina and the retinal pigment epithelium (RPE), which are mutually dependent during their development, differentiation and normal function.³¹ Both arise from the neuroectoderm-derived optic vesicle, which forms the bilayered optic cup. The inner layer evolves towards the neuroretina, whereas the outer layer forms the RPE. The RPE is physically separated from the underlying capillary vascular tissue, the choriocapillaris, by Bruch’s membrane.

1.2.1. Neurosensory retina

Vision starts with the capture of photons by the light-sensitive cells in the retina: the photoreceptors. The two main types of photoreceptors, cones and rods, convert light into an electrical neuronal signal by means of the phototransduction cascade (Fig. 1.4).^{32,33} This process takes place in the photoreceptor outer segment, that consists of up to 1000 flat membranous structures adjacent to the RPE. These photoreceptor outer segments are surrounded and eventually phagocytosed by apical extensions of the retinal pigment epithelium (Fig. 1.7). This specific arrangement of photoreceptor outer segments greatly enlarge the area and thus the efficacy of phototransduction. In rod photoreceptors, the process of phototransduction is initiated by the absorption of a photon in the

**Figure 1.4.**

Schematic representation of a rod (left) and cone (right) photoreceptor. The photoreceptor outer segments, where photo-transduction takes place, are composed of flattened structures, the discs (in rods) and lamellae (in cones). The sharp edges of these discs and lamellae are called the rim area. Contrary to cones, the disc membranes in rods are not continuous with the plasma membrane.

photoreceptor outer segments by the light-sensitive rhodopsin, which is composed of the protein opsin and the chromophore 11-cis-retinal. Upon capture of a photon, this 11-cis-retinal, which is a derivative of vitamin A, is transformed to its isomer all-trans-retinal. Through a number of intermediate steps, this ultimately leads to membrane hyperpolarization and the release of neurotransmitters at the photoreceptor terminal synapse. Regeneration of rod 11-cis-retinal takes place in the RPE, whereas the regeneration of 11-cis-retinal in cones appears to depend on both the intraretinal Müller cells and the RPE.^{34,35}

New photoreceptor outer segments evaginate from the plasma membrane around the connecting cilium, while the older outer segments at the tip are shed and processed by the RPE.^{36,37} The peripherin/RDS protein, in association with the ROM1 protein, is essential for the normal formation of the rim area of flattened photoreceptor outer segments (Fig. 1.4),³⁸ as will be discussed further in this thesis.

The cone photoreceptors function in bright light and are essential for color vision and high spatial resolution. There are three different types of cones: the S cones, which are most sensitive to short wavelength (blue) light, the M cones, which process medium wavelength (green) light, and the L cones, which are particularly sensitive to light of long wavelength (red).^{39,40} The rod photoreceptors, on the other hand, enable vision in dim light, and are particularly sensitive to contrast, brightness, as well as motion.

Cones and rods are not equally distributed throughout the retina. The peripheral retina is dominated by rods, whereas the fovea contains only cones (Fig. 1.5).⁴¹

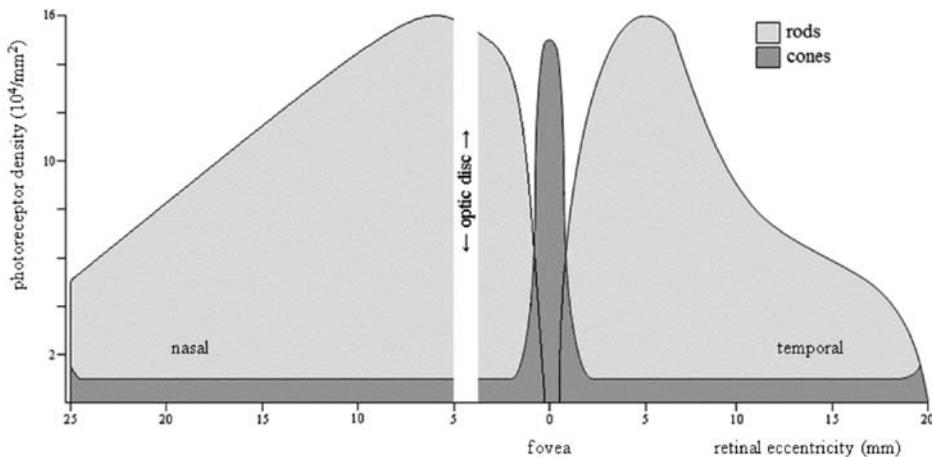


Figure 1.5.

The spatial density of cone and rod photoreceptors in the retina. The human eye contains 4–6 million cones, which are densely packed in and around the fovea. The eye contains 78–110 million rods, which are dispersed throughout the retina, except for the fovea.

Just like M and L cones, the S cones are distributed throughout the retina, except in the central fovea, where S cones are virtually absent.⁴² The fovea only contains randomly scattered M and L cones, whereas the parafovea mainly contains rods.^{41,43} Cone density is greater in the nasal than in the temporal peripheral retina.⁴⁴ The rods in the parafoveal area appear to be most vulnerable to changes related to ageing and age-related maculopathy.⁴³ Maximum foveal cone density as well as the ratio of L to M cones can show striking differences between individuals with normal color vision.^{39,44,45}

After complex processing mechanisms of the resultant photoreceptor signals by cells within the multilayered retina itself (Fig. 1.6),^{46–48} these signals are transmitted to the brain through the optic nerve. In the brain, the modulated visual information from the retina is integrated through numerous visual pathways, resulting in visual images and interpretation.^{40,49}

Apart from this complex process of image forming and processing, originating in the rod and cone photoreceptors, a subset of retinal ganglion cells is also photosensitive, expressing an opsin-like protein called melanopsin.⁵⁰ These ganglion cells mediate non-image forming visual responses. As such, they play a key role in the rapid adjustment of pupil size, in light modulation of activity, and in the adaptation and fine tuning of the circadian clock to environmental light, the latter possibly through the regulation of pineal melatonin synthesis.^{51–54} These non-image forming, adaptive ocular photoresponses are thus important in circadian rhythm entrainment.

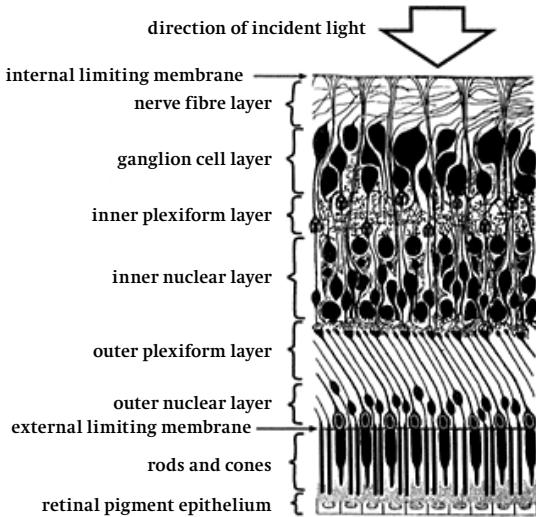


Figure 1.6.
*Schematic representation
of the retinal layers.*

1.2.2. Retinal pigment epithelium

The photoreceptors and the monolayer of RPE cells are intimately related, as numerous RPE microvilli surround the distal photoreceptor outer segments (Fig. 1.7). These apical microvilli greatly increase the surface of contact, and therefore optimize metabolic efficiency. The neuroretina is to a certain extent attached to the RPE through the so-called interphotoreceptor matrix that envelopes the cone photoreceptors.^{55,56} The proximity of photoreceptors and RPE enables two of the pivotal functions of the RPE: the regeneration of visual pigment and phagocytosis of rod and cone outer segments. Every day, the RPE phagocytoses approximately 10% of the photoreceptor outer segments. This distal 10% of the photoreceptor outer segments contains the highest concentration of by-products of the visual cycle, such as toxic radicals, photo-damaged proteins and lipids, that need to be digested by the RPE. The shed outer segments fuse with lysosomes, forming phagolysosomes that effectuate this degradation.

The pigmentation of RPE cells originates from melanin, which is mainly packed in melanosomes in the apical part of the RPE cell. The RPE melanin has a photoprotective function, through the absorption of scattered light, and also has an anti-oxidant action.⁵⁷ The concentration of RPE melanosomes is the highest in the central macula.⁵⁸ With advancing age, melanin itself may become oxidized and the number of melanosomes decreases.^{59,60} Meanwhile, an increase in the number of complex granules containing melanin associations is observed, such as melanolysosomes and the toxic melanolipofuscin.⁶¹⁻⁶³

In addition to these functions, the RPE is essential for many other processes.^{31,59} For instance, the RPE forms and maintains two extracellular matrixes, the interphotoreceptor matrix and Bruch's membrane. Transport of metabolites and ions between the

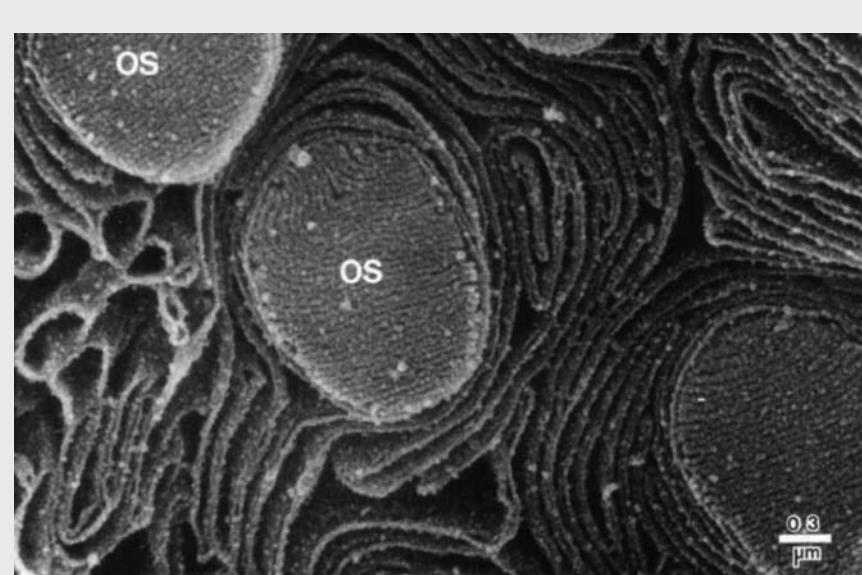


Figure 1.7.

High resolution scanning electron microscopic photograph of rod photoreceptor outer segments (OS), surrounded by membranous extensions of the apical surface of the retinal pigment epithelium (RPE). The close contact between the photoreceptor OS and the RPE enables a highly efficient visual pigment regeneration and phagocytosis of the OS. (Source: Hollenberg et al., Invest Ophthalmol Vis Sci 1988;29:1380-1390).

photoreceptors and the choriocapillaris is mediated by the RPE, which forms a selective blood-retinal barrier. Thus, a vectorial transport of nutrients from the choroid to the outer retina and the removal of waste products, water and ions in the other direction is tightly regulated. In normal conditions, the RPE efficiently transports water away from the subretinal space between the neuroretina and the RPE. In addition, the RPE appears to play a pivotal role in the immunological microenvironment of the retina.⁶⁴⁻⁶⁸ Finally, the RPE also secretes a variety of growth- and structure-regulating factors, such as vascular endothelial growth factor (VEGF) and tissue inhibitor of metalloproteinases-3 (TIMP-3).⁶⁹⁻⁷²

In the healthy eye, these factors are beneficial to the structure and function of the eye.⁷³ RPE-derived VEGF is presumed to play an important role in ocular development, especially with regard to the development and maintenance of the choriocapillaris.^{70,74} In pathological conditions, however, RPE-derived factors such as VEGF and TIMP-3 may have a detrimental influence. For instance, overexpression of VEGFs is known to promote the formation of choroidal neovascularization in age-related macular degeneration (AMD) and other eye diseases.^{75,76} Accumulation of TIMP-3 underlies Sorsby fundus dystrophy,

but is also seen in AMD.^{77,78}

Just like the photoreceptors, the RPE characteristics differ between the central and peripheral retina.^{59,79} For instance, RPE cell density decreases from the fovea to the peripheral retina,^{31,59,80} and the number of photoreceptors per RPE cell is higher in the macula.^{81,82} Macular RPE cells are taller and have a smaller diameter than peripheral RPE cells, and the macular RPE contains a larger amount of melanin granules.⁵⁹

The aforementioned functional interaction between the RPE and photoreceptors poses an enormous metabolic demand on both cell populations, which are among the highest energy consumers in the body. Although light and oxygen are essential for vision, these conditions may also promote the formation of reactive oxygen species, leading to photochemical damage to the retina.⁸³⁻⁸⁵ The number of RPE cells decreases with age, which increases the phagocytic burden on the remaining cells.⁸⁶ As a result, an age-related accumulation of lipofuscin occurs in the RPE.^{61,87,88}

Lipofuscin is a heterogeneous material composed of a mixture of lipids, proteins and different fluorescent compounds. More than 90% of the accumulated lipofuscin in the RPE originates from conjugates formed by visual cycle retinoids in photoreceptor outer segments that are incompletely digested by the phagolysosomes.⁸⁹⁻⁹¹

The exact composition of lipofuscin is still largely unknown, but an important constituent is the toxic fluorophore N-retinylidene-N-retinylethanolamine (A2E).^{92,93} The formation of A2E begins in the photoreceptor outer segments: rather than being converted back to 11-cis-retinal, two molecules of all-trans-retinal react with phosphatidylethanolamine (PE) to form A2-PE.^{92,94} A2E is subsequently formed in the RPE phagolysosomes after phosphate hydrolysis of A2-PE, where it impairs proper phagolysosomal degradation by the RPE.^{95,96}

As A2E formation depends on the availability of its precursor, all-trans-retinal, conditions that increase the amount of all-trans-retinal also increase lipofuscin formation. In turn, the availability of all-trans-retinal, a derivative of vitamin A, depends on dietary vitamin A intake.^{92,97,98} The turnover of all-trans-retinal and subsequent formation of A2E depends on the activity of the visual cycle, which is directly dependent on the amount of light exposure.^{92,92,95,99-101} Therefore, the amount of A2E formation correlates with the amount of vitamin A intake and light exposure.^{92,101,102} Once A2E is formed, the RPE appears unable to effectively get rid of this toxic waste product.¹⁰¹

Light is not only essential for the formation of A2E, but it also plays an important role in the adverse effects of A2E on the RPE cells. Light with a wavelength in the blue spectrum is especially able to excite A2E, forming highly reactive, toxic A2E-epoxides.^{103,104} In addition, lipofuscin is a photoinducible generator of free radicals, such as the superoxide anion, singlet oxygen and hydrogen peroxide.¹⁰⁵ These toxic byproducts damage DNA in the RPE cells,^{106,107} and may lead to apoptosis of RPE cells that is proportional to the amount of A2E.¹⁰⁸⁻¹¹⁰ Other unfavorable consequences of A2E accumulation include membrane destabilization and lysosomal dysfunction.^{111,112}

Interestingly, photooxidation products of A2E are also able to activate the complement system, which plays a pivotal role in the pathogenesis of AMD.^{113,114} Blue light-induced

excitation of A2E initiates photooxidative processes in the RPE that may lead to upregulation of VEGF.¹¹⁵ The degradation of photoreceptor phospholipids by the RPE is also inhibited by A2E.⁹⁶ The highest concentration of lipofuscin is found in the RPE from the parafoveal zone, where rod density is highest and where atrophic AMD often starts.^{88,116}

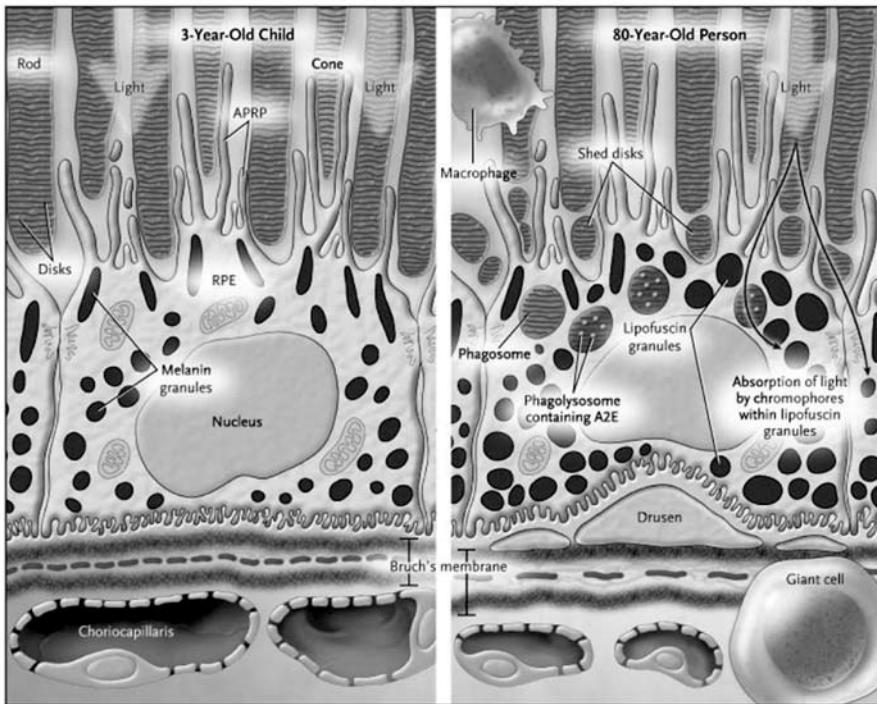
Lipofuscin and especially A2E are autofluorescent when excited with light from a specific wavelength and lie at the basis of fundus autofluorescence in normal and pathological conditions, which will be discussed extensively in this thesis (Chapter 2).

Pathologic accumulation of lipofuscin and A2E occurs in a number of retinal disorders. Examples include various retinal dystrophies such as Stargardt disease (STGD1), Best vitelliform macular dystrophy and retinal dystrophies caused by mutations in the *peripherin/RDS* gene, which is illustrated in Chapter 2.⁹³ In these cases, the disease-related lipofuscin is formed at an accelerated rate and may differ from "normal" age-related lipofuscin in the relative levels of individual fluorophores such as A2E.^{93,117} In addition to hereditary retinal dystrophies, abnormal amounts and patterns of lipofuscin accumulation have also been found in AMD, among many other retinal diseases.^{80,93,118-120}

1.2.3. Bruch's membrane

Bruch's membrane separates the RPE from the choriocapillaris and consists of three layers: a central elastic layer bordered by two collagenous layers. The thickness of the elastic layer of Bruch's membrane increases from the fovea to the periphery, and Bruch's membrane thickens with advancing age.^{121,122} In the aging Bruch's membrane, deposition of collagen, lipids and other substances such as complement components is observed.¹²³⁻¹²⁵ These accumulations may disturb Bruch's membrane integrity and cause a sharp reduction in transepithelial fluid and nutrient transport, especially in the macula, where Bruch's membrane appears to be thinnest.^{121,126-128} In addition, this Bruch's membrane fragility that increases with age may make it more prone to neovascular ingrowth.¹²¹ Subretinal deposits, such as basal laminar deposits, basal linear deposits and drusen - the precursors of age-related macular degeneration - may preferentially develop at this site, between the basement membrane of the RPE and the inner collagenous layer of Bruch's membrane (Fig. 1.8).^{125,129-131}

Drusen contain a variety of inflammatory components, among many other components, and are associated with immune-mediated processes, consistent with chronic inflammation.¹²⁵ Drusen also contain lipofuscin-rich RPE cytoplasmic fragments. Macrophages may be responsible for the removal of these lipofuscin-containing cellular derivatives.^{125,132-134} Drusen are often associated with decreased fundus autofluorescence in their central part, surrounded by a halo of increased autofluorescence.^{120,135-137} It has been speculated that this autofluorescence pattern is caused by attenuation of the RPE above the center of such a druse, together with an increased lipofuscin content and a more tangential orientation of the RPE cells at the edge of those drusen. Drusen also contain dendritic cells, which, apart from sustaining and amplifying the

**Figure 1.8.**

Age-related changes in the human macula. Due to decades of light exposure, oxidative stress, and photoreceptor outer segment phagocytosis, the retinal pigment epithelium (RPE) cells accumulate lipofuscin and its toxic constituent A2E. Drusen, which are subretinal deposits containing a mixture of cellular and inflammatory components, form between the RPE and Bruch's membrane. Bruch's membrane itself thickens and becomes more fragile with advancing age. (Source: de Jong, N Engl J Med 2006;355:1474-1485)

inflammatory process, may be involved in angiogenesis and the formation of choroidal neovascularization.^{125,138-141}

1.2.4. Choroid

The choroidal circulation shows the highest rate of blood flow in the body when compared to other tissues.^{142,143} This is especially true for the choriocapillaris, the capillary layer of the choroid which is separated from the RPE by Bruch's membrane. The choroid, with its specialized structure, plays an important role in heat dissipation and in nourishment of the RPE and outer retina.¹⁴²⁻¹⁴⁴ More than 90% of the oxygen provided by the choriocapillaris is consumed by the photoreceptors.¹⁴⁵ The choriocapillaris consists of large fenestrated capillaries, unlike the larger choroidal vessels. The capillary fenestrations allows the diffusion of small molecules such as fluorescein, which is used

in fluorescein angiography, while forming a barrier to larger molecules such as the plasma proteins albumin and IgG.^{146,147}

The architectural pattern of the choriocapillaris in the posterior pole is unique in that it consists of a mosaic of vascular lobules that operate independently.¹⁴⁸ Towards the periphery, the vascular pattern of the choriocapillaris becomes more ladder-like, with the choriocapillaris connecting the arterioles and venules at right angles. This difference in vascular architecture may largely explain the increased choroidal blood flow in the macula,¹⁴⁹ owing to the more efficient lobular pattern in this area. The structural integrity of the choriocapillaris depends on the adjacent RPE, which has a trophic effect on the choriocapillaris and also induces endothelial fenestration.^{70,73,150} As mentioned in the previous section, RPE-derived VEGFs have a trophic and structural influence on the adjacent choriocapillaris.^{70,74} Animal studies indicate that choriocapillaris atrophy occurs only after RPE degeneration.^{151,152} Choriocapillaris blood flow and choriocapillary density and diameter in the macula decreases with age, especially in AMD.^{122,153} With RPE thinning or atrophy, the underlying choriocapillaris becomes less fenestrated, reducing molecular transport.

1.3. Clinical evaluation of retinal anatomy and function

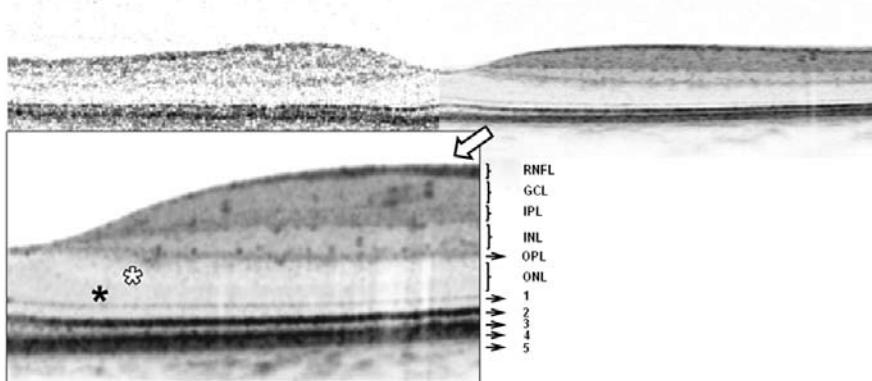
1.3.1. Introduction

The clinical evaluation of retinal dystrophies starts with careful history taking. The family history may provide valuable information about the possible mode of inheritance. Results of visual acuity measurement under standardized conditions indicate the degree of foveal involvement and are important to determine disease progression in follow-up examinations. The aspect of the retina on ophthalmoscopy often gives important clues about the nature of the disease. A thorough evaluation of retinal anatomy and function in a certain retinal dystrophy often suggests a specific diagnosis, and allows to evaluate the severity of the disease as well as the extent of the lesions. Moreover, the clinically gathered information may point to a specific underlying genetic defect. As will be illustrated in this thesis, establishing a clinical diagnosis may nevertheless be challenging, as many retinal dystrophies may have considerable overlapping features. Nowadays, the molecular genetic findings may be leading in establishing the correct diagnosis.

1.3.2. Retinal imaging

1.3.2.1. Ophthalmoscopy

A detailed examination of the retinal appearance starts with stereoscopic indirect ophthalmoscopy through well-dilated pupils. An overview of the retina may be obtained by examination with a binocular (or monocular) indirect ophthalmoscope and a +20 diopter lens. For a detailed examination of the macula, the (mid-)peripheral retina, the

**Figure 1.9.**

Comparison of images obtained with the Stratus optical coherence tomography (OCT) (left) and the Spectralis high resolution OCT (right), illustrating the difference in spatial resolution. When enlarging the central part of the high resolution OCT image, a detailed image of the retinal layers can be seen. Black asterisk, cone nuclei; white asterisk, rod nuclei; RNFL, retinal nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; 1, external limiting membrane; 2, photoreceptor inner/outer segment junction; 3, photoreceptor outer segment - retinal pigment epithelium (RPE) interdigitation; 4, RPE/Bruch's membrane complex; 5, choriocapillaris.

optic disc and the retinal vasculature, slit lamp examination with a +90, +78, or a +60 diopter lens is generally preferred. For better comparison of follow-up examinations, color fundus photographs may be taken at regular intervals.

1.3.2.2. Fluorescein angiography

Fluorescein angiography permits the study of the retinal and choroidal circulation in normal and diseased states. Photographs are taken after intravenous or oral administration of sodium fluorescein, which normally does not pass the blood-retinal barriers of the RPE and retinal vessels. Lesions on fluorescein angiography may show variable degrees of hyperfluorescence or hypofluorescence, depending on the nature and site of the lesion relative to the normal anatomical structures. To better visualize the choroidal circulation, indocyanine green angiography may be used.

1.3.2.3. Optical coherence tomography

Optical coherence tomography (OCT) offers a high resolution cross-sectional view of the retina, somewhat similar to a histopathological specimen. This high resolution is achieved through the principle of low-coherence interferometry, which is based on measurements of differences in light reflectivity of different tissue components. Most

commonly used time domain OCT scanners in clinical practice, such as the Stratus OCT, currently provide a resolution of 8–10 µm. High resolution spectral domain OCT scanners, such as the Spectralis OCT, that have more recently become commercially available that provide a maximum resolution of 3 µm or even better (Fig. 1.9). For comparison, the axial length of a photoreceptor is approximately 30 µm.

1.3.2.4. Fundus autofluorescence

Fundus autofluorescence (FAF) is a relatively new non-invasive imaging technique that allows topographical mapping of lipofuscin distribution in the RPE, when using an excitation wavelength in the short (blue) range.^{120,154} As discussed previously, RPE lipofuscin is a mixture of degradation products that include toxic retinoid fluorophores. Increased FAF is seen in areas of increased amounts of RPE lipofuscin. Decreased FAF is observed for instance in areas of RPE atrophy. FAF reflects metabolic changes at the RPE level *in vivo* and thus provides functional information that can not be obtained with conventional imaging techniques such as fluorescein angiography and OCT. Therefore, FAF may be used as an additional imaging tool in the clinical evaluation and follow-up of many retinal dystrophies that are associated with alterations in RPE lipofuscin, including those described in this thesis. The retinal diseases described in this thesis, caused by mutations in the *peripherin/RDS* gene, the *BEST1* gene, and the *CFH* gene, have all been evaluated with FAF. Therefore, FAF will be discussed more elaborately in Chapter 2.

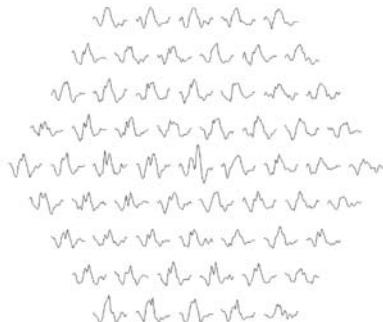
1.3.3. Retinal function tests

1.3.3.1. Electroretinography

The full-field electroretinogram (ERG) measures a mass electrical response of an action potential produced by the entire retina when stimulated by illumination. A full-field ERG can be performed under photopic (light-adapted) conditions, evoking a response that originates exclusively from the cone photoreceptors. Conversely, under scotopic (dark-adapted) conditions, the rod responses may be measured, as well as mixed cone-responses, depending on the used light stimuli. In this thesis, the scotopic ERG will be used to predominantly elicit a rod mass response. Thus, the photopic full-field ERG reflects the functioning of the entire cone photoreceptor system, whereas the scotopic full-field ERG roughly reflects pan-retinal rod photoreceptor function.

The multifocal ERG produces a topographical map of many local, simultaneously recorded, cone-derived ERG responses.¹⁵⁵ The multifocal ERG records ERG responses of the central 50° of the retina, subdivided in typically 61 or 103 small areas (hexagons) that each produce its own ERG response (Fig. 1.10). These ERG responses are not simply “small full-field ERGs”, but are derived from specific mathematical extractions. The multifocal ERG may be used for instance to specifically evaluate macular function, and may differentiate between specific areas of the macula, especially when the full-field ERG yields normal responses.

To be able to facilitate comparison of studies, test conditions of electrophysiological

**Figure 1.10.**

Multifocal electroretinography (mfERG). The mfERG is able to simultaneously record local ERG responses from specific retinal areas. Each of these areas produces a small ERG response, as shown in the figure.

tests have been standardized by the International Society for Clinical Electrophysiology of Vision (ISCEV, see www.iscev.org) for full-field ERG and multifocal ERG.^{156,157}

1.3.3.2. Electro-oculography

The electro-oculogram (EOG) measures the standing potential (a voltage difference between the RPE and the cornea), that originates from differences in the ionic composition at the apical and basal membranes of the RPE. A voltage change is seen when comparing the standard potential in the light-adapted state to that in the dark. In general, the voltage amplitude is about twice as high in light conditions compared to the dark-adapted state. The test results are usually reported as the Arden ratio, which is obtained by dividing the light voltage peak by the voltage level in the dark. The EOG also is a mass response, which originates from the entire monolayer of RPE cells instead of from the photoreceptors in full-field ERG. The EOG may be useful in evaluating RPE function in various diseases that affect the RPE, but is especially useful in the clinical diagnosis of Best vitelliform macular dystrophy.¹⁵⁸ ISCEV guidelines are also used for EOG recording.¹⁵⁹

1.3.3.3. Dark adaptation

Dark adaptation is tested by measuring the absolute thresholds of cone and rod sensitivity after exposure to light. Although the recovery of sensitivity is faster in cones than in rods, the absolute level of sensitivity is greater in rods. The sensitivity measurements are plotted against time, so that a dark adaptation curve can be drawn. In clinical practice, dark adaptation measurement is useful in the evaluation of night blindness, for instance in conditions such as retinitis pigmentosa. In some instances, dark adaptation may be more sensitive than the full-field ERG as an indicator of pathologic conditions affecting the rod photoreceptor system.

1.3.3.4. Color vision

The perception of color originates from the absorption of electromagnetic energy by the visual pigments in the cone photoreceptor outer segments, at wavelengths between 400 and 700 nm. As mentioned previously, there are three different types of cones that each have their different but overlapping spectrum of wavelength sensitivity. Clinical tests

of color vision include screening tests for rapid and coarse identification of color vision abnormalities, as well as sensitive tests of color matching and discrimination.¹⁶⁰

1.3.3.5. Contrast sensitivity

The perception of contrast is essential in daily life, for instance for the ability to discriminate and recognize objects and people's faces. Patients with relatively good visual acuity may have poor contrast vision, that may cause significant visual difficulties in daily life. Contrasts of light and/or colors are recognized after integration of these stimuli by the integrative cells of the retina and the higher visual centers. There are several chart tests of contrast sensitivity. The usefulness of each test depends on the range of spatial frequencies that has to be tested.¹⁶¹ As there are no disease-specific diagnostic patterns of deficiency in contrast sensitivity, the value of contrast sensitivity testing in a diagnostic clinical setting is limited.

1.3.3.6. Visual fields

Visual field testing is able to locate areas in the visual field that show a decreased or absent sensitivity to stimuli. If such an area of sensitivity loss is surrounded by a zone of relatively normal sensitivity, it is called a scotoma. Kinetic perimetry, for instance with the Goldmann perimeter, is able to plot the peripheral visual field with the use of moving stimuli of variable intensity, and may also roughly locate central relative or absolute scotomas. Static perimetry, for instance with the Humphrey visual field analyzer, uses non-moving stimuli of variable luminance to evaluate sensitivity defects in the central visual field in detail. These visual field test, when performed repeatedly at certain intervals, may also be useful for follow-up purposes.

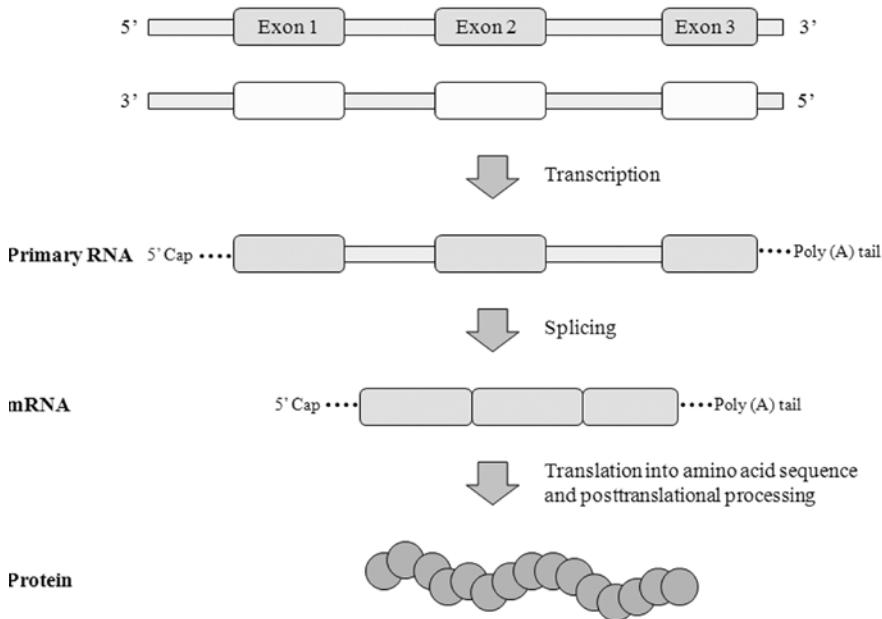
1.4. Introduction to molecular genetics

1.4.1. Basic principles of molecular genetics and heredity

1.4.1.1. Introduction

The human DNA (deoxyribonucleic acid) consists of a sequence of four different nucleotides (adenine, thymine, guanine, and cytosine), which in total is called the genome. The genome comprises approximately 3.3 billion base pairs and is organized into 46 chromosomes that reside in the cell nucleus. Two of these chromosomes are sex chromosomes: two X chromosomes in females, one X and one Y chromosome in males. The other chromosomes, also known as autosomes, form 22 different chromosome pairs. Each pair of chromosomes carries one paternal and one maternal chromosome. Each of these homologous pairs carry a DNA sequence that is almost identical. The human genome encodes approximately 20.000 to 25.000 genes, which are specific DNA sequences that encode instructions for the ultimate formation of RNA molecules and proteins.

In reproduction cycles, chromosome pairs exchange segments of DNA, a process known

**Figure 1.11.**

Summary of the process from DNA to protein formation. In the cell nucleus, the DNA is transcribed into primary RNA. Removal of the non-coding introns results in the formation of the messenger RNA (mRNA). This mRNA is transported from the nucleus to the ribosomes in the cytoplasm, where translation to the primary protein and posttranslational protein processing take place.

as recombination or “crossing over”. Genetic recombination increases genetic diversity throughout evolution. In addition to nuclear chromosomes, a small amount of circular DNA (16.569 base pairs) is also present in multiple copies in mitochondria. An allele is one of the variant forms of a gene at a particular location, or locus, on a chromosome. In general, an individual possesses two copies of a gene (one on each chromosome of a chromosome pair) and thus two alleles. The term genotype refers to the genetic constitution of an individual, whereas the observable (clinical) characteristics of this individual constitute the phenotype.

Most genes contain protein-coding regions, the exons, interspersed by non-coding regions or introns. Through the mechanism of transcription, DNA is transcribed in the nucleus into a primary RNA (ribonucleic acid) sequence that incorporates the transcript of both the exons and the introns (Fig. 1.11). The subsequent process of cleavage and splicing removes the non-coding intronic parts at the splice sites, leaving only the protein-coding exons to form the so-called messenger RNA. This messenger RNA (mRNA) also consists of a sequence of bases (the same as in DNA, except thymine, which is

replaced by uracil). The next phase is the translation of mRNA into a specific amino acid sequence that will form the primary protein. This phase is performed in the cytoplasm of the cell. Different combinations of three adjacent bases (triplets or codons) form the code for different amino acids. Three specific triplets (TAG, TAA, and TGA) do not encode an amino acid but form the code to stop the translation from mRNA to protein, and are therefore called stop codons. After several posttranslational modification processes, the final functional or structural protein is formed, with its unique three-dimensional structure.

Gene variants may be inherited in several ways. In autosomal dominant inheritance, a variant or mutation in one of the two gene copies is enough to inherit a specific trait or disease. A carrier of an autosomal dominant mutation has a probability of 50% to transmit the mutation to his or her offspring. Theoretically, 50% of his children would thus inherit the disease associated with the mutation. However, as will be illustrated in this thesis, not all carriers of a dominant mutation may develop a clinically apparent disease. This phenomenon of non-penetrance or decreased penetrance is due to additional modifying genetic and/or environmental factors that are often unknown. Such decreased penetrance may consequently mask the dominant mode of inheritance.

When a mutation on both gene copies is required to cause a disease phenotype, the mode of inheritance is called autosomal recessive. When an affected person carries two identical mutations, the mutations are called homozygous. On the other hand, an affected individual is a compound heterozygous mutation carrier when the two alleles contain two different mutations. A person carrying only one autosomal recessive mutation will not develop the disease. When two members of a couple each carry one mutation, there is a 25% chance that a child will inherit both mutations and thus develop a disease phenotype.

The X chromosome may also carry disease-associated mutations and causes an X-linked inheritance pattern. In these cases, females are carriers and are usually not affected by disease as they also carry a second, normal X chromosome. Male offspring consequently has a 50% chance of carrying a mutant gene, and affected males will develop disease as they carry only one (affected) X chromosome.

Finally, genetic disorders may also be caused by mutations in the non-chromosomal DNA of the mitochondria, which encodes 37 genes. This mitochondrial DNA is maternally inherited, but may affect both men and women. The amount of mutant mitochondrial DNA may vary considerably between persons, but also between tissues and even cells of an individual, accounting for the remarkable inter- and intraindividual variation of the phenotype.

1.4.1.2. Single nucleotide polymorphisms, haplotypes and haplotype analysis

A single nucleotide polymorphism (SNP) is a single nucleotide change in the DNA sequence of different individuals. Due to genetic variations between different populations, the frequency of a SNP may vary considerably between different geographic and ethnic groups. SNPs may be found in protein-coding DNA regions, but also in non-coding

regions that are involved in for instance gene processing (splicing) or transcription factor binding. SNPs are found at a rate of approximately 1 per 500 base pairs in the human genome, which makes it possible to use them as “landmarks” on a genetic map.¹⁶²

A haplotype is a chromosomal region with certain genetic variants that can be shared between individuals (<http://www.hapmap.org/originhaplotype.html.en>). Over the course of many generations, segments of chromosomes in a certain population are intermingled through repeated recombination events. Some of these DNA segments or haplotypes, however, remain shared by multiple individuals that are not closely related. Through various mechanisms, the frequency of haplotypes varies from region to region. As a consequence, a certain haplotype may occur at different frequencies in different populations, especially when those populations are widely separated and are thus unlikely to exchange DNA through reproduction. Haplotype studies enable the search for candidate genes that are involved in monogenic diseases, as well as the identification of genes involved in gene-gene and gene-environment interaction.

1.4.1.3. Gene identification

In monogenic disease of unknown cause, the classical first step towards identification of the underlying disease-causing gene is to determine its chromosomal location by linkage analysis. Linkage analysis is based on the observation that genes and their adjacent SNPs tend to be inherited together during segregation within a family. If a certain gene and its surrounding SNP markers cosegregate in a significant number of affected individuals, the likelihood increases that this gene is in fact the disease-causing gene. This likelihood of true linkage of the gene to the disease is reflected in the LOD (logarithm of the odds ratio) score, which is a statistical test that is often used in linkage analysis.¹⁶³ By convention, a LOD score of greater than 3.0 is considered evidence for genetic linkage.

When a disease is linked to a chromosomal region which contains several genes, a candidate gene approach may identify the specific gene that is involved. Candidate genes, for instance genes that are known to possess functions related to the disease process, may then be analysed for mutations by DNA sequence analysis. If a putative mutation is found, this mutation must segregate with disease within the family. Linkage analysis has also been successful in identifying a broad range of genes that cause autosomal dominant, autosomal recessive, as well as X-linked disease. However, the ability of linkage analysis to identify unknown genes is limited by the size of the families and number of affected persons that are required to obtain a significant LOD score.

Patients with recessive disease, who are born from parents who are to a certain degree consanguineous, are more likely to be homozygous for the disease-causing mutation and surrounding polymorphisms. The explanation for this observation is found in the fact that persons who are closely or more distantly related are more likely to share identical areas of DNA sequences. The strategy of homozygosity mapping is therefore an effective approach in addition to linkage analysis, especially in medium-sized consanguineous families in which no significant LOD score can be obtained with linkage analysis. In rare recessive diseases in families with no reported consanguinity, the frequency of

homozygous mutations may also be relatively high. This is due to a distant common ancestor of the parents, constituting a distant consanguineous link. Specific mapping strategies using high-density SNP analysis may detect homozygous regions from a common ancestor who lived as long as 10 generations ago.^{164,165} The chance to find the chromosomal region harboring the disease-causing gene is highest in the homozygous regions that are largest.^{164,166} To subsequently find the actual disease-causing gene, the candidate gene approach can be used.

1.4.1.4. Mutations, polymorphisms, and pathogenicity

A pathologic gene mutation is defined as an alteration of the DNA sequence that causes a partial or complete impairment of protein function. A mutant gene or allele is the opposite of the so-called wild-type allele, referring to the most common allele in the normal population. A mutation may cause an adverse effect through different mechanisms. A dominant negative mutation leads to a protein that adversely affects the normal, wild-type protein. Haploinsufficiency occurs when an individual has only a single functional gene. This single copy does not produce sufficient amounts of normal gene product to result in a normal phenotype, leading to disease. Gene mutations sometimes result in disease, which is appropriately referred to as a genetic disorder. If such a mutation is also present in a germ cell, it may be inherited by offspring and the disease is therefore a hereditary disorder. Mutations may result from for instance nucleotide substitutions, deletions, insertions, or duplications.

The term “polymorphism” refers to an allele that is relatively frequent in the general population, and that is not directly associated with disease. A polymorphism may concern a single nucleotide change (a SNP), but also an amino acid change (which may be caused by a single nucleotide changes in the DNA sequence), as well as larger alterations of the coding or non-coding DNA sequence.

There are several types of mutations. A missense mutation is an alteration in the DNA sequence that leads to substitution of one amino acid by another amino acid in the protein product. Even such a single amino acid change may considerably change protein structure and function, and may eventually cause disease. Missense mutations are very common in genetic disorders. The majority of mutations in the *BEST1* gene in Best vitelliform macular dystrophy, for instance, are missense mutations (see Chapter 3). A frameshift mutation is caused by insertions or deletions of a number of nucleotides that is not divisible by three (the triplet of bases coding a single amino acid). As a result, the DNA reading frame is completely changed, leading to a completely altered amino acid sequence of a protein. This obviously causes a significantly changed protein structure and functionality, often resulting in non-functional protein products. Frameshift mutations in the *peripherin/RDS* gene are common in multifocal pattern dystrophy simulating Stargardt disease/STGD1 (Chapter 4). If a mutation leads to a premature stop codon, the mutation is called a nonsense mutation. An abnormally short or “truncated” protein will be the end result, which is often associated with severe phenotypic abnormalities. A splice site mutation changes the DNA sequence that denotes the site at which splicing

of one or more introns normally takes place. Consequently, one or more introns remain in the mature messenger RNA, or one or more exons are skipped, and can disrupt the generation of a normal protein product. A null mutation, for instance a nonsense or a splice site mutation, is a mutation that results in either the absence of a gene product or the absence of any protein function.

It may be challenging to establish whether a gene sequence variant is the cause of a disease. Pathogenicity is more easily assumed in mutations that severely alter protein structure and/or length, such as nonsense mutations. It is often more difficult to determine if a single amino acid change is a disease-causing missense mutation or merely a benign polymorphism. To add proof to supposed pathogenicity of such a missense variant, additional support is often needed from different lines of evidence. First of all, the sequence variant(s) should segregate with the disease in the respective family as expected for dominant or recessive mutations. If the variant concerns a change to an amino acid with very different structural and electrical properties, this may point to significant consequences for protein structure and function. This is more likely when the missense change is located in a region of the protein that is known to be functionally important. Protein modeling by specific computer programmes may be helpful in these cases.

Another relative argument may be provided by the finding that a specific genetic variant is conserved in numerous species throughout evolution. This would then indicate that this specific variant is functionally important and that alteration of this specific sequence is disadvantageous. Another clue of pathogenicity may be provided by the absence of the variant in a considerable control group of persons without the phenotype. However, this is a statistical argument that does not unequivocally exclude the possibility that the genetic variant is a rare non-pathogenic variant.

The distinction between a polymorphism and a pathologic mutation is sometimes clear, but may also be rather artificial. For example, relatively common polymorphisms may show a strong association with certain diseases, for instance in AMD (see paragraph 6 of this introduction). On the other hand, some mutations are not invariably associated with a disease phenotype, due to non-penetrance as a result of genetic and environmental modifying factors.

1.4.2. Mutation detection in monogenic disease

The identification of the underlying mutation(s) in a monogenic disease is important for a number of reasons. Detection of the underlying mutation(s) may confirm or change a clinical diagnosis. Especially in the case of a retinal dystrophy that shows non-specific, overlapping phenotypic features, molecular genetic analysis may unequivocally point to a specific underlying defect and thus helps to establish a definite diagnosis. This is important to the patient, as genotype-phenotype correlation studies can help to provide the patient with adequate information about the prognosis of the disease. In addition, information about the underlying genetic defect facilitates genetic counseling.

To date, knowledge on the genetic cause of a retinal disease rarely has therapeutic consequences. Although the first human gene therapeutic studies in retinal dystrophies

are emerging, only patients with specific mutations in specific genes are currently eligible for inclusion in these experimental trials.^{167,168}

There are several mutation detection strategies to test the presence or absence of one or more mutations in specific genes. In general, the DNA-strands of interest are first amplified with polymerase chain reaction (PCR) technique. Sequence analysis of the possibly involved gene may be used when a small number of candidate genes with a limited number of exons are involved. In larger genes, this method is time consuming and costly, but is able to detect new mutations.

The method of single strand conformational polymorphism analysis (SSCP) was widely used until recently. This technique is based on the principle that DNA fragments, that differ as a result of a mutation, will show a different migration pattern on gel electrophoresis due to conformational differences. Comparison of affected and unaffected individuals will thus reveal DNA alterations in a specific region of a gene (often in the exons). The sensitivity of this test is approximately 80% and the test is relatively cheap, making it a good screening method prior to sequencing.¹⁶⁹

Other PCR-based mutation detection techniques include heteroduplex analysis,¹⁷⁰ denaturing gradient gel electrophoresis (DGGE),¹⁷¹ and denaturing high performance liquid chromatography (DHPLC).¹⁷² All of these techniques have their individual advantages and disadvantages, their sensitivities depending on optimization of several test conditions. As automated sequence analysis has become cheaper in the last 10 years, this is currently the preferred method for mutation analysis.

The gene microarray ("gene chip") is a method that allows fast, cheap, and reliable simultaneous screening of multiple (hundreds of) known mutations in multiple genes.¹⁷³ By using an available microarray, patients with a specific retinal dystrophy can be prescreened for already known mutations and other gene variants with this relatively cheap and fast method. If no mutations are found, more expensive methods such as DGGE and/or nucleotide sequencing can be applied to detect new mutations.

1.4.3. Molecular genetic analysis of complex multigenic disease

A complex genetic disease does not show a clearly definable genetic inheritance pattern, despite evidence of a strong genetic basis. In these cases, abnormalities in multiple genes may be suspected, possibly in conjunction with environmental factors. Family studies are essential to investigate whether a disease has a significant inherited component. Familial aggregation of disease means that the risk of a disease is higher in an individual that is related to an affected individual, compared to a person without affected family members. In this respect, studies on monozygotic twins are especially helpful, as they are genetically identical. Previous twin studies in AMD clearly alluded to the importance of genetic factors in the pathogenesis of AMD, as monozygotic twins showed high concordance of disease features in intermediate and late-stage AMD.^{174,175} The polygenic nature of such complex disease, in which multiple genes each contribute to a relatively modest extent, makes the detection of these genes more difficult. Phenotypic variability and environmental influences add up to this challenge. Some of the clinical features may

have a more significant genetic determination than others. Small peripheral drusen, for instance, appear to have a strong familial preponderance.^{176,177} How to find these disease-associated genes in complex multigenic disease?

Classical linkage studies are problematic in the analysis of complex disease, as there is often no clear inheritance pattern and an apparently low penetrance. In complex multigenic disease, large samples of families and sib-pairs are required to conduct genetic linkage analysis. Even then, it remains difficult to reach adequate statistical power.¹⁷⁸ Complex disease often shows considerable phenotypic variability. Selecting only specific phenotypic subtypes in a specific (for instance ethnic and/or geographic) population for linkage studies, in an attempt to obtain homogeneous groups, leads to further loss of statistical power. Even small degrees of misclassification can also cause substantial loss of statistical power.¹⁷⁹

Other strategies are therefore required. Progression in the study of complex multigenic diseases greatly relies on the accomplishments of large-scale genetic projects such as the Human Genome Project (<http://www.genome.gov/10001772>), which identified the entire human DNA sequence and the approximately 25.000 genes of the human genome, and the International HapMap Project (<http://www.hapmap.org/>), which develops a haplotype map of the human genome. Genetic association studies that use case-control cohorts and inherited SNP landmarks of specific haplotypes have a much greater ability to detect a genetic risk association and to find disease-associated genes. Compared to linkage studies, these association studies do not require families with specific inheritance patterns and require smaller sample sizes than linkage analysis to detect disease-associated genes.

If a particular haplotype is found more frequently in individuals affected by a specific disease, compared to healthy controls, a gene influencing this disease may be located within or near this haplotype. SNP-based genome-wide association studies use SNP landmarks in combination with haplotype information to find these genes. These strategies have become a powerful and cost-effective tool in genetic association studies, particularly in the search of genes associated with complex diseases such as AMD.^{180,181}

1.4.4. Genotype-phenotype correlation versus genetic and phenotypic heterogeneity

The association of a specific genetic defect (genotype) with a specific clinical picture (phenotype) is called genotype-phenotype correlation. In a broad sense, genotype-phenotype correlation refers to the association of alterations of a specific gene with a specific clinical picture, such as the association of mutations in the *BEST1* gene with Best vitelliform macular dystrophy and *rhodopsin* gene mutations with retinitis pigmentosa. In a strict sense, strong genotype-phenotype correlation is found when specific mutations in a specific gene are invariably associated with a specific phenotype. Some genes and their defects, for instance the *ACBA4* gene and its associated phenotypes,¹⁸² show a considerable degree of genotype-phenotype correlation.

Mutations in other genes, such as the *peripherin/RDS* and *BEST1* gene described in this thesis, are associated with striking phenotypic heterogeneity. Most mutations in these

genes show very limited genotype-phenotype correlation. Non-penetrance of a genetic defect may also be observed, meaning that an underlying mutation does not result in a corresponding phenotype. By definition, monogenic disease is caused by one or more mutations in a single gene. Nevertheless, there are many examples of so-called monogenic diseases in which the resultant phenotype is highly variable, even in individuals carrying an identical mutation. In these cases, the clinical outcome is strongly influenced by additional modifying factors, such as modifying genes,¹⁸³⁻¹⁸⁶ and possibly environmental modifying factors such as nutritional factors,^{98,187} light exposure,⁸⁵ and smoking.¹⁸⁸⁻¹⁹⁰ The genes associated with the retinal diseases described in this thesis are examples of such a variability. Retinal disorders (for instance retinitis pigmentosa) may also show considerable genetic heterogeneity, meaning that mutations in different genes result in clinically similar phenotypes.

1.5. Clinical and molecular genetic characteristics of hereditary retinal dystrophies

Monogenic hereditary retinal dystrophies affect approximately one in every 3000 individuals.¹⁹¹ The group of retinal dystrophies shows broad genetic and phenotypic heterogeneity.¹⁹²⁻¹⁹⁵ Clinical classifications encounter difficulties, as there is an ongoing debate concerning the clinical parameters that should be used for characterization. Some classifications use ophthalmoscopic features, others use electrophysiologic findings, whereas some classifications include both. Moreover, newer techniques such as FAF and OCT provide further clinical information that may strengthen or weaken existing classifications. On the other hand, a classification based solely on the underlying molecular genetic defect(s) is at present also unpractical. Mutations in several genes, such as for instance the *BEST1* gene, the *peripherin/RDS* gene, and the *ABCA4* gene are associated with remarkable clinical heterogeneity. As indicated in the previous section, the clinical outcome of a single genetic defect is modified by additional genetic factors in these cases. Moreover, environmental factors may play a modifying role. Therefore, a genetic classification is an oversimplification of the clinical reality until we are able to map most of the influencing genetic and environmental interactions in an individual. Still, a correct clinical diagnosis is very important, not only to give direction to the search for the underlying genetic defect, but also to be able to provide the patient with accurate information about the disease and its prognosis. For this reason, the current classification of retinal dystrophies is based on the clinical findings, whenever possible supplemented by the underlying genetic cause.

1.5.1. Macular dystrophies

Patients with a macular dystrophy typically experience loss of visual acuity, sometimes accompanied by metamorphopsia and/or central scotomata. The term “macular” dystrophy suggests pathology limited to the macular area of the retina. It should be

noted, however, that many of these dystrophies may be considered disorders that affect the entire retina on the molecular level. In addition, electrophysiological examination (full-field ERG and EOG), and sometimes ophthalmoscopy, may also indicate pan-retinal photoreceptor dysfunction, especially when the disease progresses. In this thesis, a macular dystrophy will be broadly defined as a retinal phenotype that is largely confined to the central fundus on ophthalmoscopy, with a generally normal photopic and scotopic full-field ERG earlier in the course of the disease. In macular dystrophies, the macular region often merely shows a greater susceptibility to clinically manifest degeneration than the peripheral retina.

Macular dystrophies are associated with a broad range of underlying genetic causes. Most macular dystrophies, for instance those caused by *BEST1* (Chapter 3) and *peripherin/RDS* mutations (Chapter 4), are inherited in an autosomal dominant fashion. However, the most frequent macular dystrophy, Stargardt disease (STGD1), is caused by autosomal recessively inherited mutations in the *ABCA4* gene. As mentioned previously, it is important to realize that dystrophies associated with a single genetic defect may still show considerable phenotypic variability.

On the other hand, different macular dystrophies with different underlying genetic causes may share phenotypic features. For example, drusen are a rather non-specific ophthalmoscopic finding. Drusen may be described as round, white to yellow accumulations between the RPE and Bruch's membrane, sometimes with a crystalline aspect, that are often most prominent in the macula. They are not only the hallmark lesions of AMD, but drusen-like lesions may also be encountered in several autosomal dominant macular dystrophies, such as central areolar choroidal dystrophy (caused by *peripherin/RDS* mutations),^{196,197} Sorsby fundus dystrophy (caused by mutations in the *TIMP3* gene),¹⁹⁸⁻²⁰⁰ malattia leventinese (also known as Doyne's honeycomb retinal degeneration, caused by *EFEMP1* mutations),²⁰¹⁻²⁰³ North Carolina macular dystrophy (linked to the *MCDR1* locus),^{204,205} as well as in retinal disorders such as basal laminar drusen (see Chapter 5).^{206,207} The term "dominant drusen" is a generalizing description, referring to a broad range of macular diseases of autosomal dominant inheritance associated with drusen, including some of the aforementioned phenotypes.²⁰⁸ These phenotypes may also share the complication of choroidal neovascularization, which is also seen in neovascular AMD.^{197,199,205,209}

Another example are the yellow-white, "pisciform" lesions in the central retina in Stargardt disease/STGD1. Virtually identical lesions may also be observed in autosomal dominant STGD3 (caused by mutations in the *ELOVL4* gene on chromosome 6),²¹⁰⁻²¹² STGD4 (mapped to chromosome 4),^{213,214} as well as in multifocal pattern dystrophy simulating STGD1 (see Chapter 4, section 4.3).^{215,216}

Areas of chorioretinal atrophy may be encountered in the advanced stages of many macular dystrophies, such as STGD1, central areolar choroidal dystrophy, Best vitelliform macular dystrophy, and North Carolina macular dystrophy.

In summary, the aforementioned monogenic phenotypes may all share ophthalmoscopic features such as drusen, choroidal neovascularization, yellow-white flecks, as well

as chorioretinal atrophy, features that are also observed in AMD. Since many of these dystrophies may occur in late-onset variants,^{182,202,217} and may show overlapping and/or atypical phenotypic features with AMD, differentiation with AMD is sometimes difficult. These findings and the studies that will be described in this thesis illustrate that the dividing line between many macular dystrophies and AMD is not as clear and straightforward as once thought.

However, AMD has been conclusively shown to be associated with many genes that all increase the risk of AMD to a certain degree, but neither of these genetic variants on itself has been shown to be the direct underlying cause of AMD. Environmental influences, such as smoking and dietary factors, are significant modifiers of disease risk in AMD. Due to this complex multifactorial etiology, AMD is generally not considered to be a macular dystrophy, as the latter group consists of diseases that are in principle monogenic and have an earlier mean age at onset than AMD.

An overview of all macular dystrophies is beyond the scope of this general introduction. A summary of macular dystrophies and associated genes is given in Table 1.1. For a comprehensive overview, the reader is advised to consult the following references in superscript.^{194,218-220}

1.5.2. Progressive cone and cone-rod dystrophies

The group of progressive cone and cone-rod dystrophies also shows marked clinical and genetic heterogeneity.¹⁹⁵ Progressive cone and cone-rod dystrophies usually present in childhood or early adult life, as opposed to the stationary cone dysfunction syndromes that often present shortly after birth or in infancy. In general, patients experience loss of visual acuity, photophobia, color vision abnormalities, and may develop nyctalopia (night blindness) in the later stages of the disease. Patients rarely display a fine pendular nystagmus, although this feature is more typical for stationary cone dysfunction syndromes such as achromatopsia. The aforementioned symptoms may precede the fundus abnormalities. Fundus examination typically show a “bull’s eye” maculopathy or, less frequently, more discrete RPE alterations in the fovea. In some cases, sparing of the fovea is observed on ophthalmoscopy as well as on central visual field testing. In progressive cone dystrophy, the full-field ERG shows markedly abnormal cone-mediated (photopic) responses with normal rod-mediated (scotopic) responses.¹⁹⁵ Many patients that initially display a progressive cone dystrophy phenotype, eventually also develop generalized rod dysfunction, reflected in an abnormal scotopic full-field ERG. This is reflected in the finding that several genes are associated with both progressive cone and cone-rod dystrophy. Many cases of progressive cone and cone-rod dystrophy are sporadic, but autosomal dominant, autosomal recessive, and X-linked inheritance have also been described.

1.5.3. Retinitis pigmentosa (rod-cone dystrophy)

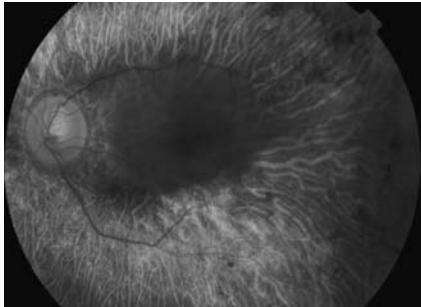
Retinitis pigmentosa (RP) is a generic term, encompassing a group of progressive retinal dystrophies in which -in early stages- rod photoreceptor dysfunction is equally or more pronounced than cone dysfunction.¹⁹³ Although clinical findings in this group of retinal

Table 1.1.*Summary of the identified genes and loci in macular dystrophies.*

Disease	OMIM number	Mode of inheritance	Associated gene or locus	Reference(s)
adult-onset foveomacular vitelliform dystrophy	608161	AD	<i>peripherin/RDS</i> <i>BEST1</i>	221 222
autosomal dominant bull's eye macular dystrophy	608051	AD AR	<i>PROM1</i> <i>ABCA4</i>	218,223 224
Best vitelliform macular dystrophy	153700	AD	<i>BEST1</i>	27
central areolar choroidal dystrophy	215500	AD	<i>peripherin/RDS</i> 17p13	26 225
dominant cystoid macular dystrophy	153880	AD	7p15.3	226
Doyne honeycomb retinal dystrophy (malattia leventinese)	126600	AD	<i>EFEMP1</i> (fibulin-3)	203
juvenile retinoschisis	312700	XL	XLR5	227
North Carolina macular dystrophy	136550	AD	unknown (MCDR1 locus)	228-230
pattern dystrophy	169150	AD	<i>peripherin/RDS</i> 5q21.2-q33.2	231 232
progressive bifocal chorioretinal atrophy	600790	AD	6q14-q16.2	233
Sorsby fundus dystrophy	136900	AD	<i>TIMP3</i>	200
Stargardt disease (STGD1)	248200	AR	<i>ABCA4</i>	234
Stargardt-like macular dystrophy (STGD3)	600110	AD	<i>ELOVL4</i>	212
Stargardt-like macular dystrophy (STGD4)	603786	AD	<i>PROM1</i>	213,218

AD, autosomal dominant; AR, autosomal recessive; OMIM, Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>); XL, X-linked.

dystrophies may be highly variable, patients with this disease generally experience night blindness, associated with progressive loss of the (mid)peripheral visual field. Typically, ophthalmoscopy shows (mid)peripheral areas of intraretinal hyperpigmentation, often in a “bone-spicule” configuration, together with a pale, waxy optic disc and attenuated retinal arterioles (Fig. 1.12). The full-field ERG is crucial for the diagnosis of RP, showing abnormal rod photoreceptor responses that predominate cone dysfunction earlier in the course of the disease. In cases of RP that are caused by defects in genes that are found

**Figure 1.12.**

Retinitis pigmentosa. Note the bone-spicule hyperpigmentation outside the macula, a waxy pale optic disc, and attenuated retinal arteries.

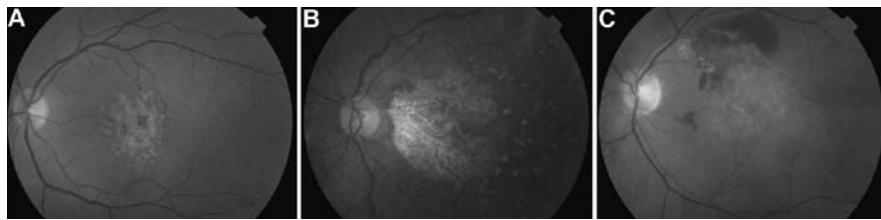
exclusively in rods, secondary cone photoreceptor loss was postulated to be caused by the sharp decrease of rod-derived cone viability factor.^{235,236} In later stages, both cone and rod responses may become non-recordable, and widespread chororetinal atrophy may occur. In end-stage RP, patients may retain only a small residual area of central visual field ("tunnel vision"). Visual acuity may also show a progressive decline, as central cone function also becomes compromised later in the course of the disease.^{193,237} Besides phenotypic heterogeneity, RP also shows marked genetic heterogeneity. The disease can be inherited as an autosomal recessive (50-60% of cases), autosomal dominant (30-40%), or X-linked (5-15%) trait, besides a small proportion of other modes of inheritance.¹⁹³ In addition to isolated RP, the condition may also be associated with more than 30 different syndromes.¹⁹³ A certain degree of genotype-phenotype correlation is observed in RP, that may be useful in the classification of various forms of RP.²³⁸

1.5.4. Leber congenital amaurosis

Leber congenital amaurosis is considered the most severe form of inherited retinal blindness, as this photoreceptor dystrophy usually presents within the first weeks of life.²³⁹ In most cases, the visual acuity is below 20/200 early in the course of the disease. Leber congenital amaurosis is a genetically and clinically heterogeneous disease, that is often inherited in an autosomal recessive fashion. For an extensive overview, the reader is advised to consult an extensive review by den Hollander and colleagues.²³⁹

1.6. Clinical and molecular genetic characteristics of age-related macular degeneration

Inherited retinal degenerations may share clinical features with age-related macular degeneration (AMD), the leading cause of blindness in people above 60 years of age in the Western world.²⁴⁰ The characteristic signs of AMD are drusen, deposits in Bruch's membrane that underlie the RPE.²⁴⁰ Drusen appear as yellow-white dots concentrated in the macula and sometimes the peripheral retina on ophthalmoscopy (Fig. 1.13A). Several clinical classification systems for AMD have been proposed.²⁴¹⁻²⁴⁴ These classification systems use several characteristics to grade AMD, including the size, number, and

**Figure 1.13.***

Age-related macular degeneration (AMD). A. Soft drusen in the macula, that merge to a larger pigment epithelial detachment with hyperpigmentation. B. Advanced atrophic AMD, showing profound chorioretinal, “geographic” atrophy of the macula, surrounded by large drusen. C. Neovascular AMD, associated with hemorrhages within the lesion.

type of drusen, as well as pigmentary abnormalities, resulting in the development of severity scales. Late stages of AMD, when visual loss becomes manifest, are subdivided into two broad categories. Atrophic (“dry”) AMD is characterized by a certain degree of chorioretinal atrophy (in advanced stages known as “geographic atrophy”) in the macula, without the presence of choroidal neovascularization (Fig. 1.13B). In neovascular (“wet”) AMD, new vessels arise from the choriocapillaris, that overcome Bruch’s membrane and sometimes the RPE barrier, to grow under or into the neuroretina (Fig. 1.13C). This results in subretinal and/or intraretinal fluid accumulation, hemorrhages and scar formation, leading to a relatively rapid drop in visual acuity. Atrophic AMD may evolve towards neovascular AMD, and vice versa.²⁴⁵ As atrophic and neovascular AMD may resemble end stages of other retinal diseases, including several retinal dystrophies, late AMD could be considered a diagnosis of exclusion, although it is much more frequent than those retinal dystrophies.²⁴⁰

Before the finding of significant gene associations in AMD, several twin-based and other familial studies already indicated that AMD has an important genetic component: close relatives were shown to have a strongly increased risk of developing AMD.^{174,246,247}

Mutations in a broad range of genes cause phenotypes that may closely mimic AMD. In general, these retinal disorders occur at an earlier age and follow a specific inheritance pattern. Logically, the search for AMD-associated genetic factors started with the screening of genes that were known to be involved with monogenic retinal dystrophies. Studies have implicated a broad range of those genes in AMD (Table 1.2), but many studies remained inconclusive or controversial.²⁴⁸

The great breakthrough in the quest for AMD-associated genes came in 2005, when four papers simultaneously provided compelling evidence that a specific SNP in the *Complement Factor H (CFH)* gene significantly increases the risk of AMD.²⁹³⁻²⁹⁶ This polymorphism encodes a tyrosine to histidine amino acid change at position 402 (p.Tyr402His) of the CFH protein. In brief, the CFH protein is a major inhibitor of the alternative pathway of the complement system. The complement system is an important

Table 1.2.*Genes associated with age-related macular degeneration.*

Gene	Normal function of protein product	Reference(s)
<i>CFH</i>	inhibition of alternative complement pathway	249-253
<i>CFB</i>	activation of alternative complement pathway	254-258
<i>C2</i>	activation of classical complement pathway	254-256,258
<i>C3</i>	central complement activator	254,259-262
deletion of <i>CFHR1</i> and <i>CFHR3</i>	overlapping function with CFH?	263-265
<i>SERPING1</i>	inhibition of classical complement pathway	266
<i>HTRA1</i>	serine protease	267-271
<i>ARMS2</i> (<i>LOC387715</i>)	unknown function in mitochondria?	272-275
<i>PLEKHA1</i>	unknown	269,273,276
<i>TLR3</i>	host defense against viruses	277
<i>APOE</i>	lipid transport and metabolism	278-282
<i>ABCA4</i>	retinoid transporter in photoreceptors	283,284
<i>fibulin-5</i>	extracellular matrix glycoprotein involved in elastogenesis, present in Bruch's membrane	285-287
<i>fibulin-6</i> (<i>hemicentin-1</i>)	unknown	288-291
<i>VEGF</i>	angiogenesis	292-294
<i>HLA</i>	antigen presentation	339,340

component of innate immunity, consisting of three pathways including the alternative pathway.²⁹⁷ This alternative pathway is a safeguard system of the human body that recognizes, attacks, and eliminates microbes and modified tissue cells,²⁹⁷ which is spontaneously activated throughout the body under normal conditions.²⁹⁸ To prevent overactivation, several factors are present that inhibit this activation of the alternative pathway under circumstances where complement activation is unwanted. The CFH protein is a central player in this down-regulation of alternative pathway complement activation.²⁹⁹ A detailed discussion of the structure, function and disease associations of CFH will be presented in Chapter 5.

To date, the *CFH* p.Tyr402His variant has been shown to be the most important genetic risk factor in AMD in the Caucasian population. The odds ratios for the development of AMD (soft drusen, geographic atrophy, as well as neovascular AMD) range from 2.45 to 7.4, depending on the study design and population, as well as the heterozygous or homozygous presence of the p.Tyr402His risk allele.^{293,295,296,300} Other *CFH* haplotypes may also increase susceptibility to AMD independently of the p.Tyr402His variant, although the effect still appears less pronounced than that of p.Tyr402His.²⁹⁷ Further support for the role of dysregulation of the alternative complement pathway in AMD comes from the finding that variants in other complement genes also alter the risk of AMD (Table 1.2).

Other genes of largely unknown function, *LOC387715/ARMS2*,^{273,275} *HTRA1*,^{268,271} and *PLEKHA1*,²⁷³ that lie in the vicinity of each other on chromosome 10q26 confer a strong risk for the development of AMD. Both the *CFH* p.Tyr402His and the *LOC387715/ARMS2* p.Ala69Ser risk variant are associated with progression of AMD.³⁰¹ Several studies indicate that additional polymorphisms in *LOC387715/ARMS2* may also increase the risk of AMD.^{274,302} A frequent variant in the *LOC387715/ARMS2* gene leads to the deletion of the polyadenylation signal and consequent instability of the mRNA, resulting in the absence of ARMS2 protein expression in homozygotes.^{272,303} The ARMS2 protein has been proposed to play a role in mitochondrial function,^{272,274} although this is contradicted by other findings.³⁰⁴

Genetic susceptibility to AMD shows important ethnic variations. For instance, the *CFH* p.Tyr402His risk variant is not significantly associated with AMD in the Japanese population,^{305,306} and plays only a modest role in the Chinese population.³⁰⁷ Other coding and non-coding *CFH* variants do appear to moderately influence the risk of AMD in these ethnic groups.³⁰⁸ In contrast, several polymorphisms in the *LOC387715/HTRA1* gene loci are strongly associated with AMD in both Japanese and Chinese.^{189,309,310} These different genetic backgrounds, different risk factor associations, as well as the different prevalence numbers of risk modifying polymorphisms,³¹¹ may account for the different patterns of disease and phenotypic subgroups in different ethnic subgroups.^{312,313}

While the population risk of AMD is 3%, individuals carrying all risk genotypes in 5 variants in *CFH*, *LOC387715*, and *C2/CFB* were calculated to have an absolute AMD risk of approximately 35%.^{314,315} In contrast, those who carry none of these risk genotypes have an absolute AMD risk of 0.17%. Specific clinical features of AMD appear to show a stronger genetic association. For example, large numbers of hard drusen and the presence of peripheral drusen (such as in some basal laminar drusen phenotypes) have a strong familial preponderance.^{176,177,316}

Over the last decades, various animal models have been developed, displaying features of human AMD.³¹⁷ Although there still is no exact animal model of AMD, these models have been able to increase our understanding of the underlying molecular mechanisms in AMD. Also, these animal models are invaluable for the development of new therapeutic approaches.

1.7. Aims and outline of this thesis

This thesis aims to add to the insight in the clinical and genetic characteristics of retinal diseases caused by mutations in the *BEST1* gene and the *peripherin/RDS* gene. In addition, it aims to evaluate the role of the *CFH* gene in the phenotype of basal laminar drusen, an early-onset drusen phenotype that may closely resemble age-related macular degeneration. As will be illustrated, *BEST1* mutations, *peripherin/RDS* mutations, as well as variants in the *CFH* gene, are associated with specific phenotypic spectra. These phenotypic spectra show a certain degree of genotype-phenotype correlation. However, as will be discussed, a striking phenotypic variability is also seen, that often cannot simply be explained by the specific type of mutation.

Chapter 1 serves as a general introduction, providing the reader with information on the basic aspects of retinal anatomy and function, as well as the clinical and molecular genetic methods that may be applied in the analysis of retinal disease. The general aspects of retinal dystrophies are highlighted. In addition, the general characteristics of age-related macular degeneration are described, as several of the retinal diseases described in this thesis may closely resemble this most common macular disease.

Chapter 2 describes the principles of fundus autofluorescence, a relatively new imaging technique, which is highly relevant to the clinical evaluation of the retinal dystrophies that are studied in this thesis. An overview of the fundus autofluorescence findings in several retinal dystrophies is given, including those caused by mutations in the *BEST1* and *peripherin/RDS* gene.

Chapter 3 concerns the clinical and genetic analysis of retinal dystrophies caused by mutations in the *BEST1* gene. It starts with a description of the *BEST1* gene and its protein product, followed by an overview of the broad clinical spectrum of ocular diseases associated with *BEST1* mutations, as well as a discussion of the pathophysiologic mechanisms in these diseases (Section 3.1). Section 3.2 offers an analysis of the usefulness of fundus autofluorescence, optical coherence tomography, and fluorescein angiography in Best vitelliform macular dystrophy. The clinical variability of Best vitelliform macular dystrophy is discussed, as well as the variability in the underlying *BEST1* mutations. Finally, possible genotype-phenotype correlations in Best vitelliform macular dystrophy are evaluated. Section 3.3. describes the clinical characteristics and evaluation of the *BEST1* gene in multifocal vitelliform dystrophy.

Chapter 4 contains a clinical and genetic analysis of retinal dystrophies caused by mutations in the *peripherin/RDS* gene. The chapter starts with a description of the *peripherin/RDS* gene and its protein product, as well as an overview of the striking variety of phenotypes caused by *peripherin/RDS* mutations. Data from animal models are discussed, providing evidence for specific pathophysiologic mechanisms (Section 4.1). Section 4.2 discusses central areolar choroidal dystrophy, a phenotype caused by specific *peripherin/RDS* mutations, that may closely mimic atrophic age-related macular degeneration. Section 4.3 describes the phenotype of multifocal pattern dystrophy simulating Stargardt disease (STGD1)/fundus flavimaculatus, caused by *peripherin/RDS* mutations, which shows

close resemblance to Stargardt disease.

Chapter 5 first discusses the complement factor H (*CFH*) gene and the structure and function of its gene product, the CFH protein. The broad range of diseases associated with *CFH* gene variants is reviewed, as well as the genotype-phenotype correlations (Section 5.1.). In Section 5.2, the phenotype of basal laminar drusen is discussed, as well as the results of analysis of the *CFH* gene in this early-onset drusen phenotype that strongly resembles age-related macular degeneration.

The studies described in this thesis are further discussed and integrated in *Chapter 6*, which serves as a general discussion. A synopsis of the findings in this thesis is provided in *Chapter 7*.

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Chapter 2

Fundus autofluorescence imaging of retinal dystrophies

Section 2.1 is partially adapted from:

Analysis of visual pigment by fundus autofluorescence. T. Theelen, T.T.J.M. Berendschot, **C.J.F. Boon**, C.B. Hoyng, B.J. Klevering.
Exp Eye Res 2008;86:296-304.

Fundus autofluorescence in patients with inherited retinal diseases: Patterns of fluorescence at two different wavelengths. T. Theelen*, **C.J.F. Boon***, B.J. Klevering, C.B. Hoyng.
Ophthalmologe 2008;105:1013-1022. [in German]. (* joint first authors)

Section 2.2 is adapted from:

Fundus autofluorescence imaging of retinal dystrophies. **C.J.F. Boon**, B.J. Klevering, J.E.E. Keunen, C.B. Hoyng, T. Theelen.
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2.1. Fundus autofluorescence

Introduction

Fluorescence is an optical phenomenon, in which absorption of light of a given wavelength by a certain exogenous substance results in the emission of light of a longer wavelength by the same substance. In contrast to fluorescence, autofluorescence is an intrinsic property of cells. In the case of autofluorescence, exposure of cells to a specific wavelength results in the emission of light of a higher wavelength, arising from endogenous fluorophores. Such autofluorescence phenomena may also be observed in the human eye.

Origin of fundus autofluorescence

Short-wavelength fundus autofluorescence

The primary source of fundus autofluorescence (FAF) is lipofuscin within the retinal pigment epithelium (RPE). Although the RPE is very efficient in the phagocytosis and degradation of photoreceptor outer segments (OS), a small fraction of incompletely digestible products accumulates in the RPE lysosomes. This mixture of substances forms lipofuscin. Over the course of a lifetime, a single RPE cell is estimated to phagocytose more than 3.000.000.000 photoreceptor OS.¹ Granules containing lipofuscin and melanofuscin, a compound product of lipofuscin and melanin, may occupy as much as 20 to 33% of the free cytoplasmic space of an RPE cell above the age of 70.²

Lipofuscin comprises autofluorescent fluorophores, that mostly originate from the light- and vitamin A-dependent visual cycle that takes place in the photoreceptor OS.³ Some of these fluorophores have toxic properties. The exact composition of lipofuscin is still largely unknown, but an important constituent is the toxic fluorophore N-retinylidene-N-retinylethanolamine (A2E).^{3,4} The formation of lipofuscin and A2E is dependent on light exposure and the dietary intake of vitamin A, the precursor of 11-cis-retinal.⁵⁻⁷ With age, as the total amount of light exposure and vitamin A-intake increases, the amount of lipofuscin accumulation in the RPE increases,⁸⁻¹⁰ corresponding with an increase of FAF.¹¹ Besides A2E, several other constituents of RPE lipofuscin have been described that may contribute to the FAF signal.¹² Iso-A2E is formed by photo-isomerization of A2E.¹³ All-trans-retinal dimer phosphatidylethanolamine (atRAL dimer-PE) and all-trans-retinal dimer ethanolamine (atRAL dimer-E) are formed by pathways distinct from that of A2E, but are also formed from two all-trans-retinal molecules that condensate.^{12,14} Just like A2E, atRAL dimer-PE and atRAL dimer-E are also present at elevated levels in the lipofuscin-engorged RPE of *Abca4* mutant mice, as a result of disturbed retinoid transport.¹⁵⁻¹⁷

Lipofuscin and especially A2E are autofluorescent when excited with light from a specific wavelength and lie at the basis of FAF in normal and pathological conditions. FAF from

RPE lipofuscin can be excited with light of a wavelength between 430 and 600 nm, with a peak of excitation efficiency reached with excitation wavelengths between 480 and 510 nm (blue-green light).^{18,19} The emission spectrum of lipofuscin is also broad (480–800 nm), with a maximum in the 600 to 640 nm range.^{1,18} Other unidentified lipofuscin fluorophores, besides A2E and its derivatives that are described in the following section, also likely contribute to the FAF signal.¹⁸

The excitation wavelength that is generally used in clinical practice is 488 nm, generated by an argon laser or an optically pumped solid state laser in a confocal scanning laser ophthalmoscope (cSLO). FAF imaging can be performed at low excitation energies, that are well below the maximum safety limits of laser light exposure.^{1,20–22}

In normal individuals, retinal FAF images show a typical pattern (Fig. 2.1). A dark-grey area is seen in the fovea, due to the absorption of FAF by macular pigment and attenuation of FAF by RPE melanin.^{18,23} In addition, the rate of lipofuscin formation as a result of cone OS shedding may be considerably slower, compared to rods, which are located extrafoveally.²⁴ Accordingly, a histological study has revealed a dip in lipofuscin concentration just below the fovea, together with an increase of RPE melanin.²⁵ FAF gradually increases to lighter grey, with a maximum at 7–13° from the fovea, after which it decreases towards the peripheral retina.⁸ The optic disc normally shows absence of FAF due to a lack of RPE and lipofuscin. FAF is also absent at the site of the retinal vessels, because of the absorption of the light by blood.

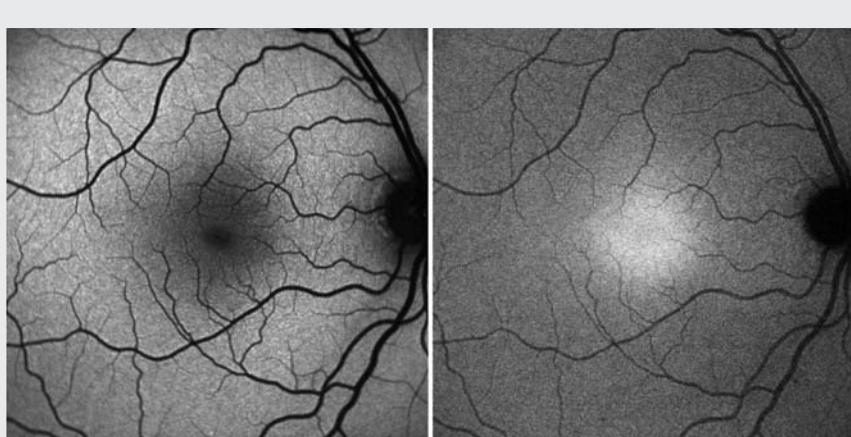


Figure 2.1.

Normal short-wavelength fundus autofluorescence (FAF) image (left), showing decreased FAF in the fovea, and absent FAF corresponding to the retinal vasculature and optic disc. On normal near-infrared FAF (right image), the fovea shows the highest signal, as opposed to short-wavelength FAF.

Near-infrared fundus autofluorescence

FAF images may be obtained not only with short-wavelength excitation (488 nm with the cSLO, see section 2.1), but also with near-infrared excitation (787 nm with the cSLO). Such near-infrared FAF (NIR-FAF), excited with a wavelength of 787 nm, and detected above 810 nm, recorded with the cSLO, produces a signal that is 60-100 times less intense than that obtained with short-wavelength FAF.^{18,26}

Although little is known about the exact origin of NIR-FAF, its main source is thought to arise from melanin and its derivatives,^{18,25-29} such as melanolipofuscin and oxidized melanin.^{2,18,30-33} Contrary to short-wavelength FAF, high NIR-FAF is seen in the fovea of normal subjects (Fig. 2.1), corresponding to higher RPE melanin in this area. Higher NIR-FAF is also seen in association with increased pigment, for instance in naevi (Fig. 2.2).

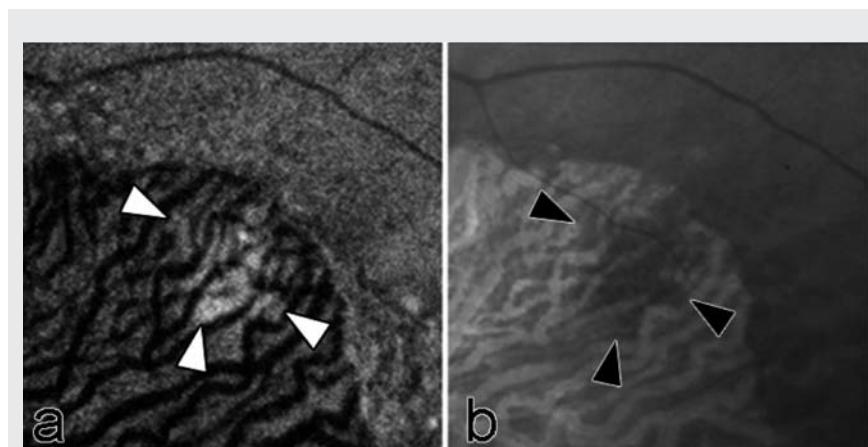


Figure 2.2.*

Pigmented choroidal naevus (black arrowheads), corresponding to focally increased near-infrared fundus autofluorescence (NIR-FAF, white arrowheads). The overlying retinal pigment epithelium (RPE) in atrophic in this patient with central areolar choroidal dystrophy. This RPE atrophy corresponds to decreased NIR-FAF, bordered by flecks of mildly increased NIR-FAF.

Abnormal NIR-FAF may be observed in a broad range of pathological retinal and/or choroidal dystrophic or degenerative conditions, such as Stargardt disease/STGD1 (Fig. 2.3A-C), multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus (Fig. 2.3D-E), central areolar choroidal dystrophy (Fig. 2.2). In STGD1 and multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus, lesions on NIR-FAF seem to exceed those on short-wavelength FAF both in size and number (Fig. 2.3). This could indicate that changes in melanin are more widespread than lipofuscin abnormalities and possibly even precede lipofuscin changes in these conditions. Distinct patterns of NIR-

FAF abnormalities are also seen in retinitis pigmentosa,³⁴⁻³⁷ chloroquine retinopathy,³⁸ as well as in age-related macular degeneration.²⁸

In the following sections, the term FAF will be reserved for short-wavelength FAF. NIR-FAF will not be discussed in further detail, as relatively little is known about its origin and clinical applications.

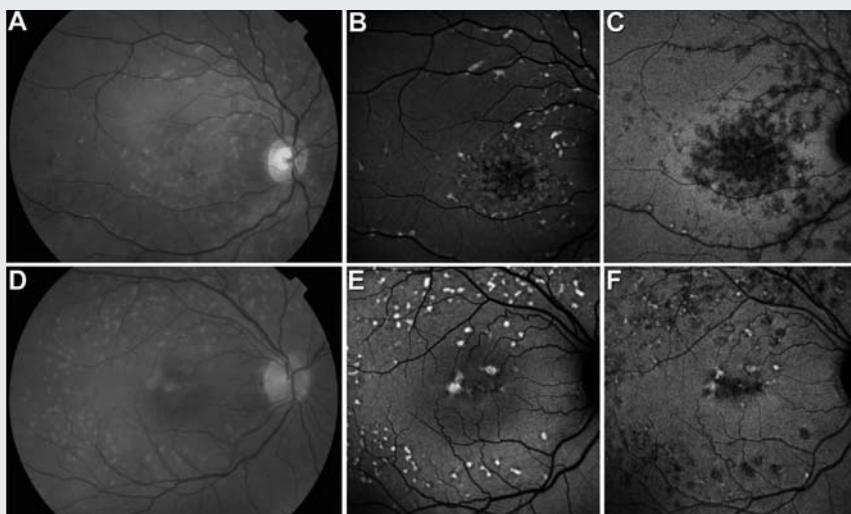


Figure 2.3.*

(**A-C**) Stargardt disease (*STGD1*)/fundus flavimaculatus, caused by autosomal recessive *ABCA4* gene mutations. **A.** Fundus photograph of *STGD1*, showing multiple yellowish, irregular flecks throughout the fundus, as well as a “beaten bronze” chorioretinal atrophy of the macula. **B.** Short-wavelength fundus autofluorescence (FAF) shows increased FAF of the flecks, and mottled areas of decreased FAF in the macula. **C.** On near-infrared fundus autofluorescence (NIR-FAF), the lesions are clearly larger and more numerous. On NIR-FAF, most lesions have a decreased intensity. (**D-F**) Multifocal pattern dystrophy simulating *STGD1*/fundus flavimaculatus, caused by an autosomal dominant mutation in the peripherin/RDS gene. The irregular yellowish flecks are highly similar to those in *STGD1*, on ophthalmoscopy (**D**), short-wavelength FAF (**E**), as well as NIR-FAF (**F**). The macular lesion in this patient, however, appears smaller and less atrophic.

Methods of fundus autofluorescence measurement

Fundus autofluorescence imaging with the confocal scanning laser ophthalmoscope

The use of confocal optics enables the suppression of out-of-focus light, and therefore enhances the image contrast.³⁹ FAF imaging with the cSLO was first described by von Rückmann and colleagues in 1995.⁴⁰ The FAF signal has a low intensity in comparison to fluorescein angiography and fundus reflectance. Therefore, a series of single FAF images is generally recorded, a mean image is subsequently calculated from these images, and pixel values are normalized, resulting in a sharp and detailed FAF image (Fig. 2.1A).¹ As mentioned previously, the cSLO uses an excitation wavelength of 488 nm generated by an argon laser or a solid-state laser. With the use of a band-pass barrier filter, with a short wavelength cut-off point at 500-525 nm (depending on the type of cSLO system), most of the reflected light is blocked, whereas autofluorescence light is allowed to pass and reach the detector. In ideal conditions (good patient cooperation, clear optical media, and sufficient amounts of lipofuscin accumulation in RPE cells), modern cSLO systems are able to visualize the human polygonal RPE cell pattern *in vivo*.^{41,42} The cSLO obtains monochromatic images, as it translates the polychromatic FAF signal, elicited by a single excitation wavelength, to an image composed of grey values. As a result, the image contrast is enhanced, although this implies a loss of information on the differentially emitted wavelengths. This technical limitation currently precludes the acquisition of color images with the cSLO. Using other techniques, this polychromatic signal may be recorded, and signals that arise from different fluorophores may be separated.⁴³ The practical details of acquiring FAF images with the cSLO can be found in section 2.2 of this chapter.

Fundus autofluorescence imaging with the fundus camera

A conventional fundus camera, using the excitation and emission filters as in fluorescein angiography, results in FAF images of poorer quality compared to cSLO FAF images.⁴⁴ This suboptimal FAF quality is caused by non-confocality of the camera system, which increases the unwanted registration of scattered light, a relatively low FAF signal, absorption of short-wavelength light by the crystalline lens, and fluorescence from the crystalline lens. However, modifications of the conventional fundus camera, by means of changing the excitation and barrier filters by moving their wavelengths to the higher (“red”) end of the spectrum, are able to limit the adverse effects of for instance the crystalline lens on the FAF image.^{1,44-46} Compared to this modified fundus camera-based acquisition of FAF images, the mean image obtained with the cSLO still results in FAF images with a better signal-to-noise ratio and better visualization of details, and less pseudofluorescence.⁴⁴ As the modified fundus camera uses longer excitation wavelengths (500-610 nm) than the cSLO, FAF from other fluorophores than those seen with the cSLO may also be detected.⁴⁴

Clinical applications of fundus autofluorescence

Introduction

Since the introduction of FAF in clinical practice, it has been used in a wide range of ocular diseases affecting the posterior segment of the eye. These diseases not only include age-related macular degeneration and retinal dystrophies, which will be discussed separately, but also conditions such as central serous chorioretinopathy,^{47,48} choroidal melanocytic lesions,⁴⁹⁻⁵² macular hole,^{53,54} as well as various forms of posterior uveitis.⁵⁵⁻⁵⁹ In many of these diseases, FAF is often able to visualize more detailed and/or widespread abnormalities than would be suspected by other means of imaging. However, the clinical implications and relevance of FAF in most of these diseases has not yet been firmly established.

Retinal dystrophies

The broad range of retinal dystrophies is associated with an equally broad range of genetic causes, which may markedly influence the phenotypic outcome and prognosis. The impact of the underlying genetic defect on the function of cones, rods, as well as the RPE, may affect the FAF signal and the pattern of FAF abnormalities. Depending on the genetic defect, FAF abnormalities of lesions in predominantly macular phenotypes such as STGD1 (Fig. 2.3A-C, 2.4), multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus (Fig. 2.3D-F), Best vitelliform macular dystrophy, and central areolar choroidal dystrophy, may only be observed in the centre of the macula, or may extend well beyond the anatomical borders of the macula. This will be illustrated and discussed more elaborately in section 2.2 of this chapter, as well as in the chapters dealing with several of these phenotypes separately.

Conversely, FAF abnormalities may also be predominantly present in the peripheral and midperipheral retina, while appearing to relatively spare the macula, such as in various forms of retinitis pigmentosa. Ring-shaped areas of increased FAF, that do not have a visible correlate on ophthalmoscopy, may be observed within the macular area that appears to be spared, for instance in retinitis pigmentosa and cone-rod dystrophy of different genetic backgrounds.^{37,60,61} These ring-shaped zones may migrate with time, narrowing concentrically in many cases of retinitis pigmentosa, and apparently demarcate areas of impaired retinal function.^{61,62} These findings point to a common downstream pathway in these diseases, involving a “wavefront” of degeneration of lipofuscin-filled RPE. Therefore, FAF in these retinal conditions has a clear functional and prognostic correlate that may prove to be of use in the follow-up of these diseases.

Some hereditary retinal dystrophies are associated with a markedly decreased to absent FAF signal. A striking example in this regard is early-onset severe rod-cone dystrophy, also known as Leber congenital amaurosis type 2, caused by autosomal recessive mutations in the RPE65 gene.⁶³ This gene defect leads to a virtual absence of rhodopsin, all-trans-retinal, 11-cis-retinal, and - consequently - an absence of lipofuscin fluorophores, despite a relative sparing of photoreceptor viability.⁶⁴ Interestingly, recent pioneering studies

have been published on gene therapy in such patients with severe RPE65 mutations.⁶⁵⁻⁶⁷ In these patients, *RPE65* gene delivery was achieved by subretinal injection of viral vectors loaded with this gene. In none of these preliminary studies, FAF was an outcome parameter, although one study measured FAF before starting therapy.⁶⁵ It would be interesting to see if an increase of FAF intensity may be noted in these or future cases, which could be regarded as an indirect indication of successful gene transfer. However, such measurements would ideally require reliable quantification of FAF, which is at present insufficiently possible, as discussed further on in this introduction.

Age-related macular degeneration

Lipofuscin accumulation is the characteristic feature of RPE cell aging. Excessive lipofuscin accumulation and its previously discussed adverse effects has been shown to be linked with complement activation, inflammation, and oxidative damage.⁶⁸ As all of these factors play a role in age-related macular degeneration (AMD), FAF appears a suitable imaging modality to evaluate AMD.

In early AMD, alterations of FAF do not necessarily correlate with lesions on ophthalmoscopy and/or fluorescein angiography, such as drusen.⁶⁹⁻⁷¹ Drusen may show either increased, decreased, or normal FAF intensity.^{69,72,73} Larger, soft and confluent drusen often show a patchy, mildly increased FAF pattern.^{69,70,74} Such a FAF pattern appears to be associated with an especially high risk for the development of choroidal neovascularization.^{70,74,75} Increased FAF intensities in areas of hyperpigmentation are thought to be at least partially due to the accumulation of melanolipofuscin, whereas a decreased FAF signal would correspond to absence of RPE cells or a decrease in their lipofuscin content as a result of degenerative processes.^{71,73} Different patterns of FAF can be observed in early AMD, although it is unknown if these patterns are associated with a different genetic background, and their possible prognostic implications are largely unclear.⁶⁹ Areas of increased FAF in patients with early AMD and a normal visual acuity correspond to decreased sensitivity on microperimetry and fine matrix mapping.^{76,77} Geographic atrophy, characterized by areas of profound chorioretinal atrophy that often initially occur in the parafoveal area, tends to spare the fovea for a prolonged period during progression.^{78,79} Such foveal sparing may not only be observed in atrophic AMD, but also in macular dystrophies such as central areolar choroidal dystrophy, pattern dystrophy associated with maternally inherited diabetes and deafness, and Stargardt disease (Fig. 2.4).

Histopathological studies have shown that ophthalmoscopically visible areas of geographic atrophy correspond to atrophy of the photoreceptor-RPE-choriocapillaris complex.⁸⁰⁻⁸³ Such an area of profound atrophy is generally bordered by a junctional zone of lipofuscin- and melanolipofuscin-filled RPE cells. These observations correspond to FAF findings, often showing absence of FAF in the area of geographic atrophy, which is bordered by a junctional zone of increased FAF.^{84,85} This zone of high FAF, which usually does not have a visible correlate on ophthalmoscopy or other imaging methods, often precedes a loss of FAF and enlargement of the atrophic area.^{85,86} The zone of high FAF also

correlates functionally with already decreased retinal sensitivity, affecting rods more than cones.^{77,87} This area of increased FAF may therefore represent a “wavefront” of RPE and photoreceptors that is still functional, but already going through a degenerative process involving lipofuscin accumulation. These findings are reminiscent of the aforementioned rings of increased FAF in some retinal dystrophies that also demarcate areas of impaired retinal function, indicating some common downstream pathway that is visualized by FAF.

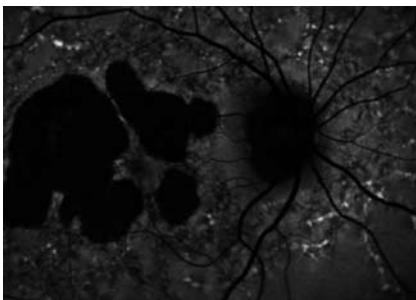


Figure 2.4.

Fundus autofluorescence image of foveal sparing in a patient with Stargardt disease. An island of fovea remains in the macula, in the midst of an area of profound atrophy of the retinal pigment epithelium.

Distinct FAF patterns of the zone of increased FAF surrounding the geographic atrophy may be observed, which show high intraindividual symmetry.^{84,88,89} There is a high interindividual variability in the rate of geographic atrophy progression in atrophic AMD.⁹⁰ These variable growth rates significantly correlate with the underlying different FAF patterns of the surrounding zone.⁹¹ At present, it is unknown if these different patterns relate to different genetic backgrounds. These findings underscore the importance of lipofuscin accumulation in the pathogenesis of atrophic AMD, as well as the role of FAF in the identification of prognostic markers and high-risk characteristics for progression of geographic atrophy.⁹¹ In this sense, FAF may prove to be a useful tool not only in natural history studies, but possibly also in future interventional clinical trials,⁹⁰⁻⁹² provided that reliable quantification becomes possible.⁹³⁻⁹⁵

FAF and its implications have been less well characterized in neovascular AMD compared to atrophic AMD. An increased FAF signal appears to be less common in choroidal neovascularization,^{96,97} feeding the speculation that RPE lipofuscin may not play an important role in the formation of choroidal neovascularization. In early choroidal neovascularization, normal FAF correlates with visual acuity, lesion size, and symptom length.⁹⁸ Certain FAF patterns in choroidal neovascularization may predict the visual outcome better than fluorescein angiography.⁹⁹ These findings indicate that there may be a role for FAF in future treatment protocols in neovascular AMD. FAF may also be useful in the follow-up after autologous transplantation of RPE and choroid in atrophic and neovascular AMD.¹⁰⁰⁻¹⁰²

Future perspectives in fundus autofluorescence

Combination of fundus autofluorescence and high-resolution spectral domain optical coherence tomography

Further development of the cSLO has led to the recent commercial availability of the Spectralis HRA/optical coherence tomography(OCT)(Heidelberg Engineering, Heidelberg, Germany). This instrument combines FAF imaging and high-resolution spectral domain OCT in one, together with an eye tracking system that minimizes movement artefacts. As such, this technique allows the simultaneous recording and accurate orientation of FAF and high-resolution OCT sections at anatomic sites of interest. A pixel-to-pixel correlation may be achieved, allowing *in vivo* high-resolution, three-dimensional mapping and follow-up of retinal structures and their abnormalities.^{103,104} Structures on FAF may be reliably compared with their correlate on the high-resolution OCT, making this combined imaging technique very promising to obtain further insight into the pathogenesis of a multitude of retinal diseases. Owing to the eye tracking system, specific lesion sites may be exactly located and compared on follow-up images.

Quantification of fundus autofluorescence

The FAF signal of a certain location is generally categorized as normal, increased, or decreased, compared to the normal background signal of the same image. A major challenge remains the exact quantification of absolute FAF intensity.^{95,105} As the cSLO normally processes and normalizes FAF images, which are therefore not suitable for quantitative FAF analyses, the image processing software should be turned off for quantitative measurement purposes. When comparing absolute FAF intensities between different examinations and different patients, several variables must be taken into account.¹⁰⁵ First, variations in technical image acquisition such as laser power and detector sensitivity must be avoided and standardized. In addition, patient variables such as eye movements, positioning, pupil size, refractive error, and the degree of lens and vitreous opacities must be taken in account. Finally, dynamic metabolic processes of lipofuscin accumulation, dark adaptation, and bleaching effects may represent confounding factors. It appears impossible to standardize all these factors. It is only when most of these factors can be dealt with sufficiently that reliable FAF signal quantification comes within reach.

Analysis of visual pigment by fundus autofluorescence

Irradiation with short-wavelength visible light is able to bleach retinal photopigments, starting with the dissociation of rhodopsin into opsin and 11-cis-retinal, which changes the optical density of the photoceptors.^{11,106,107} During this photopigment bleaching with light from a wavelength of 488 nm, using the cSLO, an increase of FAF is observed, according to an asymptotical, illumination-dependent kinetics (Fig. 2.5).¹⁰⁸ Such an increase in the level of FAF is presumed to originate from the reduced absorption of light by the bleached photoreceptor pigments. In retinal dystrophies, visual pigment density

may be altered, which may be objectively analyzed by such visual pigment measurement techniques.¹⁰⁸ However, a number of difficulties in these measurements remain to be resolved, such as incomplete bleaching of the fovea due to absorption of light by macular pigment, the difficulty of accurate visual pigment density calculations in large areas of RPE atrophy, and the previously discussed problems in quantification of FAF.¹⁰⁸ Should these difficulties be overcome, analysis of visual pigment by FAF may prove to be of value in the early diagnosis, an improved understanding of pathophysiologic mechanisms, as well as a better follow-up in retinal dystrophies.

Other imaging techniques

Adaptive optics technology is able to improve the spatial resolution by reducing the effects of rapidly changing optical distortion.^{109,110} Using adaptive optics, individual cellular structures, such as the photoreceptors and RPE, may be visualized with a lateral resolution of $\sim 2 \mu\text{m}$.^{22,110-114} Adaptive optics imaging in various retinal dystrophies indicates that macular cones may display distinct characteristics that appear to depend on the genetic background of the underlying disease.¹¹⁵ The adaptive optics technique may therefore provide additional ultrahigh resolution insight into the mechanisms of photoreceptor and RPE degeneration.

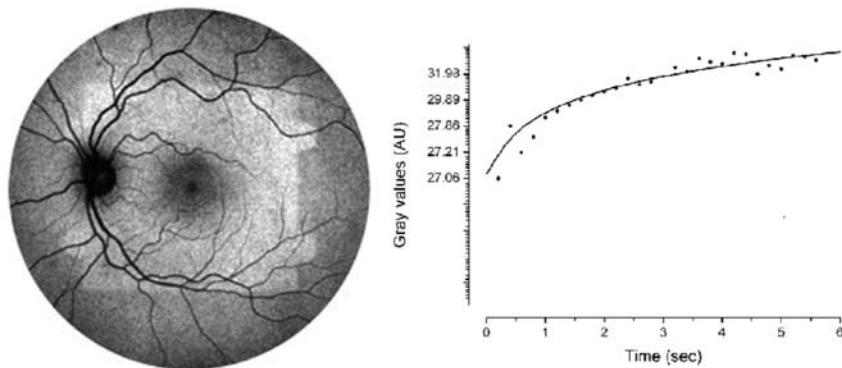


Figure 2.5.

Left image: short-wavelength fundus autofluorescence (FAF) image recorded with the Heidelberg Retina Angiograph 2 (HRA 2) (using a 55° lens), taken immediately after an initial 30° exam with the same illumination settings. The site of the previous FAF examination is outlined as a bright area with the shape of the internal mirror of the HRA 2 camera. This area corresponds to bleached visual pigments. Right image: during short-wavelength FAF illumination, FAF intensity increases during photopigment bleaching, as depicted by this gray value plot. The FAF increase due to bleaching is most pronounced in a circular area around the fovea.

Metabolic mapping with FAF imaging of fluorophores involved in cell metabolism may give information on the metabolic state of healthy and diseased retinal cells.^{43,105,116,117} To be able to record such a very weak FAF signal, it must be separated from lipofuscin-derived FAF and influences from the crystalline lens.¹⁰⁵

Two-photon excited fluorescence imaging, using infrared light, is able to more effectively concentrate excitation energy both spatially and temporally.¹⁰⁵ This technique may therefore potentially result in a better signal-to-noise ratio and better image contrast, although little is known about the exact structures and fluorophores that are visualized. In addition, a biologically safe level of laser energy is not yet available with this technique.

Blood flow characteristics may be measured when Doppler flowmetry can be added to FAF and/or OCT imaging modalities.^{109,118} A combination of FAF, OCT, and electrophysiological information from multifocal electroretinography, could represent an elegant means to simultaneously obtain detailed morphological and functional information in different retinal conditions.^{109,119}

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2.2. Fundus autofluorescence imaging of retinal dystrophies

Abstract

Objective: To illustrate the spectrum of short-wavelength fundus autofluorescence (FAF) abnormalities in a variety of retinal dystrophies.

Methods: We examined 112 patients with retinal dystrophies, including Stargardt disease (8 patients), Best vitelliform macular dystrophy (27 patients), central areolar choroidal dystrophy (48 patients), and various pattern dystrophies (29 patients). All retinal dystrophies were confirmed by molecular genetic analysis. FAF imaging was performed with a confocal scanning laser ophthalmoscope.

Results: A broad range of characteristic FAF patterns was observed in Stargardt disease, Best vitelliform macular dystrophy, central areolar choroidal dystrophy, as well as the pattern dystrophies. FAF imaging provided qualitative information, reflecting the degree of accumulation of lipofuscin and its related fluorophores.

Conclusion: FAF imaging constitutes a useful additive tool in the diagnosis and follow-up of various retinal dystrophies.

Introduction

During the last decade, fundus autofluorescence (FAF) imaging of the retina has emerged as a useful, non-invasive imaging technique in the diagnosis and follow-up of several retinal dystrophies.¹⁻⁵ Obtaining sufficient-quality FAF images generally becomes feasible starting from 5 years of age.⁵ Short-wavelength excited FAF origins from fluorophores within the retinal pigment epithelium (RPE), and RPE lipofuscin distribution is the principal cause for the observed FAF patterns.⁶ Lipofuscin contains a mixture of fluorescent molecules that are by-products of the visual cycle, many of which have not been thoroughly characterized to date.⁶⁻⁹

The major lipofuscin fluorophore is N-retinylidene-N-retinylethanolamine (A2E), which exerts diverse toxic effects on the RPE.⁹⁻¹⁸ While the slight and diffuse increase of lipofuscin with ageing is physiologic, extensive and focal accumulation of lipofuscin is pathologic and may be encountered in a variety of retinal dystrophies.^{13,14,17,19} Histopathological studies have found an excessive accumulation of lipofuscin and/or A2E in several retinal dystrophies, including those associated with mutations in the ABCA4 gene, the BEST1 (VMD2) gene, and the *peripherin/RDS* gene.²⁰⁻²² The present study illustrates the appearance of characteristic FAF patterns in selected, relatively frequent retinal dystrophies.

Material and methods

Autofluorescence imaging

FAF imaging was performed with a confocal scanning laser ophthalmoscope (cSLO, Heidelberg Retina Angiograph 2; Heidelberg Engineering, Dossenheim, Germany) and all images were acquired by the same operator (C.J.F. Boon). After pupil dilation to at least 6 mm with one drop of phenylephrine and one drop of tropicamide, FAF imaging was performed using the 30° field of view and a resolution of 1536 x 1536 pixels, using an optically pumped solid state laser (488 nm) for excitation. A barrier filter (500 nm) suppressed blue argon excitation light at 488 nm, so that reflectance signals did not contribute to the FAF image obtained from the posterior pole of the examined eye.

For the acquisition of FAF images, a standard procedure was followed, which included focusing of the retinal image in the infrared reflection mode at 820 nm, sensitivity adjustment at 488 nm and acquisition of 9 single 30° x 30° FAF images which encompassed the entire macular area and at least part of the optic disc. In order to improve the signal-to-noise ratio of the FAF signal, the nine single images were aligned and a mean image was calculated after detection and correction of eye movements using the software provided by the manufacturer (Heidelberg Eye Explorer, Heidelberg Engineering, Dossenheim, Germany). Images were digitized and saved on hard disc for further analysis and processing. In general, areas of increased FAF are believed to represent lipofuscin increase either within or outside RPE cells, whereas decreased FAF may indicate RPE cell death and atrophy of the RPE-photoreceptor complex.

Patients

A total of 112 patients with a variety of retinal dystrophies participated in the study. All examinations were undertaken with the understanding and written consent of each subject, and all procedures were conducted in accordance with the Declaration of Helsinki. All patients were consecutively seen in a tertiary referral setting at the Department of Ophthalmology of the Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands.

We included 8 patients with autosomal recessive Stargardt disease/fundus flavimaculatus (STGD1), 27 patients with Best vitelliform macular dystrophy (BVMD), 48 patients with central areolar choroidal dystrophy (CACD), and 29 patients with some form of pattern dystrophy: 9 of these patients had the adult-onset foveomacular vitelliform dystrophy (AFVD) subtype, 16 patients were diagnosed with multifocal pattern dystrophy simulating STGD1, one patient had butterfly-shaped pattern dystrophy, and 3 patients had pattern dystrophy related to maternally inherited diabetes and deafness (MIDD).

In all STGD1 patients, the diagnosis was confirmed by the identification of 2 compound heterozygous mutations in the *ABCA4* gene. In all 27 BVMD patients, the diagnosis was confirmed by an absent light rise on the electro-oculogram and the finding of a specific variant in the *BEST1* gene. All patients with CACD and multifocal pattern dystrophy simulating STGD1, as well as the patient with butterfly-shaped pattern

dystrophy, carried mutations in the *peripherin/RDS* gene. The study population of patients with multifocal pattern dystrophy simulating STGD1 was a subset of recently reported patients.²³ In the AFVD patients, no mutations in *BEST1* or *peripherin/RDS* were identified. This is in accordance with a recent study that found that mutations in these genes are relatively rare in AFVD patients.²⁴ The patients with MIDD-related pattern dystrophy carried a mitochondrial m.3243A>G mutation.

After their medical history was obtained, all patients underwent an extensive ophthalmic examination, including best-corrected Snellen visual acuity, indirect ophthalmoscopy and fundus photography. In addition to FAF imaging, subsequent examination included optical coherence tomography and, in selected cases, fluorescein angiography, electroretinography and electro-oculography.

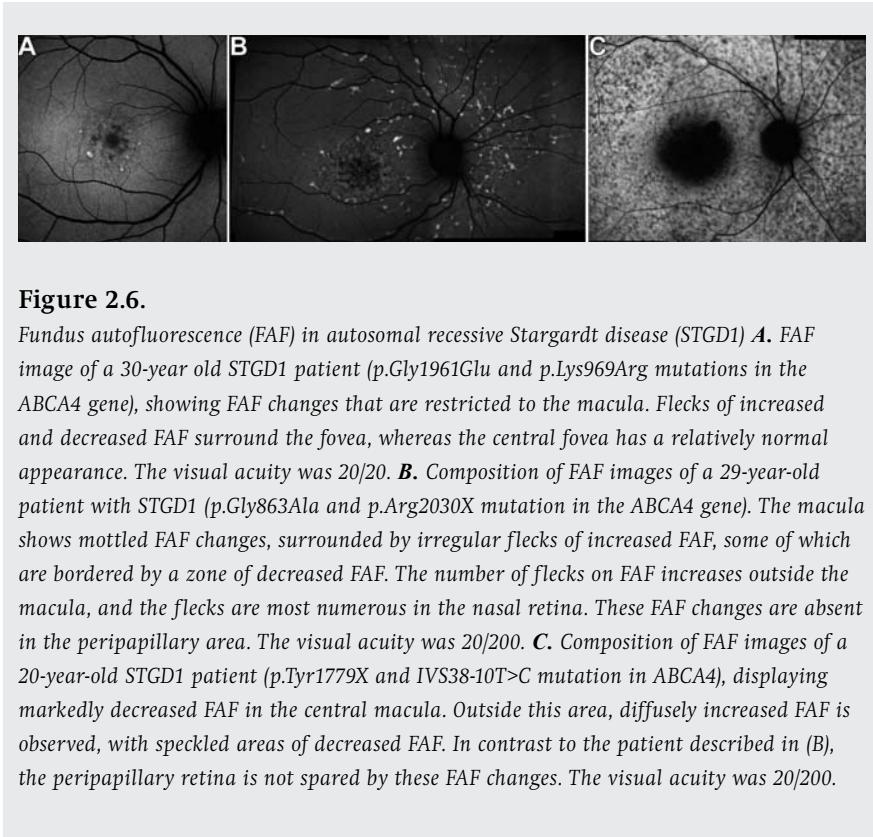
Results

Autosomal recessive Stargardt disease / fundus flavimaculatus (STGD1)

In our study group, all STGD1 patients displayed FAF abnormalities. Six STGD1 patients showed areas of focally increased FAF, sometimes bordered by a zone of decreased FAF. In addition, spots of decreased FAF were visible in these patients. In two patients, these areas of altered FAF were restricted to the macula (Fig. 2.6A), whereas four patients showed a distribution of these flecks throughout or beyond the posterior pole. Many of these flecks corresponded with irregular yellowish flecks on ophthalmoscopy, but some flecks of increased FAF were not seen on ophthalmoscopy. The highest number of these extramacular flecks was seen in the nasal retina (Fig. 2.6B). Two patients showed a diffusely increased FAF pattern throughout the fundus, with a speckled pattern of small areas of decreased FAF. In these patients, a large area of markedly decreased FAF could be observed in the macula (Fig. 2.6C). Seven patients showed relatively normal FAF in the parapapillary zone, but one patient showed parapapillary FAF abnormalities (Fig. 2.6C).

Best vitelliform macular dystrophy

All patients with Best vitelliform macular dystrophy in our study group displayed FAF abnormalities, except both patients who were in the carrier stage, in whom FAF abnormalities were either very discrete or absent (Fig. 2.7A). In lesions in earlier stages, in which vitelliform material appeared more abundant on ophthalmoscopy, FAF imaging displayed areas of markedly increased FAF. In the vitelliform stage, the round lesions showed homogeneously increased FAF (Fig. 2.7B). In the pseudohypopyon stage, FAF was visible mainly in the inferior portion of the lesion, co-locating with the ophthalmoscopically visible pseudohypopyon (Fig. 2.7C). In the vitelliruptive or scrambled-egg stage, the amount of autofluorescent material was reduced to a certain extent and condensed mainly at the borders of the lesion, whereas the central part of the lesion showed slightly decreased FAF (Fig. 2.7D). In the atrophic stage, areas corresponding with chorioretinal atrophy corresponded with a major decrease of the FAF signal (Fig. 2.7E).

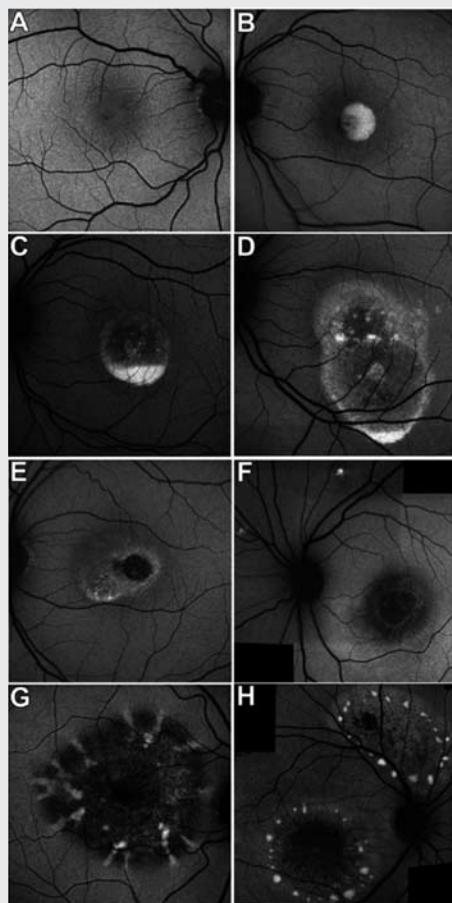
**Figure 2.6.**

Fundus autofluorescence (FAF) in autosomal recessive Stargardt disease (STGD1) **A.** FAF image of a 30-year old STGD1 patient (*p.Gly1961Glu* and *p.Lys969Arg* mutations in the ABCA4 gene), showing FAF changes that are restricted to the macula. Flecks of increased and decreased FAF surround the fovea, whereas the central fovea has a relatively normal appearance. The visual acuity was 20/20. **B.** Composition of FAF images of a 29-year-old patient with STGD1 (*p.Gly863Ala* and *p.Arg2030X* mutation in the ABCA4 gene). The macula shows mottled FAF changes, surrounded by irregular flecks of increased FAF, some of which are bordered by a zone of decreased FAF. The number of flecks on FAF increases outside the macula, and the flecks are most numerous in the nasal retina. These FAF changes are absent in the peripapillary area. The visual acuity was 20/200. **C.** Composition of FAF images of a 20-year-old STGD1 patient (*p.Tyr1779X* and *IVS38-10T>C* mutation in ABCA4), displaying markedly decreased FAF in the central macula. Outside this area, diffusely increased FAF is observed, with speckled areas of decreased FAF. In contrast to the patient described in (B), the peripapillary retina is not spared by these FAF changes. The visual acuity was 20/200.

Reduced FAF also dominated in the cicatricial stage. However, some spots of discretely increased FAF were present within these areas of decreased FAF (Fig. 2.7F). In general, FAF imaging was able to visualize even very small amounts of vitelliform material that were not readily seen on ophthalmoscopy. Finally, one patient displayed a spoke-like pattern of FAF changes simulating macular pattern dystrophy (Fig. 2.7G), and another patient had multifocal vitelliform lesions (Fig. 2.7H).

Central areolar choroidal dystrophy

In central areolar choroidal dystrophy (CACD), we will describe FAF characteristics in the stages of CACD as described by Hoyng and Deutman (1996). In stage I CACD, a discrete speckled increase of FAF was observed in the parafoveal area (Fig. 2.8A). The FAF abnormalities corresponding with early stage II involved a speckled, round to oval area of predominantly increased FAF. This area of abnormal FAF enlarged towards late stage II, and the spots of increased FAF within the lesion decreased in favor of speckles of decreased FAF. Stage III was characterized by the appearance of well-defined areas of absent FAF outside the fovea, which corresponded with chorioretinal atrophy on ophthalmoscopy

**Figure 2.7.**

Fundus autofluorescence (FAF) in Best vitelliform macular dystrophy (BVMD).

A. Patient in the carrier stage, with no evident FAF abnormalities. The visual acuity (VA) was 20/20. A p.Thr6Pro variant in the BEST1 gene was found in this patient. **B.** Vitelliform stage of BVMD, showing a round macular lesion with an intensely increased FAF, corresponding with the yellow subretinal material seen on ophthalmoscopy.

This patient, who carried a p.Thr6Pro variant in BEST1, had a VA of 20/20.

C. In the pseudohypopyon stage, material of highly increased FAF is seen inferiorly. Some small spots of increased FAF are also observed in the superior part of the lesion. This patient, carrying a p.Thr6Pro variant in BEST1, had a VA of 20/20.

D. Vitelliruptive or "scrambled-egg" stage, showing a more dispersed localization of material of increased FAF. This material is mainly situated at the borders of the lesion, whereas the central part of the lesion shows some foci of increased FAF.

A p.Lys194_Alala195insVal mutation in BEST1 was identified in this patient, who had a VA of 20/20. **E.** Example of the atrophic stage, showing a well-demarcated area absent FAF, corresponding with chorioretinal atrophy. This patient had a VA of 20/32, and carried a p.Leu82Val variant in BEST1. **F.** Composition of FAF images of a patient in the cicatricial stage of BVMD, showing a central lesion of reduced FAF, bordered by a ring-shaped zone of slightly increased FAF. Two small extramacular lesions of increased FAF can also be observed. The VA was 20/125, and a p.Thr6Pro variant in BEST1 was found in this patient.

G. Patient with BVMD showing a spoke-like distribution of FAF changes adjacent to a central area of decreased to absent FAF. A p.Ala243Val variant was found in BEST1 in this patient, who had a VA of 20/25. **H.** Example of FAF abnormalities in a patient with multifocal vitelliform dystrophy, who carried a p.Gly299Ala variant in the BEST1 gene.

A large suprapapillary lesion shows FAF changes that resemble those in the central lesion. The VA was 20/80.

(Fig. 2.8B). These areas enlarged and multiplied on follow-up (Fig. 2.8C). Stage IV CACD was characterized by foveal involvement of the area of absent FAF. Eventually, a round to oval area of virtually absent FAF was seen in patients with end-stage CACD, bordered by a small band of increased FAF (Fig. 2.8D).

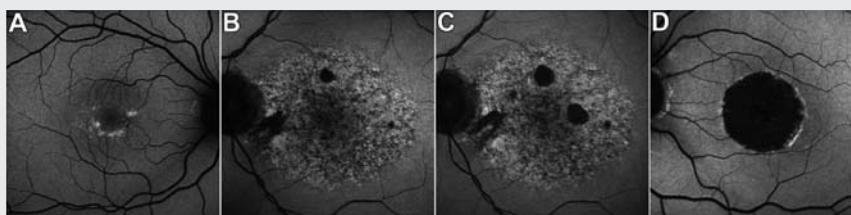


Figure 2.8.

Fundus autofluorescence (FAF) in central areolar choroidal dystrophy (CACD). All patients carried a p.Arg142Trp mutation in the peripherin/RDS gene. **A.** FAF image of stage I CACD with a diffuse parafoveal increase of FAF. The visual acuity was 20/20. **B.** Early stage III CACD, showing areas of absent FAF outside the fovea, corresponding with profound chorioretinal atrophy, on a background of a speckled changes of increased and decreased FAF. **C.** Same patient as in panel (B), showing enlargement of the existing atrophic area after a follow-up period of 1.5 years. In addition, new regions of absent FAF have appeared. The visual acuity was still 20/20. **D.** Stage IV CACD, with a round macular lesion of absent FAF, bordered by a small band of increased FAF. Note the discrete FAF of larger choroidal vessel underlying the lesion. The visual acuity was 20/800.

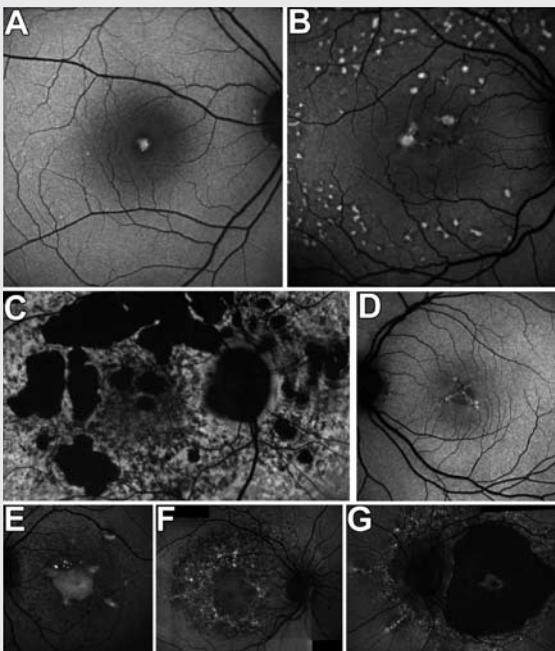
Pattern dystrophies

Adult-onset foveomacular vitelliform dystrophy

The vitelliform lesions in the patients with adult-onset foveomacular vitelliform dystrophy corresponded with round to oval lesions with increased FAF in 8 patients (Fig. 2.9A), sometimes with a small spot of reduced FAF. Two patients had unilateral disease. In two patients, zones of increased and decreased FAF were seen within the same lesion. One of these patients had lesions with characteristics of the scrambled-egg and pseudohypopyon stage as seen in Best vitelliform macular dystrophy. In another patient, the lesion in one eye showed decreased FAF, corresponding with chorioretinal atrophy on ophthalmoscopy. Small multifocal lesions of increased FAF were seen in the macular area of one patient. In all patients, lesions were smaller than one disc diameter on FAF.

Multifocal pattern dystrophy simulating STGD1

In general, FAF in patients with multifocal pattern dystrophy simulating STGD1 shows changes that are very similar to the FAF changes seen in patients with the fundus

**Figure 2.9.**

Fundus autofluorescence (FAF) in various pattern dystrophies. **A.** FAF image of adult-onset foveomacular vitelliform dystrophy, showing a small foveal lesion of increased FAF. This patient had a visual acuity (VA) of 20/32. No mutations were found in peripherin/RDS or BEST1. **B.** FAF of a patient with multifocal pattern dystrophy simulating STGD1, displaying irregular flecks of markedly increased FAF, many of which show small adjacent zones of decreased FAF. The FAF characteristics

of these lesions in the macular area and along the retinal vascular arcades are very similar to those observed in STGD1 (See Fig. 2.6B). The VA was 20/20. This patient carried a p.

Asp145fsX30 mutation in the peripherin/RDS gene. **C.** Advanced multifocal pattern dystrophy simulating STGD1, with a large area of speckled increase and decrease of FAF extending beyond the retinal vascular arcades and optic disc. Multifocal, well-circumscribed patches of absent FAF are observed within this area, corresponding with RPE atrophy on ophthalmoscopy. This patient had a VA of 20/25, and carried a p.Pro147fsX4 mutation in peripherin/RDS.

D. FAF image of a patient with butterfly-shaped pigment dystrophy. The clinically visible butterfly-shaped macular changes are also seen on FAF. The pigmented part of the lesion showed increased FAF, surrounded by decreased FAF. This patient, who carried a p.

Arg220fsX34 mutation in peripherin/RDS, had a VA of 20/20. **E.** Patient with maternally inherited diabetes and deafness (MIDD)-associated pattern dystrophy (m.3243A>G mutation), showing a large pseudovitelliform macular lesion of increased FAF, surrounded by satellite lesions of increased FAF and dispersed spots of decreased FAF. The visual acuity was 20/40.

F. Pattern dystrophy in a MIDD patient (m.3243A>G mutation) with speckled changes of increased and decreased FAF in the macula and surrounding the optic disc. The foveal area shows a relatively normal FAF aspect. The visual acuity was 20/20. **G.** Advanced pattern dystrophy in a MIDD patient carrying the m.3243A>G mutation, showing irregular changes of increased and decreased FAF along the temporal retinal vascular arcades and surrounding the optic disc. The macula shows a large area of markedly decreased to absent FAF with the exception of a foveal island, which seems remarkably spared. The visual acuity was 20/20.

flavimaculatus subtype of STGD1. In multifocal pattern dystrophy simulating STGD1, the STGD1-like flecks displayed highly increased FAF, often with small adjacent zones of decreased FAF (Fig. 2.9B). In a later stage, these flecks of increased FAF showed confluence towards a large ring-shaped area of mottled FAF abnormalities surrounding the macula and optic disc, without sparing of the parapapillary retina (Fig. 2.9C). The changes could gradually involve the macula (Fig. 2.9C). Within this ring-shaped area of increased FAF, granular zones of substantially decreased FAF appeared. After one or more decades of disease, profound chorioretinal atrophy of the posterior pole could occur, which corresponded with a severely decreased or absent FAF signal. In the macular area, FAF changes were variable. In 8 eyes, discrete spots of either increased or decreased FAF were seen in the fovea. A group of irregular flecks of mostly increased FAF, comparable to but often larger than the peripheral flecks, was seen in 7 eyes.

In 8 eyes of 5 patients, large, irregularly shaped lesions were seen, showing spots of increased and decreased FAF. In one eye, a butterfly-shaped lesion was seen on FAF as well as on ophthalmoscopy. One or more well-defined areas of severely decreased FAF could be observed in 8 eyes of 5 patients, corresponding with profound chorioretinal atrophy (Fig. 2.9C).

Butterfly-shaped pigment dystrophy

We examined one patient with butterfly-shaped pigment dystrophy, in whom the butterfly-shaped lesions on ophthalmoscopy were also observed on FAF (Fig. 2.9D). The pigmented part of the butterfly-shaped lesion showed increased FAF, and was surrounded by decreased FAF.

Pattern dystrophy associated with maternally inherited diabetes and deafness (MIDD)

In one MIDD patient, spots of decreased and increased FAF were scattered throughout the macula, in one eye with a central area of increased FAF corresponding with a pseudovitelliform lesion at ophthalmoscopy (Fig. 2.9E). Another patient showed the same speckled aspect of spots of increased and decreased FAF, with a relatively normal FAF aspect of the fovea (Fig. 2.9F). The third MIDD patient who displayed advanced FAF changes in the posterior pole and around the optic nerve, showed apparently normal FAF of both foveas, corresponding with a normal visual acuity (Fig. 2.9G).

Discussion

In the present study, FAF abnormalities were observed in all examined eyes with ophthalmoscopically visible retinal dystrophies. Focally increased FAF was the most common finding, frequently accompanied by areas of decreased or even absent FAF. We observed specific FAF abnormalities that were dependent on the examined retinal disorder.

Fundus autofluorescence in STGD1

Autosomal recessive Stargardt disease/fundus flavimaculatus (STGD1) is caused by mutations in the *ABCA4* gene.²⁵ With disease progression, excessive increase of lipofuscin and especially A2E can be observed, which is accelerated by light exposure.²⁶⁻²⁸ This abnormal increase of RPE lipofuscin is probably one of the first detectable pathophysiological changes in STGD1.²⁶ Therefore, FAF imaging appears a promising method to monitor patients with STGD1.²⁹

In our study, central macular lesions showed largely decreased FAF, reflecting chorioretinal atrophy. In most patients, these central lesions were surrounded by smaller, well-circumscribed lesions of predominantly increased FAF, largely corresponding with the irregularly shaped yellowish flecks that were observed at ophthalmoscopy. However, some of these lesions on FAF were not readily seen at ophthalmoscopy. This emphasizes the potential of FAF to detect pathologic changes in STGD1 in advance to clinically visible abnormalities. Lesions were sometimes confined to the macula, but an innumerable amount of flecks of increased FAF, scattered throughout the retina, could also be observed. Like in the normal retina,¹⁹ the rate of lipofuscin accumulation in STGD1 shows considerable intraretinal variation.^{26,30} A disease sequence of six stages in STGD1 has been proposed by Cideciyan and colleagues,²⁶ based on lipofuscin accumulation, retinoid cycle kinetics, as well as rod, cone and RPE cell loss. Stage I shows entirely normal results, whereas the only detectable abnormality in stage II is an increase in mean FAF intensity. In stage III, these changes are superimposed by a focal increase of FAF intensity (Fig. 2.6A and B). Different FAF patterns have been described in STGD1, which appear to correspond with functional abnormalities.^{29,31} Lesions tend to enlarge and show confluence, while the initially elevated FAF signal evolves towards decreased to absent FAF intensities. These decreasing FAF intensities are due to the loss of lipofuscin-laden RPE cells, which is accompanied by increasing photoreceptor degeneration and slowing of the retinoid cycle. In stage VI (end-stage) *ABCA4*-related retinal degeneration, variably sized regions of absent FAF are observed as a consequence of complete RPE and photoreceptor degeneration.

In cases of STGD1 with abnormal cone and rod responses on electroretinography, most patients show a decreased FAF signal at the macula, sometimes in combination with patches of decreased FAF beyond the vascular arcades, corresponding with chorioretinal atrophy. This group of STGD1 patients also tends to show more visual loss.²⁹ Although there is no direct evidence that conventional short-wavelength FAF accelerates the disease process of *ABCA4*-related retinal dystrophies, there are indications that some retinal degenerative diseases like STGD1 may have lower light damage thresholds.^{21,30,32,33} Therefore, some authors advocate the use of reduced intensity FAF imaging.^{32,33}

Fundus autofluorescence in Best vitelliform macular dystrophy

Best vitelliform macular dystrophy (BVMD) is an autosomal dominant retinal dystrophy that predominantly affects the macula. BVMD is caused by mutations in the *BEST1* gene.³⁴ Typically, BVMD patients show bilateral round to oval yellow subretinal lesions in the

macula that resemble an egg-yolk at a certain point of time.³⁵ In a minority of cases, multifocal vitelliform lesions may be encountered.^{36,37} Most histopathological studies have found an abnormal increase of RPE lipofuscin and A2E in the RPE of donor eyes of BVMD patients.^{20,37-41}

In our study, FAF imaging allowed a detailed appreciation of the lesions in all BVMD patients. FAF was also able to visualize small amounts of vitelliform material that were not seen at ophthalmoscopy. In early stages, BVMD lesions showed a predominantly increased FAF, which decreased towards later stages. This increased FAF signal may not only find its origin in RPE lipofuscin, but may also be derived from autofluorescent fluorophores within the subretinal vitelliform material. Previous studies indicate that BVMD lesions may remain relatively constant in size,⁴ but they may also enlarge when evolving to later stages.^{42,43} In virtually all patients, internal FAF characteristics change substantially during follow-up.⁴ Lower visual acuity appears to be associated with a more irregular pattern of increased FAF within the lesion, due to scarring or hemorrhage.^{4,44} In our study, lesions in later stages of BVMD were characterized by a generally decreased FAF. This gradual loss of FAF in later disease stages indicates that this subretinal material of increased FAF gradually disappears when RPE cell death progresses and atrophy and/or scars develop.

Fundus autofluorescence in central areolar choroidal dystrophy

Most cases of autosomal dominant CACD appear to be associated with mutations in the *peripherin/RDS* gene, but genetic heterogeneity has been described.⁴⁵⁻⁴⁷ In our study, patients with autosomal dominant CACD showed increased FAF in the macular area early in the course of the disease, suggesting lipofuscin accumulation in the macular RPE. In late stage II CACD, zones of decreased FAF may become more prominent, which probably corresponds to a gradual loss of RPE cells. Thus, our FAF findings indicate a progressive lipofuscin accumulation and consecutive RPE damage during the first two stages. With time, lesions may extend beyond the retinal vascular arcades and optic nerve head.^{45,48} FAF in stage III CACD shows sharply demarcated areas of severely decreased to absent FAF. These atrophic areas develop within the areas of granular FAF changes. Therefore, the initial diffusely affected RPE may finally result in regions of total loss of RPE. These regions gradually enlarge and finally involve the foveal RPE in end-stage CACD. In this stage, a well-defined, round to oval area of absent FAF is seen, surrounded by a small band of increased FAF. Interestingly, FAF abnormalities may precede ophthalmoscopically visible changes in CACD.⁴⁵ This makes FAF imaging especially suitable for non-invasive screening of families with CACD.

Fundus autofluorescence in pattern dystrophies

The phenotypically and genetically heterogeneous group of pattern dystrophies is characterized by a variety of deposits of yellow, orange or gray pigment, predominantly in the macular area.⁴⁹⁻⁵² The classification of Gass discriminates between five main categories of pattern dystrophies: adult-onset foveomacular vitelliform dystrophy

(AFVD), butterfly-shaped pigment dystrophy, reticular dystrophy of the pigment epithelium, multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus (MPD), and fundus pulverulentus.³⁵ In the group of AFVD patients in the present study, all lesions were smaller than one disc diameter in size and generally showed increased FAF. These results correspond with previous FAF findings in AFVD patients and with the histopathological finding of increased lipofuscin in AFVD lesions.⁵³⁻⁵⁵ Like in BVMD, AFVD patients may show small multifocal lesions of increased FAF, as well as a scrambled-egg and pseudohypopyon aspect.

In patients with MPD, which is often caused by *peripherin/RDS* mutations, the posterior pole may be scattered with irregular flecks resembling those in STGD1 on FAF, as well as on ophthalmoscopy and optical coherence tomography.²³ Like in STGD1, the flecks in MPD may extend beyond the macular area. In later stages, these flecks may show confluence to a ring-shaped area of abnormal FAF, surrounding the macula and optic disc. Central macular lesions show a broad range of FAF changes, often with predominantly increased FAF, especially in earlier stages. In time, macular lesions may also show markedly decreased FAF due to profound chorioretinal atrophy. Therefore, it may be difficult to discriminate between STGD1 and MPD on the basis of the FAF aspect. Distinguishing features between MPD and STGD1 are the autosomal dominant pattern of inheritance, the relatively late age at onset, the comparatively good and stable visual acuity, and the absence of a “dark choroid” on fluorescein angiography.²³ Molecular genetic analysis may aid in the differential diagnosis between these different entities.

In butterfly-shaped pigment dystrophy, patients display macular lesions with a spoke-like pigment pattern surrounded by a zone of depigmentation, somewhat resembling the shape of a butterfly.⁵⁶ The phenotype of butterfly-shaped pigment dystrophy is genetically heterogeneous.^{57,58} In a case caused by a *peripherin/RDS* missense mutation (p.Cys213Tyr), increased amounts of lipofuscin have been found in the RPE on histopathologic examination.²² This corresponds with the finding in our study of increased FAF of the pigmented part of the lesion in the patient with butterfly-shaped pigment dystrophy.

Pattern dystrophy phenotypes may also be associated with syndromes, such as myotonic dystrophy, pigment dispersion syndrome, and mitochondrial syndromes like maternally inherited diabetes and deafness (MIDD) and MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome.⁵⁹⁻⁶³ MIDD and MELAS are associated with mutations in the mitochondrial DNA, with the m.3243A>G point mutation being the most frequently identified mutation.⁶⁴ As much as 86% of patients with maternally inherited diabetes and deafness (MIDD) may show some form of pattern dystrophy. In our study, RPE changes in MIDD patients were easily defined on FAF as speckled areas of increased and decreased FAF in the macula and surrounding the optic disc. The intriguing feature of foveal sparing can be observed on FAF in a MIDD patient with advanced pattern dystrophy,⁶⁵ which is illustrated in Fig. 2.9G.

Conclusions

FAF imaging is a useful tool for the identification and follow-up of lesions associated with lipofuscin accumulation or RPE cell loss. As such, FAF imaging may yield important additional information in a diagnostic setting in relatively frequent retinal dystrophies, such as STGD1, BVMD and in retinal dystrophies associated with *peripherin/RDS* mutations. FAF may visualize more lesions compared to ophthalmoscopy. Moreover, FAF imaging provides qualitative information, as it reflects the lipofuscin content in lesions, and FAF changes appear to relate to functional abnormalities in at least some retinal dystrophies.^{1,29,66} However, non-lipofuscin accumulating parts of a lesion may be missed with FAF. Therefore, FAF alone may not be useful for lesion size measurement. Retinal dystrophies may present with a considerable clinical variability. Late stages with RPE cell loss may look similar in various retinal dystrophies and in other retinal disorders such as age-related macular degeneration. In this respect, FAF may be of limited use in the differential diagnosis between different forms of retinal dystrophy. Molecular genetic testing may be very helpful in these cases. FAF imaging is a non-invasive imaging modality that is a straightforward and relatively patient-friendly means to get an overview of the accumulation of fluorophores like lipofuscin and atrophic changes within the RPE-photoreceptor complex.^{2,6} Therefore, FAF imaging may constitute a convenient tool for cross-sectional and family studies, as well as in the follow-up of various retinal dystrophies. In addition, FAF imaging may also play a role as a parameter for the evaluation of therapeutic effects in future clinical treatment trials.

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Chapter 3

Clinical and molecular genetic analysis of phenotypes associated with mutations in the *BEST1* gene

Section 3.1 is adapted from:

The spectrum of ocular phenotypes caused by mutations in the *BEST1* gene. **C.J.F. Boon**,
B.J. Klevering, B.P. Leroy, C.B. Hoyng, J.E.E. Keunen, A.I. den Hollander.
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T. Theelen, E.H. Hoefsloot, M.J. van Schooneveld, J.E.E. Keunen, F.P.M. Cremers, B.J. Klevering,
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Arch Ophthalmol 2007;125:1100-1106.

3.1. The spectrum of ocular phenotypes caused by mutations in the *BEST1* gene

Abstract

Bestrophin-1 is an integral membrane protein, encoded by the *BEST1* gene, which is located in the basolateral membrane of the retinal pigment epithelium. The bestrophin-1 protein forms a Ca^{2+} activated Cl^- channel and is involved in the regulation of voltage-dependent Ca^{2+} channels. In addition, bestrophin-1 appears to play a role in ocular development. Over 120 different human *BEST1* mutations have been described to date, associated with a broad range of ocular phenotypes. The purpose of this review is to describe this spectrum of phenotypes, ranging from Best vitelliform macular dystrophy and adult-onset foveomacular vitelliform dystrophy, autosomal dominant vitreoretinochoroidopathy, the microcornea, rod-cone dystrophy, cataract, posterior staphyloma (MRCS) syndrome, and autosomal recessive bestrophinopathy. The genotype-phenotype correlations that are observed in association with *BEST1* mutations are discussed. In addition, *in vitro* studies and animal models that clarify the pathophysiological mechanisms are reviewed.

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Abbreviations: ADVIRC, autosomal dominant vitreoretinochoroidopathy; AFVD, adult-onset foveomacular vitelliform dystrophy; AMD, age-related macular degeneration; ARB, autosomal recessive bestrophinopathy; BVMD, Best vitelliform macular dystrophy; Cav, voltage-dependent calcium; CERES, composite exonic regulatory element of splicing; CNV, choroidal neovascularization; EOG, electro-oculography; ERG, electroretinography; ESE, exonic splicing enhancer; ESS, exonic splicing silencer; FAF, fundus autofluorescence; MRCS, microcornea, rod-cone dystrophy, early-onset cataract, and posterior staphyloma; OCT, optical coherence tomography; RPE, retinal pigment epithelium.

1. Introduction

Best vitelliform macular dystrophy (BVMD) is among the most frequently encountered autosomal dominant retinal dystrophies and predominantly affects the macula. BVMD was the first disease shown to be caused by mutations in the *BEST1* gene, which encodes the bestrophin-1 protein that localizes to the retinal pigment epithelium (RPE).¹ Subsequent studies showed that *BEST1* gene mutations may also be found in patients with adult-onset foveomacular vitelliform dystrophy (AFVD).²⁻⁴ BVMD and AFVD are related phenotypes, with abnormalities that are generally restricted to the macula. However, more widespread ocular abnormalities may arise in association with specific *BEST1* gene mutations that cause autosomal dominant vitreoretinochoroidopathy (ADVIRC) and the autosomal dominant microcornea, rod-cone dystrophy, early-onset cataract, and posterior staphyloma (MRCS) syndrome.⁵⁻⁸ The same applies to autosomal recessive bestrophinopathy (ARB), the human null phenotype of bestrophin-1, which is associated with high hyperopia and shallow anterior chambers.⁹ Therefore, ADVIRC, the MRCS syndrome, as well as ARB belong to a spectrum of diseases with ocular developmental abnormalities that extend beyond the retina.

In this paper, we aim to review the characteristics of the *BEST1* gene and its multifunctional protein product bestrophin-1, with an emphasis on the broad spectrum of ocular phenotypes associated with mutations in this gene. The effects of different *BEST1* mutations are discussed, as well as their genotype-phenotype correlations. Available *in vitro* and animal models are addressed, as well as the histopathological observations in *BEST1*-related diseases, that expand our insight in the pathogenesis. Finally, perspectives on future therapeutic strategies are discussed.

2. Molecular biology of the human *BEST1* gene

2.1. The *BEST1* gene

The human *BEST1* gene was identified in 1998 by Petrukhin and colleagues.¹ *BEST1* is located on chromosome 11q12, spans 15 kilobases of genomic DNA and contains 11 exons of

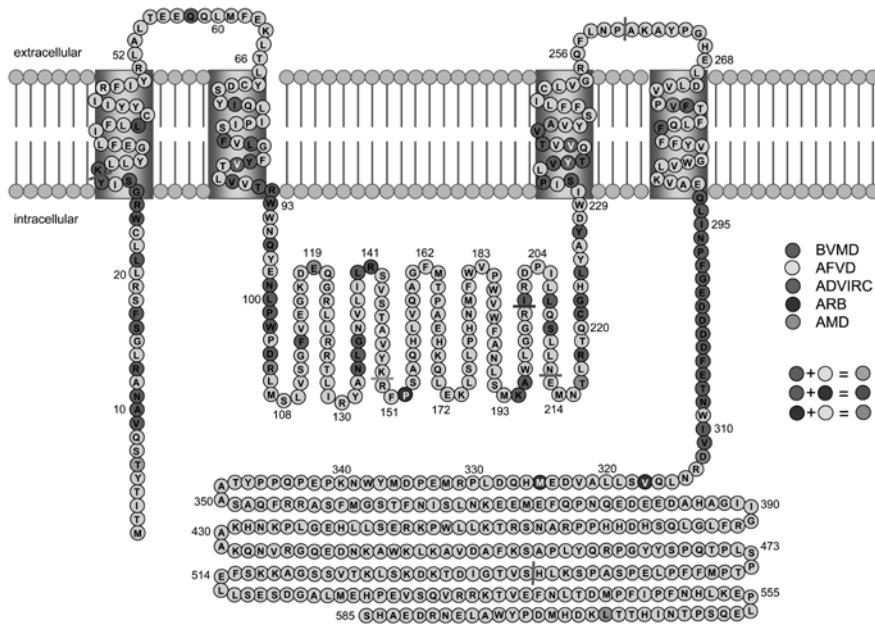
which 10 are protein-coding.^{1,10} Eight years later, the mouse ortholog was characterized.¹¹ An alternative name for *BEST1* is *VMD2*, but the Human Genome Organisation and the Mouse Genome Database nomenclature committees recently recommended the *BEST* (in humans) and *Best* (in mice) nomenclature. The *BEST1* gene is the founding member of a family of four paralogs, the other three called *BEST2*, *BEST3*, and *BEST4*.¹²⁻¹⁴ The *BEST1* gene is expressed predominantly in the retinal pigment epithelium (RPE), although some expression has also been detected in kidney, brain, spinal cord, and testis.^{1,10} The RPE-specific expression is driven by a region within -154 to -104 base pairs of the *BEST1* promoter, which contains two E-boxes.¹⁵⁻¹⁷ These E-boxes are able to bind transcription factors, such as OTX2 and MITF.¹⁵⁻¹⁷

2.2. The bestrophin-1 protein

Bestrophin-1 is an integral membrane protein consisting of 585 amino acids.^{14,18,19} The bestrophin-1 protein localizes to the basolateral plasma membrane of the RPE, but may also be present intracellularly.²⁰ The regional expression level of bestrophin-1 is higher in the RPE outside the macula compared to the macular RPE.²¹ Consequently, it has been speculated that the macular phenotype associated with *BEST1* mutations might be related to a relative insufficiency of wild-type bestrophin-1 in the macula.²¹

Two topological models of human bestrophin-1 have been proposed that are partly contradictory. In a model constructed by Tsunenari and colleagues, bestrophin-1 has 5 transmembrane domains.¹⁴ In contrast, the protein is predicted to have 4 transmembrane domains in the model of Milenkovic and colleagues (Fig. 3.1).¹⁹ The major difference between these models is that amino acids ~199-233 are situated in an extracellular loop in the Tsunenari model, whereas these amino acids are located in a large cytoplasmic loop in the model by Milenkovic. Bestrophin-1 most likely oligomerizes to dimers, or possibly to tetramers/pentamers.^{12,22,23} Several splice variants of bestrophin-1 have been described. The mRNA from isoform 1 results from the transcription of all 11 exons and leads to the translation of wild-type bestrophin-1. Isoform 2 results from alternative splicing of exon 7, causing premature termination of the protein at amino acid 435, should the mRNA escape nonsense-mediated decay.^{1,12} In isoform 3 (498 amino acids) and 4 (604 amino acids), exon 2 is skipped.^{1,12} Isoform 3 shows an additional skipping of exon 8, which deletes a highly conserved transmembrane domain that is presumed to be essential for bestrophin Cl⁻ channel activity.^{1,12} Isoform 4 produces an elongated protein due to additional alternative splicing of exon 10.^{1,12}

Bestrophin-1 is a multifunctional protein, with activity both at the basolateral plasma membrane of the RPE and intracellularly.¹² Several lines of evidence strongly indicate that bestrophin-1 functions as a Cl⁻ channel,^{12,14,20,23-30} activated by intracellular Ca²⁺.^{12,20,23,25,26,30,31} Human bestrophin-1 is also highly permeable to HCO₃⁻, indicating that it may also function as a HCO₃⁻ channel.^{32,33} Recent experiments suggest that the bestrophin-1 Cl⁻ channel is gated through the binding of Ca²⁺ by the C-terminal protein region ranging from amino acids 312 to 323.³¹ This region is called EF1 and was postulated to be an EF hand, which is a Ca²⁺ binding helix-loop-helix structural protein domain.^{31,34}

**Figure 3.1.***

Protein model of bestrophin-1 (adapted from Milenkovic¹⁹). The model of Milenkovic and colleagues was used, as their experiments have shown that only the four domains depicted in the figure are located in the cell membrane. The known human disease-associated mutations are indicated with colours. The protein variants found in age-related macular degeneration are also shown, although functional studies question their significance in disease pathogenesis. Coloured residue: missense mutation or in-frame deletion. Coloured bar: nonsense or frameshift mutation. Abbreviations: BVMD, Best vitelliform macular dystrophy; AFVD, adult-onset foveomacular vitelliform dystrophy; ADVIRC, autosomal dominant vitreoretinochoroidopathy; ARB, autosomal recessive bestrophinopathy; AMD, age-related macular degeneration.

Two additional C-terminal domains appear to be required for this Ca^{2+} regulated Cl^- channel gating.³¹ First, the acidic amino acid-rich region 293-308, which is located in the cytosol, adjacent to the last transmembrane domain. The second relatively acidic region, ranging from amino acids 350-390, also likely influences Ca^{2+} regulated Cl^- channel gating, possibly in the oligomerized state and/or through phosphorylation processes.³¹ Besides activation by Ca^{2+} , *in vitro* experiments also suggest that bestrophin-1 Cl^- channel activity is regulated by cell volume.^{12,35-37} The presumed Cl^- channel function is further

supported by the observation that many disease-causing *BEST1* mutations in BVMD and ARB result in a dysfunction of bestrophin-1 Cl⁻ channel function.^{9,12,20,23,30,38}

Bestrophin-1 also acts as an inhibitor of intracellular voltage-dependent Ca²⁺ (Cav) channels,³⁹⁻⁴² through the interaction with the β subunit of these Cav channels.⁴² The proline-rich region ranging from amino acids 330-370, especially the Pro₃₃₀XXXPro₃₃₄ domain, in the cytoplasmic C-terminus of bestrophin-1, appears important for the modulation of Cav channels.⁴² Evidence on the type of effect of bestrophin-1 on Cav channels is somewhat conflicting. Rosenthal and colleagues found that human bestrophin-1 regulates Cav channels in RPE-J cells by altering their voltage dependence and accelerating their activation, without changing the Ca²⁺ current.⁴⁰ In contrast, Yu and co-workers did find a decrease in endogenous Ca²⁺ current in both RPE-J cells and Human Embryonic Kidney (HEK)-293 cells, without a significant effect on Cav channel voltage dependence.⁴² Possibly, the level of inhibition of Cav channels by bestrophin-1 is dependent on bestrophin-1 Cl⁻ channel closure.^{31,42} Opening of these Cl⁻ channel channels would consequently relieve this inhibition, receiving positive feedback by the subsequent Ca²⁺ influx.^{31,42}

The electro-oculogram (EOG) reflects the ion conductance of the RPE and its light peak response has been presumed to be generated by activation of a Ca²⁺-sensitive Cl⁻ conductance.^{12,27} In BVMD, the EOG typically shows a markedly decreased light peak response,⁴³ which may at least partly be due to bestrophin-1 dysfunction.¹² However, in mice, disruption of *Best1* does not diminish the RPE-derived light peak on the electroretinogram (ERG), nor does it substantially alter Ca²⁺-sensitive Cl⁻ conductances.³⁹ These results thus challenge the hypothesis that bestrophin-1 is a classical Ca²⁺-sensitive Cl⁻ channel and is responsible for the abnormal EOG in BVMD through this mechanism.^{12,39,44} Rather than bestrophin-1 Ca²⁺-sensitive Cl⁻ channel function, bestrophin-1 modulation of intracellular Cav channels seems to be necessary for the generation of a normal RPE-generated ERG light peak response in mice and rats.³⁹⁻⁴¹ Of note, it is unclear to what extent such a RPE-specific ERG light peak in mice and rats can be compared to the human EOG light peak.

Clinical findings in ADVIRC, the MRCS syndrome, and ARB patients that carry *BEST1* mutations indicate that bestrophin-1 may also be required for normal ocular development, although the mechanisms are as yet unclear.⁶⁻⁹ In mice, bestrophin-1 mRNA expression is relatively high during the late phase of the embryonic development and the early postnatal period.⁴⁵ Bestrophin-1 protein expression occurs considerably later in mice, and appears to be a good marker of RPE differentiation.⁴⁵ The onset of bestrophin-1 protein expression in the mouse starts at postnatal day 10, and coincides with the visual competence of photoreceptors.

The RPE is known to be essential in the regulation of growth factor signaling to the choroid and sclera, and bestrophin-1 may also prove to be of influence on these mechanisms.^{46,47} In addition to the proposed functions as a Cl⁻ channel, a regulator of voltage-dependent Ca²⁺ channel, and the role in ocular development, bestrophin-1 may also play a role in epithelial secretion, like several other Ca²⁺-sensitive Cl⁻ channels.^{24,26,48,49}

Bestrophin-1 possibly also exerts its influence on intracellular mechanisms. The bestrophin-1 Cl⁻ channel function as well as its regulating influence on intracellular Ca²⁺ currents may be able to influence intracellular processes, for instance phagocytosis and lysosomal function.^{40,46,50-52} The C-terminal cytoplasmic domain of bestrophin-1 may be phosphorylated and is able to interact with protein phosphatase 2A,⁵³ which plays a role in intracellular signaling.⁵⁴

Bestrophin homologs have been detected in animals, fungi, and prokaryotes, but not in protozoans or plants.⁵⁵ Phylogenetic analysis suggests that the bestrophins originated by duplication and divergence of a common protein at the base of the eukaryotic tree.⁵⁶ The function of the other three bestrophin paralogs in humans is unclear. In mice, Best2 mRNA has been identified in the eye, colon, nasal epithelia, trachea, brain, lung, and kidney. Bestrophin-2 has been identified in mouse colon and the nonpigmented ciliary epithelium of the ciliary body of the eye.⁵⁷ Consequently, in the latter case, there is a possible regulatory role in the formation of aqueous humor.⁵⁷ In addition, bestrophin-2 may have a function in the olfactory epithelium,⁵⁸ although studies are contradictory in this regard.⁵⁷ The function of human bestrophin-3 and bestrophin-4 is unknown, but in mice and rats, bestrophin-3 splice variants have been found in exocrine glands, heart, vascular smooth muscle cells, lung, testis, and kidney.^{59,60} Bestrophin-3 is also expressed in the mouse heart.⁶¹

2.3. Human *BEST1* gene mutations

2.3.1. Best vitelliform macular dystrophy

The BVMD phenotype is inherited in an autosomal dominant fashion. The cloning of the *BEST1* gene was facilitated by the identification of a large 12-generation Swedish family consisting of more than 250 individuals affected with BVMD.⁶² The causative gene was subsequently mapped to chromosome 11q13 by linkage analysis,^{63,64} and was characterized in 1998 by positional cloning of a retina-specific expressed sequence tag.^{1,10} It was suggested that BVMD might be genetically heterogeneous,⁶⁵ but this does not seem to be the case.⁶⁶ Mutations in *BEST1* are detected in nearly all BVMD cases with a positive family history.^{3,67,68} Those BVMD cases without *BEST1* mutations did not have a positive family history, and may have either been misdiagnosed, or may represent phenocopies.^{3,67}

To date, more than 100 different *BEST1* mutations have been reported in BVMD.^{1,3,10,67,69-81} An overview of *BEST1* mutations can be found in the VMD2 database of the University of Regensburg (http://www-huge.uni-regensburg.de/VMD2_database) and on the Retina International website (<http://www.retina-international.com/sci-news/vmd2mut.htm>), and is depicted in Fig. 3.1. The majority (92%) of mutations identified in BVMD are missense mutations located in the N-terminal half of the protein (Fig. 3.1). This part of the protein exhibits the highest evolutionary conservation, while the C-termini differ substantially between paralogs.⁵⁶ Only one nonsense mutation,⁷⁹ one splice site mutation,³ two frameshift mutations,^{72,74} and three in-frame deletions have been reported.^{3,10,67} Of the

latter mutations, only the in-frame deletion p.Ile295del and the c.1574delCA frameshift mutation were found in cases with well-described, stringent clinical inclusion criteria, as well as a positive family history of BVMD and/or segregation of the mutation in these families.^{10,72} The majority (61%) of *BEST1* mutations have been found uniquely in one family. Seven mutations (p.Thr6Pro, p.Arg218Cys, p.Tyr227Asn, p.Ala243Val, p.Ile295del, p.Glu300Asp, p.Asp301Glu) have been found in more than three families.^{1-3,10,38,67,71,72,74,76,82} The p.Thr6Pro mutation, for instance, is notably frequent in the Netherlands,^{1,83} which may be due to a founder effect. Three *BEST1* mutations have been reported to have arisen *de novo*.^{69,78,81}

Multifocal vitelliform dystrophy, a clinically and genetically heterogeneous condition, may be caused by *BEST1* mutations in approximately 60% of cases.⁸³ To date, 9 mutations have been described in association with multifocal vitelliform dystrophy, including 7 missense mutations (p.Thr6Pro, p.Ser16Tyr, p.Arg92Ser, p.Ala195Val, p.Tyr227Asn, p.Asn296Lys, and p.Phe298Ser), one in-frame deletion (p.Asp302_Asp304del), and one in-frame insertion (p.Lys194_Ala195insVal).⁸²⁻⁸⁴ Most of these mutations were also seen in patients with unifocal, central BVMD and/or AFVD, indicating that *BEST1*-related multifocal vitelliform dystrophy is a multifocal variant of BVMD and/or AFVD.

2.3.2. Adult-onset foveomacular vitelliform dystrophy

Fundoscopic findings in AFVD overlap with BVMD,⁸⁵ but AFVD is characterized by a later age at onset, a smaller lesion size, a slower progression, and a slightly subnormal to normal EOG.^{86,87} Many cases appear to be sporadic, but a small number of families with an autosomal dominant inheritance pattern have been reported.^{85,88,89} AFVD is genetically heterogeneous: mutations have also been reported in the *peripherin/RDS* gene.^{90,91} In a group of 32 AFVD patients, 8 (25%) had *BEST1* mutations.³ Two of these mutations, p.Thr6Pro and p.Ala243Val, have also been detected in families with BVMD. Two other mutations, p.Arg47His and p.Asp312Asn, have only been identified in AFVD. In another series of 28 patients with various macular diseases, mutations were detected in one of three families with AFVD and in one patient with bull's eye maculopathy.^{2,4} These two mutations, p.Ala146Iys and p.Glu119Gln, have not been identified previously. In two other studies, no *BEST1* mutations were identified in 10 and 12 AFVD patients, respectively.^{92,93}

2.3.3. Autosomal dominant vitreoretinochoroidopathy (ADVIRC) and the MRCS syndrome

To date, three heterozygous *BEST1* missense mutations have been found in ADVIRC: p.Val86Met,⁸ p.Val235Ala,⁵ and p.Tyr236Cys.⁸ One mutation, p.Val239Met, has been described in the MRCS syndrome, a genetically heterogeneous disease.⁶⁸ These four mutations in ADVIRC and the MRCS syndrome all appear to alter normal splicing of *BEST1*. *In vitro* splicing assays suggest that affected individuals heterozygous for p.Val86Met, p.Tyr236Cys and p.Val239Met produce three different *BEST1* isoforms. In addition to the product of the wildtype allele, each mutant allele is capable of producing two abnormal proteins, one containing a missense mutation, while the other one contains

an in-frame deletion upon exon-skipping.⁸ The p.Val86Met mutation leads to a deletion of exon 4, whereas p.Tyr236Cys and p.Val239Met cause a deletion of exon 6 and exon 7, respectively.^{5,8} *In vitro* splicing assays indicate that the p.Val235Ala mutation causes a duplication of exon 6 by a rare phenomenon called trans-splicing, but it cannot be excluded that the duplication is an artefact of the splicing assay. The splicing defects caused by these mutations are likely due to the disruption of exonic splice enhancers (ESEs),⁸ which is further described in paragraph 4.1.

2.3.4. Autosomal recessive bestrophinopathy

ARB is an autosomal recessive disease caused by homozygous or compound heterozygous mutations in the *BEST1* gene. To date, eight patients from six different families have been described.^{9,94} Two patients from the same family carried a homozygous p.Arg200X nonsense mutation.⁹ Six patients carried compound heterozygous missense mutations. The following combinations were encountered: p.Arg141His and p.Val317Met (one patient), p.Leu41Pro and p.Pro152Ala (one patient), p.Leu41Pro and p.Arg141His (one patient), p.Asp312Asn and p.Met325Thr (in two patients from the same family), and p.Leu88del17 combined with p.Ala195Val (one patient).^{9,94} In addition, two individuals from a single family were compound heterozygous for p.Arg141His and p.Tyr29X, with a phenotype described as atypical BVMD.⁷⁹ However, the description of the phenotype is highly suggestive of ARB. Three of the ARB mutations, p.Leu41Pro, p.Arg141His, and p.Ala195Val, in a single heterozygous state, have also been reported in BVMD,^{3,67,74,83} whereas p.Asp312Asn has also been reported as a single heterozygous mutation causing AFVD.³

2.3.5. Age-related macular degeneration

BEST1 mutation analysis in patients with age-related macular degeneration (AMD) has led to the identification of a small number of sequence variants with questionable significance. Three studies have screened the *BEST1* gene in large numbers of AMD patients. One study did not detect any disease-associated variants in 200 AMD patients.³ In another study, two different alterations, p.Thr216Ile and p.Leu567Phe, were detected in 3 (1.1%) of 259 AMD patients.^{2,4} In a third study, 5 different alterations were detected in 5 (1.5%) of 321 AMD patients.⁶⁷ Three patients carried a missense change (p.Arg105Cys, p.Glu119Gln, p.Val275Ile), whereas two patients carried a heterozygous nonsense mutation (p.Lys149X). These patients may have been misdiagnosed, representing phenocopies that are caused by *BEST1* mutations but mimic AMD.⁶⁷ The p.Glu119Gln substitution has also been detected in a patient with bull's eye maculopathy.²

3. The clinical spectrum of ocular diseases associated with *BEST1* gene mutations

3.1. Best vitelliform macular dystrophy

BVMD was first described in 1905 by the German ophthalmologist Friedrich Best (1871–1965).⁹⁵ Most BVMD patients experience a reduction of visual acuity as the presenting symptom, although photophobia, metamorphopsia, and night blindness may also be noted.⁸⁴ The age at onset in BVMD is highly variable, ranging from the first decade to beyond the sixth decade, with a mean age at onset in the fourth decade.^{84,96–99} There is a significant correlation between patient age and visual acuity.¹⁰⁰ Mild to marked hypermetropia is a common associated finding.¹⁰¹

Several classifications of BVMD have been proposed, based on the aspect of the lesions on ophthalmoscopy. In these classifications, the first stage is generally considered the carrier or previtelliform stage (Fig. 3.2A), in which the fovea is normal or shows discrete RPE alterations, in combination with an abnormal EOG. This stage may be followed by the vitelliform stage (Fig. 3.2B and G), in which a slightly elevated macular lesion is observed, that is completely filled by yellowish material, resembling an egg yolk. In the next stage, the previously homogeneous vitelliform material breaks up, resulting in the vitelliruptive or “scrambled-egg” stage (Fig. 3.2C and H). This may then be followed by the pseudohypopyon stage, in which a horizontal level of the yellowish vitelliform material is seen in the inferior part of the cystic lesion (Fig. 3.2D and J). Above this level of vitelliform material, the lesion contains relatively transparent fluid. With time, chorioretinal atrophy ensues, in the so-called atrophic stage (Fig. 3.2E). When subsequent scarring within the lesion appears, which is sometimes associated with choroidal neovascularization, this is considered the final, cicatricial and/or neovascular stage by most classifications (Fig. 3.2F, I and K). The most frequently cited classifications are those by Deutman,¹⁰¹ Mohler and Fine,⁹⁷ and Gass.¹⁰² Although these classifications largely correspond, Gass positions the pseudohypopyon stage before the vitelliruptive stage. However, a study by Clemett showed that this sequence may be reversed, and that these stages may even evolve back and forth for several years.⁹⁶ Many patients show a different stage in each eye.⁹⁶ Even eyes with lesions in the cicatricial stage often retain a remarkably good visual acuity, despite impressive central scarring.^{97,103}

Choroidal neovascularization (CNV) occurs in 2–9% of BVMD cases.^{96,97,103–105} CNV may be difficult to recognize in BVMD lesions, and the (former) presence of CNV in such lesions is often inferred from the presence of a subretinal hemorrhage or a greyish-green scar within a lesion, with or without leakage on fluorescein angiography.^{96,97,103,105} Some reports suggest that CNV in BVMD lesions is more likely to occur after ocular trauma.^{103,106,107} In the long term, eyes with BVMD lesions and CNV are generally associated with a drop in visual acuity, but patients eventually often retain a visual acuity of 20/50 or better without treatment,¹⁰³ unlike for instance AMD.¹⁰⁸ Case reports indicate that CNV in BVMD lesions may respond to various treatments, including argon laser photocoagulation,^{109,110} photodynamic therapy,¹¹¹ and intravitreal injection of vascular endothelial growth

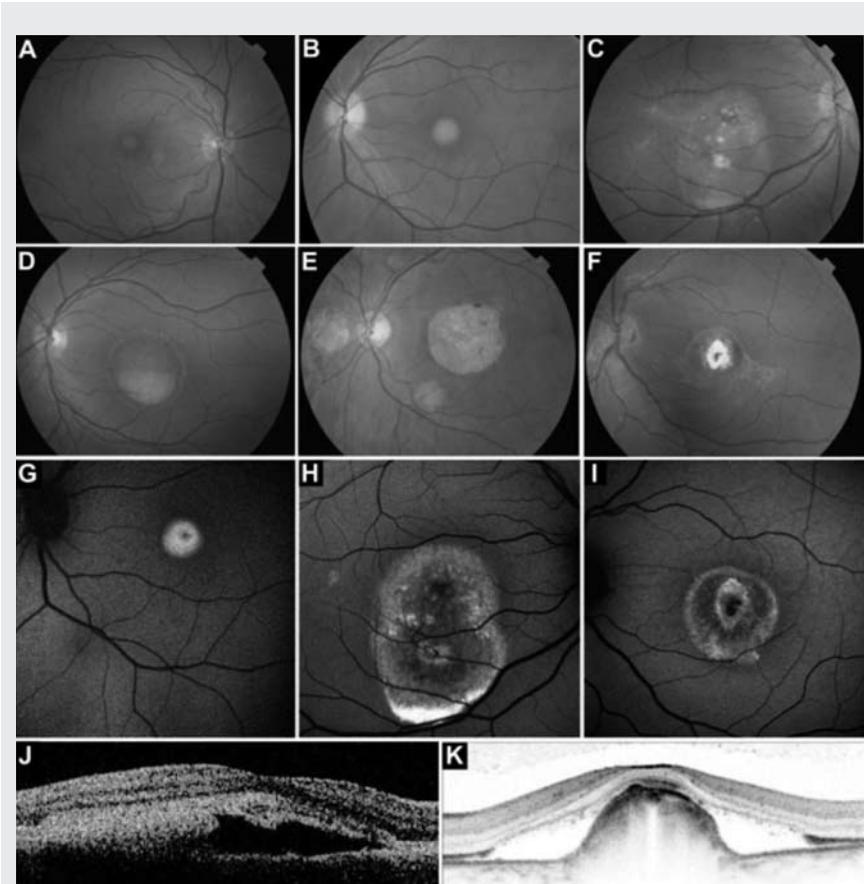


Figure 3.2.*

Best vitelliform macular dystrophy (BVMD). All patients in this figure had an abnormal electro-oculogram and a positive family history for BVMD. **A.** Previtelliform or carrier stage in a 40-year-old patient carrying a p.Tyr227Asn mutation in the BEST1 gene. The visual acuity (VA) was 20/16. Hypopigmented, slightly atrophic retinal pigment epithelial changes are seen in the fovea, which remained stationary during a follow-up period of 30 years, starting at the age of 10. **B.** Vitelliform stage in a 41-year-old patient carrying a p.Arg25Trp mutation in BEST1. The lesion is entirely filled with yellowish material. **C.** Vitelliruptive or "scrambled-egg" lesion in a 12-year-old patient with a VA of 20/25. This patient carried a p.Lys194_Ala195insVal mutation in BEST1. Scattered yellow-white vitelliform deposits are observed throughout the lesion. **D.** Pseudohypopyon stage in a 11-year-old patient carrying a p.Thr6Pro BEST1 mutation. The VA was 20/25. **E.** Atrophic stage in a 60-year-old patient who carried a p.Thr6Pro mutation in BEST1. Note the multifocal atrophic lesions besides the central, large atrophic lesion in this patient, who had a VA of 10/100. **F.** Cicatricial stage in

a 17-year-old patient, who also carried a p.Thr6Pro mutation in the BEST1 gene, with a VA of 20/100. **G.** Fundus autofluorescence (FAF) image of the vitelliform lesion in the patient described with image (B), showing an intensely increased FAF signal. **H.** FAF image of the vitelliruptive lesion of the same patient as on image (C), showing a dispersion of the material of increased FAF. In addition, a small pseudohypopyon with increased FAF can be seen in the inferior part of the lesion. **I.** FAF image of the same cicatricial stage lesion as on image (F), showing decreased to absent FAF in the center of the scar, and increased FAF at the edge of the scar, as well as at the edge of the lesion. **J.** Vertical optical coherence tomography (OCT) section of the pseudohypopyon lesion as depicted on image (D). The vitelliform material in the inferior part of the lesion is hyperreflective on OCT. Deposition of hyperreflective material is also seen on the outer retinal surface under the fovea. The transparent fluid in the upper part of the lesion corresponds with hyporeflectivity on the OCT image. **K.** High-resolution spectral domain-OCT image of the same cicatricial lesion as on images (F) and (I), revealing an elevation of the macula with a highly reflective subfoveal mass, corresponding to the scar. All retinal layers and RPE seem to be preserved over the entire area. The thickened photoreceptor layer at the borders of the retinal detachment corresponds to an outer ring of increased FAF in panel (I). An area of partial photoreceptor loss around the central mass co-locates to decreased perifoveal FAF.

factor-inhibitors such as bevacizumab or ranibizumab.^{112,113} A rare complication in BVMD is retinal detachment, which may be successfully treated with cryotherapy and retinal indentation surgery.^{36,114,115}

A characteristic finding in BVMD is a severely decreased to absent light rise on the EOG.⁴³ An abnormal EOG has been considered an absolute prerequisite to establish the diagnosis of BVMD in patients with vitelliform lesions. In addition, it allows detection of asymptomatic carriers. However, although the great majority of BVMD patients and carriers meet this criterium, several studies indicate that the EOG may initially be normal or even remain normal in *BEST1* mutation carriers, even in those who are clinically affected.^{81,83,84,99,116} Thus, only mutation analysis of the *BEST1* gene allows confirmation of a clinical diagnosis of BVMD.

Photopic and scotopic full-field electroretinography (ERG) is generally normal in BVMD patients,^{83,84,99,117} although the 30-Hz flicker and dark-adapted ERG may be abnormal.^{84,118} The multifocal ERG is abnormal in the majority of BVMD patients.^{73,84,99,117,119,120} In these patients, reduced amplitudes as well as increased implicit times of the multifocal ERG P1 and N1 components are often observed, especially in the central stimulus rings.^{73,84,99,117,119,120} These findings are presumed to reflect abnormal cone photoreceptor and bipolar cell function.¹²¹ Dark adaptation measurements in BVMD patients are generally normal.¹²²

Fluorescein angiography often shows hyperfluorescence in the early phase, due to a variable degree of RPE and/or chorioretinal atrophy, with evidence of subtle fluorescein

accumulation within the lesion in the late phase.^{84,102,123} However, the accumulation of vitelliform material in the vitelliform and pseudohypopyon stage blocks fluorescence, especially in the early phase.^{84,102,123}

Indocyanine green angiography may be able to more accurately locate possible choroidal neovascularization, which is often difficult to identify on fluorescein angiography.¹⁰³ In addition, indocyanine green angiography may show hyperfluorescent changes in the (mid-) peripheral retina in BVMD that are not seen on ophthalmoscopy and fluorescein angiography.¹²⁴

On fundus autofluorescence (FAF), the yellowish subretinal vitelliform material in BVMD lesions shows an intensely increased FAF signal (Fig. 3.2G, H, and I).^{83,84,99,123,125-131} As BVMD lesions evolve towards later stages, this material of increased FAF becomes dispersed throughout the lesion in a granular fashion, with or without a pseudohypopyon of increased FAF in the inferior part of the lesion (Fig. 3.2H). This autofluorescent material consists of toxic autofluorescent components of lipofuscin that are waste products of the visual cycle, such as A2E and its precursors and derivatives.¹³²⁻¹³⁷ Such material of increased FAF may not only be located in the RPE,^{133,137} but also in the photoreceptors and the photoreceptor outer segments that are shed into the subretinal space in BVMD lesions.^{123,129,138-140} In the cicatricial and atrophic stage, areas of decreased FAF become more prominent, due to RPE atrophy and/or blockage of FAF by scar tissue, as well as a loss of autofluorescent material (Fig. 3.2I).^{99,123,125} Such a decreased, irregular FAF signal in BVMD lesions is generally associated with a lower visual acuity.^{99,141} FAF may be a helpful tool in the follow-up of BVMD lesions, especially as it is able to visualize abnormalities and changes within a lesion that are not evident on ophthalmoscopy.^{99,125,126}

Optical coherence tomography (OCT) enables the high-resolution visualization of BVMD lesions in the antero-posterior dimension.^{83,117,123,126,142-145} In the vitelliform, pseudohypopyon, and vitelliruptive stages, OCT shows a central macular detachment of the neurosensory retina.^{123,126,142-145} The space between the neuroretina and RPE may contain hyperreflective material, corresponding to the vitelliform material seen on ophthalmoscopy, and/or hyporeflective material, corresponding with transparent fluid on ophthalmoscopy (Fig. 3.2J).^{123,126,142-145} Scars and choroidal neovascularization in a BVMD lesion also appear hyperreflective on OCT (Fig. 3.2K).

Follow-up studies indicate that patients in the carrier stage or mild RPE changes often remain asymptomatic for a prolonged period of time, with a stable ophthalmoscopic aspect (Fig. 3.2A).^{96,97} In contrast, lesions in the vitelliform, pseudohypopyon, and vitelliruptive stages may evolve into one another within a few years.^{96,97,99} Up to 88% of BVMD patients with a follow-up period of at least 5 years may retain a visual acuity of more than 20/40 in the better eye.^{84,96,97} In patients with early-onset BVMD, visual acuity often deteriorates after the fourth decade, especially in patients with long-standing cicatricial and/or atrophic lesions.^{96,97} Beyond the age of 50, many BVMD patients show a deterioration of the visual acuity to less than 20/40 in the better eye.⁹⁶⁻⁹⁸

Considerable variability of lesions on ophthalmoscopy can make a clinical diagnosis of BVMD challenging. This is not only the case in for instance mild cases with relatively

discrete RPE alterations, but also in advanced cases with scarred and/or atrophic lesions. The latter lesions may be mistaken for AMD, especially in elderly patients from families with *BEST1* mutations that show decreased penetrance and variable expression.^{2,4,82,105} Lesions may display a spoke-like configuration of pigmentary abnormalities, which can mimic pattern dystrophy.^{114,123,125,126,146} Some patients display multifocal vitelliform lesions.⁸³ In these cases, the eccentric lesions are often located superonasal to the optic disc and/or adjacent to the temporal retinal vascular arcades.⁸³ These smaller extramacular lesions often resemble the central lesion both on ophthalmoscopy, as well as on FAF and OCT.⁸³ Findings within a single family with BVMD patients carrying the same *BEST1* mutation may be highly variable, ranging from asymptomatic carriers without EOG abnormalities, to typical BVMD,^{72,83,98,126,147-149}

Vitelliform lesions, comparable to those seen in BVMD, may also be encountered in several other clinical entities. Vitelliform paraneoplastic retinopathy, for instance, is a clinical picture that is highly comparable to BVMD, with typical vitelliform lesions,¹⁵⁰⁻¹⁵³ and sometimes an abnormal EOG.¹⁵⁰ This rare condition, which has been described in patients with metastatic choroidal or cutaneous malignant melanoma, may be associated with auto-antibodies against bestrophin-1.¹⁵⁰ Other diseases with vitelliform lesions include AFVD (with or without *BEST1* mutations),^{86,154,155} “pseudovitelliform” RPE detachments caused by confluence of macular drusen in basal laminar drusen and AMD,^{86,154,156} as well as acute exudative polymorphous vitelliform maculopathy.^{157,158} In the first two diseases, the EOG is generally normal, whereas the latter shows an abnormal EOG as well as multifocal vitelliform lesions.

3.2. Adult-onset foveomacular vitelliform dystrophy

AFVD, first described by Gass in 1974,⁸⁵ is considered one of the pattern dystrophies according to the classification of Gass.¹⁰² The term AFVD represents a clinically and genetically heterogeneous group of disorders that may indeed have features of pattern dystrophy,^{3,92,93,159} but AFVD may also closely resemble BVMD or AMD, and may thus be misdiagnosed.^{92,154,160-162} There is large variation in the terminology of this disorder, with names ranging from “adult vitelliform macular dystrophy” to “pseudovitelliform macular degeneration”.^{163,164} Most cases of AFVD are isolated,^{92,93} although an autosomal dominant inheritance and familial cases have also been described.^{85,88,89,165}

As in BVMD, the age at onset in AFVD is variable, with a mean beyond the fifth decade, which is later than in BVMD.^{88,92,102,155} Females appear to be somewhat more frequently affected.^{92,166} Patients often present with a relatively mild loss of visual acuity and metamorphopsia, sometimes accompanied by mild photophobia and/or (para-)central visual field defects.⁹² Visual acuity often remains fairly good and constant for a prolonged period of time, but may also show a progressive and severe decline after several years, due to chorioretinal atrophy or CNV.^{92,166-168} The typical ophthalmoscopic aspect of AFVD is a solitary, yellow-white, round to oval, slightly elevated lesion, often with a central pigmented spot, in the fovea of both eyes.^{92,102} These lesions are usually one-third to one disc diameter in size. However, lesions may be considerably larger and may

then be indistinguishable from those seen in BVMD, and lesions may also be unilateral or multifocal.^{83,92,102} CNV is an infrequent complication in AFVD, although it has been reported in up to 15% of cases.^{92,161,166,169,170}

The EOG in AFVD is normal or slightly subnormal in virtually all patients, which is especially important in differentiating AFVD from BVMD.^{88,92,167,171} The full-field ERG in AFVD is also generally normal, although the 30 Hz flicker response may be mildly reduced.⁹² Multifocal ERG may show an abnormal P1 amplitude and implicit time in all rings, but mostly in the central two rings.^{92,172} Color vision abnormalities, as well as a central scotoma on perimetry, are noted in up to half of the patients.⁹²

Fluorescein angiography in AFVD typically shows a central non-fluorescent area, surrounded by a small, irregular hyperfluorescent ring, but lesions may also display central, patchy hyperfluorescence with discrete late dye leakage like in BVMD.^{92,102,167} AFVD lesions show variable patterns of increased FAF in most patients, especially in yellowish vitelliform lesions, corresponding to lipofuscin accumulation.^{92,173-175} OCT, and more specifically high-resolution OCT, often yields additional information on the structural abnormalities associated with AFVD.^{172,176-179} Smaller lesions appear as hyperreflective structures between the neuroretina and RPE. Larger vitelliform lesions in AFVD -like BVMD - show a central macular detachment of the neuroretina, with a variable amount of hyperreflective material (corresponding to the vitelliform material) and/or hyporeflective material (corresponding to translucent fluid) within the space between neuroretina and RPE. On high-resolution OCT, reduced thickness of the neuroretina in the foveola correlates with reduced visual acuity.¹⁷⁹

The aforementioned characteristics of the clinical spectrum of AFVD are derived from studies on relatively large groups of AFVD patients, in which *BEST1* mutations were either not found or accounted only for a small proportion of patients. Due to this low number of clinically specified *BEST1*-related AFVD cases, it is difficult to determine to what extent the aforementioned clinical findings may be applied to the *BEST1*-related AFVD group. As mentioned previously, AFVD may present with BVMD-like vitelliform lesions (i.e., yellowish “egg-yolk” lesions larger than one disc diameter). In cases that combine such an appearance with the presence of a *BEST1* mutation, the only feature that distinguishes these *BEST1*-related AFVD cases from BVMD is an EOG that is within normal limits. Even then, the EOG may gradually become abnormal, illustrating the thin line between AFVD and BVMD in these cases.^{99,116} Conversely, the EOG in BVMD can be normal.^{83,99,116} Again, this indicates that the value of the EOG in establishing a specific diagnosis has been overestimated. In *BEST1*-related AFVD patients, the phenotype might actually be regarded as an exceptionally mild form of BVMD caused by *BEST1* mutations with variable expressivity. Examples of such *BEST1* mutations include p.Ala243Val and p.Thr6Pro.³

AFVD not only borders with BVMD, but may also be confused with (“pseudo”) vitelliform RPE detachments as a result of confluent drusen, which may be regularly noted in the phenotype of basal laminar drusen.^{156,180,181} The differential diagnosis can be made on ophthalmoscopy, which shows large and small drusen surrounding the lesion,

as well as on fluorescein angiography, typically showing a “stars-in-the-sky” pattern of surrounding drusen.^{156,180}

3.3. Autosomal dominant vitreoretinochoroidopathy (ADVIRC) and the MRCS syndrome

Autosomal dominant vitreoretinochoroidopathy (ADVIRC) was first described in 1982 by Kaufman, and is characterized by a peripheral circumferential retinal band of pigmentary alterations (Fig. 3.3A), midperipheral and peripapillary chorioretinal atrophy, punctate white pre- or intraretinal deposits, and fibrillary vitreous condensations.^{8,182-184} Most ADVIRC patients retain a fairly good visual acuity throughout life,^{182,184,185} although visual acuity may decrease considerably due to macular edema, chorioretinal atrophy, or - rarely - retinal detachment and vitreous hemorrhage.¹⁸³⁻¹⁸⁵ A discrete rotatory nystagmus can be present.¹⁸⁴ The majority of patients with ADVIRC that was genetically confirmed to be caused by BEST1 mutations, developed congenital or early-onset cataract, that may be located posterior subcapsular.^{8,182,184} Microcornea is common in ADVIRC (Fig. 3.3B), and many patients are hypermetropic or have nanophthalmos, as well as a shallow anterior chamber, although myopia may also be seen.^{5,8,182,184} As a result, ADVIRC patients

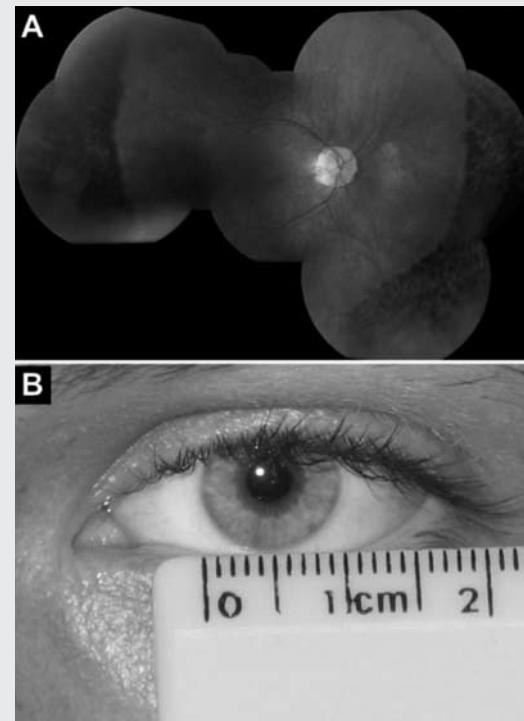


Figure 3.3.*
Autosomal dominant vitreoretinochoroidopathy (ADVIRC), in a 25-year-old patient carrying a heterozygous p.Val86Met (c.256G>A) mutation in the BEST1 gene. **A.** On a composition image of colour fundus photographs, a circumferential hyperpigmented band is seen in the (mid-) periphery. The optic disc has a slightly pale aspect, and peripapillary chorioretinal atrophy is noted. **B.** Photograph of the same patient, showing microcornea with a corneal diameter of less than 10 mm, as a sign of ocular developmental abnormalities in ADVIRC.

have a relatively high incidence of subacute and acute angle-closure glaucoma.^{8,182,184} Some patients display retinal arteriolar narrowing and pale optic discs. Retinal edema due to vascular incompetence may also be observed, as well as preretinal and/or prepapillary neovascularization and fibrosis.^{183,184} Such neovascularization may regress after panretinal photocoagulation, even without demonstrable retinal ischemia on the fluorescein angiogram.¹⁸⁴

The EOG is severely abnormal in most *BEST1*-related ADVIRC patients, but may be normal in exceptional cases.^{8,184,186} The full-field ERG may show normal to severely reduced rod and cone responses.^{8,182-184} Goldmann perimetry is often initially normal, but tends to constrict mildly with age.¹⁸⁴

It has been suggested that *BEST1* mutations may also be the cause of autosomal dominant nanophthalmos with high hyperopia and angle-closure glaucoma, a condition that was mapped to the *NN01* locus on chromosome 11 (like *BEST1*) and appears highly similar to ADVIRC.^{8,187}

The *BEST1*-associated MRCS syndrome caused by a *BEST1* mutation, which was described in only one family, is characterized by microcornea, rod-cone dystrophy, early-onset pulverulent cataract, and posterior staphyloma.⁷ In the first decades, visual acuity may be fairly good, but beyond the age of 30 years visual acuity often becomes poor (less than 20/100 to absent light perception).⁷ Patients typically start experiencing night blindness in their teens. A decrease of visual acuity, presumably due to progressive cataract and possibly photoreceptor dysfunction, may be noted before the age of 30 years, often leading to cataract surgery in the second or third decade.⁷ On ophthalmoscopy, peripheral RPE atrophy and retinal pigmentary abnormalities are seen anterior to the posterior staphyloma in younger patients, whereas the atrophy and pigmentary changes may extend to the posterior pole and staphyloma with advancing age.⁷

The EOG is abnormal in all patients with the MRCS syndrome.^{7,8} The full-field ERG shows subnormal photopic and scotopic responses in the first two decades, with the scotopic responses being more abnormal than the photopic responses, corresponding to rod-cone dystrophy.^{7,8} With time, the full-field ERG becomes extinguished, reflecting severe rod and cone photoreceptor dysfunction throughout the retina.^{7,8} Patients may be moderately myopic due to the posterior staphyloma and cataractous lens. Most patients with MRCS syndrome have posterior staphyloma in association with otherwise normal axial lengths.^{7,8} However, two patients in a family with MRCS syndrome caused by the p.Val239Met *BEST1* mutation had nanophthalmos instead of posterior staphyloma, thus largely overlapping with the ADVIRC phenotype.^{7,8} Therefore, although the MRCS syndrome is generally more severe than ADVIRC, these conditions overlap and likely form a continuum, as both of these *BEST1*-related conditions show retinal pigmentary abnormalities, retinal dystrophy, microcornea, and early-onset cataract.^{7,8,184}

3.4. Autosomal recessive bestrophinopathy

The phenotype of ARB has been recently described by Burgess and colleagues.⁹ This condition usually starts with central visual loss, with an age at onset ranging from 4 to 40

years, and a mean age at onset of approximately 23 years.⁹ Visual acuity often deteriorates to less than 20/60 in both eyes.⁹ Patients are generally hyperopic and show shallow anterior chambers, with a correspondingly increased risk of (sub)-acute angle-closure glaucoma.⁹ On ophthalmoscopy, irregular RPE alterations with whitish subretinal deposits are seen throughout the retina, with a preference for the macula and midperipheral retina (Fig. 3.4A and B). Retinal edema and a neurosensory retinal detachment with subretinal fluid may be observed in the macula, which may be confirmed on OCT (Fig. 3.4C), but patients do not appear to develop vitelliform lesions.⁹ Macular lesions may evolve towards scars, causing a further decrease in visual acuity.

Like in BVMD, ADVIRC and the MRCS syndrome, the EOG shows a severely reduced to absent light rise.⁹ The pattern ERG in ARB patients is markedly abnormal, indicating

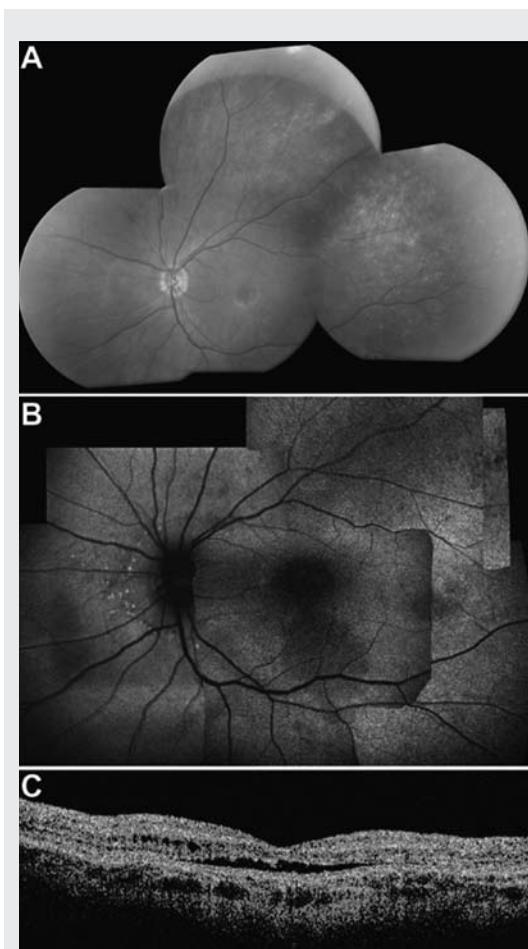


Figure 3.4*
Autosomal recessive bestrophinopathy (ARB) in a 45-year old patient who carried the compound heterozygous mutations p.Asp312Asn (c.934G>A) and p.Met325Thr (c.974T>C).
A. Composition image of colour fundus photographs, showing widespread irregular retinal pigment epithelial alterations with whitish subretinal deposits throughout the retina. In the macula, deposition of yellow-white material is seen. **B.** Fundus autofluorescence (FAF) image, showing diffuse, discrete small areas of increased and decreased FAF. **C.** On a horizontal optical coherence tomography (OCT) section of the macula, hyporeflective subretinal fluid between the retinal pigment epithelium and the neurosensory retina is seen, as well as small hyporeflective cysts of intraretinal fluid.

severe macular dysfunction.⁹ Central multifocal ERG responses are also reduced.⁹⁴ Contrary to most BVMD cases, the full-field ERG also shows reduced and delayed rod and cone responses,^{9,94} which indicates panretinal photoreceptor dysfunction. On fluorescein angiography, widespread patchy hyperfluorescence is seen, due to RPE atrophy and retinal edema.⁹ The widespread RPE abnormalities and subretinal deposits correspond to irregular areas of increased FAF, reflecting lipofuscin accumulation in the RPE, as well as areas of decreased FAF, corresponding to a loss of RPE (Fig. 3.4C).⁹ High-resolution OCT imaging in an 11-year-old asymptomatic patient with ARB showed RPE abnormalities, photoreceptor detachment, an elongated and thickened photoreceptor outer segment layer, and a preserved inner retinal layer.⁹⁴ Family members who carried one *BEST1* mutation heterozygously were entirely normal, both clinically and electrophysiologically.⁹

4. From gene mutation to disease

4.1. Impact of *BEST1* mutations on protein structure and function

There is a large body of evidence indicating that bestrophin-1 is a multifunctional protein in the RPE, functioning as a Ca^{2+} -activated Cl^- channel,^{12,20,23-25,27-30} as well as a modulator of intracellular Ca_v channels.³⁹⁻⁴² A summary of the functional consequences of *BEST1* mutations in BVMD, AFVD, ADVIRC, the MRCS syndrome, and ARB, is given in Table 3.1.

In general, mutant proteins in BVMD are trafficked normally into the RPE plasma membrane.²⁰ Several *in vitro* studies have focused on the effect of *BEST1* mutations on bestrophin-1 Cl^- channel function in BVMD. Most mutations are associated with a greatly reduced or absent Cl^- current, which is often due to a dominant negative mechanism.^{9,20,23,31,38,42} A milder Cl^- current reduction to 10-40% of the wild-type current is seen in mutations that lead to haploinsufficiency.^{20,30,42} Both dominant negative and haploinsufficiency mechanisms may also be found in AFVD.^{20,30,31,42} In HEK-293 cells, bestrophin-1 mutations in the C-terminal region, which is important for Ca^{2+} binding (regions 293-308 and 312-323), disrupt normal Ca^{2+} binding to bestrophin-1 and abolish or greatly reduce the normal Cl^- current.³¹

Two *BEST1* variants, p.Thr216Ile and p.Leu567Phe, identified in AMD patients, did not affect the Cl^- channel function of bestrophin-1,²⁰ questioning their relevance in disease pathogenesis. The p.Glu119Gln variant, detected in bull's eye maculopathy and in AMD, produced currents similar to those of wild-type bestrophin-1, but had altered relative permeability to large anions.²⁰

As mentioned previously, bestrophin-1 also functions as an inhibitor of Ca^{2+} currents produced by Ca_v channels, through the interaction with the subunit of these channels.³⁹⁻⁴² Mutations that disrupt bestrophin-1 Cl^- channel function may have different effects on Ca_v channels.⁴² While some mutations have an inhibitory effect on Ca_v channels that is comparable to wild-type bestrophin-1, others have a decreased inhibitory effect on these Ca_v channels (Table 3.1).

Table 3.1.
Summary of the BEST1 mutations that were tested for their impact on bestrophin-1 function.

Mutation	Associated disease	Mutation effect	Mechanism	Test setting	Reference(s)
p.Thr6Pro	BVMD	↓ absent Cl ⁻ current	U	HEK293 cells	23
p.Ala10Val	BVMD	↓ absent Cl ⁻ current	U	HEK293 cells	23
p.Ile73Asn	BVMD	defective membrane integration	U	canine pancreatic microsomes	19
p.Tyr85His	BVMD	↓ absent Cl ⁻ current -defective membrane integration	dom. negative -defective membrane integration	HEK293 cells -canine pancreatic microsomes	19,23
p.Val86Met	ADViRC	in-frame deletion	exon skipping	splice assays	8
p.Arg92Cys	BVMD	↓ absent Cl ⁻ current	dom. negative	HEK293 cells	23
p.Arg92Ser	BVMD	↓ absent Cl ⁻ current -normal inhibitory effect on Cav channels	U	HEK293 cells	23,42
p.Trp93Cys	BVMD	↓ Cl ⁻ current -no effect on Cav activation ↓ light peak amplitude on RPE-derived ERG	dom. negative	-HEK293 cells -mutant overexpression in AAV-transfected rats	9,23,40
p.Asn99Lys	BVMD	↓ absent Cl ⁻ current	U	HEK293 cells	23
p.Asp104Glu (p.Glu119Gln)	AMD, Bull's eye maculopathy	↓ absent Cl ⁻ current -normal Cl ⁻ current -altered anion permeability	U	HEK293 cells	23
p.Ala141His	ARB without WT bestrophin-1	↓ Cl ⁻ current -no inhibitory effect on Cav activation	null phenotype	HEK293 cells	20
					9

Table 3.1. *continued*

Mutation	Associated disease	Mutation effect	Mechanism	Test setting	Reference(s)
p.Ala146Lys	AFVD	-20-40% of WT Cl current -altered anion permeability → inhibitory effect on Cav channels	haploinsufficiency	HEK293 cells	20,42
p.Pro152Ala without WT bestrophin-1 (p.Thr216Ile)	ARB	↓ Cl ⁻ current -no inhibitory effect on Cav activation	null phenotype	HEK293 cells	9
p.Arg218Ser	AMD	-normal Cl ⁻ current -normal anion permeability	U	HEK293 cells	20
p.Arg218Cys	BVMD	↓-absent Cl ⁻ current	dom. negative	HEK293 cells	23
	BVMD	↓ Cl ⁻ current -no effect on Cav activation → light peak amplitude on RPE-derived ERG	dom. negative	-HEK293 cells -mutant overexpression in AAV-transfected rats	9,40
p.Gly222Glu	BVMD	-absent Cl ⁻ current → inhibitory effect on Cav channels	U	HEK293 cells	20,42
p.Tyr227Asn	BVMD	↓-absent Cl ⁻ current	U	HEK293 cells	23
p.Ala243Thr	BVMD	↓-absent Cl ⁻ current	U	HEK293 cells	23
p.Ala243Val	BVMD	~10% of WT Cl ⁻ current altered	haploinsufficiency	HEK293 cells	30
AFVD	ADVIRC	anion permeability			
p.Val235Ala	ADVIRC	in-frame duplication	exon skipping	splice assays	5,8
p.Tyr236Cys	ADVIRC	in-frame deletion	exon skipping	splice assays	5,8
p.Val239Met	MRCS syndrome	in-frame deletion	exon skipping	splice assays	8

Spectrum of *BEST1*-associated phenotypes

p.Phe281del	BVMD	defective membrane integration	U	canine pancreatic microsomes	19
p.Glu293His	BVMD	↓absent Cl ⁻ current	dom. negative	HEK293 cells	38
p.Glu293Lys	BVMD	↓↓absent Cl ⁻ current Cl ⁻ current	U	HEK293 cells	23
p.Ile295del	BVMD	absent Cl ⁻ current	dom. negative	-HEK293 cells -RPE-J cells	20
p.Gly299Arg	BVMD	-absent Cl ⁻ current ↓ inhibitory effect on Ca ²⁺ channels	U	-HEK293 cells -RPE-J cells	42
p.Gly299Glu	BVMD	-absent Cl ⁻ current -normal inhibitory effect on Ca ²⁺ channels	dom. negative	HEK293 cells	23,42
p.Glu300Asp	BVMD	↓absent Cl ⁻ current	dom. negative	HEK293 cells	23
p.Asp301Glu	BVMD	↓absent Cl ⁻ current	dom. negative	HEK293 cells	23
p.Thr307Ile	BVMD	↓absent Cl ⁻ current	dom. negative	HEK293 cells	23
p.Asp312Asn	AFVD	-absent Cl ⁻ current -normal inhibitory effect on Ca ²⁺ channels ↓ sensitivity of bestrophin-1 to Ca ²⁺	dom. negative	HEK293 cells	20,31,42
(p.Leu567Phe)	AMD	-normal Cl ⁻ current -normal anion permeability	U	HEK293 cells	20

Abbreviations: ↓, markedly decreased; ↓, decreased; Ca²⁺ channel, voltage-dependent calcium channel; dom. negative, dominant negative; ERG, electroretinography; RPE, retinal pigment epithelium; U, unknown; WT, wild-type.

The experiments mentioned above illustrate the various effects caused by different *BEST1* mutations. Such functional differentiation in the impact of mutations could be intuitively expected, based on the observation of variable clinical severity and different influences on the EOG. However, the reason why some mutations cause BVMD whereas others cause AFVD is far from elucidated. The establishment a genotype-phenotype correlation model is challenging, as the phenotypic description is incomplete in several studies. Moreover, the explanation for the marked phenotypic variability that may be observed in association with a single mutation in BVMD families also remains largely obscure, as will be discussed in section 4.2.

It should be noted that it is unclear to what extent *in vitro* experiments, as well as rodent studies, may be extrapolated to the function of human RPE cells *in vivo*. Mouse bestrophin-1, for instance, is only 63% identical to human bestrophin-1,⁴⁵ lacking for instance the C-terminal Pro₃₃₀XXXPro₃₃₄ domain that is important for the modulation of Ca_v channel regulation in humans.⁴² In addition, mice do not have a macula, and *Best1* knockout mice do not show obvious retinal pathology or visual abnormalities.³⁹ Rats overexpressing *BEST1* mutations that cause BVMD in humans, do not show lipofuscin accumulation, nor do they develop vitelliform lesions.¹⁸⁸

In ADVIRC and the MRCS syndrome, the *BEST1* mutations p.Val86Met, p.Val235Ala, p.Tyr236Cys, and p.Val239Met disrupt normal splicing, resulting in abnormal bestrophin-1 isoforms.^{5,8} Alternative splicing of pre-mRNA leads to the production of different protein isoforms and is orchestrated by the spliceosome. Normally, the spliceosome, which consists of small nuclear RNA proteins (snRNPs) and other associated proteins, recognizes exon-intron boundaries in pre-mRNA.¹⁸⁹ This spliceosome subsequently induces removal of the introns and proper ligation of the exons, creating mRNA that encodes the wild-type protein and potentially other isoforms.¹⁸⁹ Exonic regions may contain elements that either enhance or decrease splicing, called exonic splicing enhancer (ESE) and silencer (ESS) sites.¹⁹⁰⁻¹⁹³ If such a site has overlapping ESE and ESS functions, it is called a composite exonic regulatory element of splicing (CERES).¹⁹⁴ Although alternative splicing has important physiological functions in creating the complex human proteome,¹⁹⁵ abnormal alternative splicing due to mutations in ESE/ESS regions can cause diseases such as ADVIRC and the MRCS syndrome. Experiments on the effect of the *BEST1* p.Val235Ala (c.704T>C) and p.Tyr236Cys (c.707A>G) mutations indicate that they are located in an area (from c.704 to c.709) in *BEST1* that has a CERES function.⁵ These mutations may affect proper splicing through the altered binding of modulating proteins (so-called serine-arginine-rich RNA binding proteins, shortly named SR proteins) of the spliceosome to this CERES, therefore altering exonic splicing.⁵ The result is an mRNA that either lacks exon 4, 6, or 7, or contains a duplication of exon 6.⁵ This most likely results in an in-frame alteration of bestrophin-1. In ADVIRC patients, three protein isoforms may thus be encountered: the wild-type protein, the protein containing a missense substitution, and the protein with an in-frame deletion or duplication.

The impact on bestrophin-1 structure and function of these *BEST1* mutations in ADVIRC and the MRCS syndrome is unclear. Several mechanisms may be possible. The shortened

or elongated protein may not be properly trafficked to the basolateral membrane of the RPE and remain intracellularly, where it may be degraded. On the other hand, should the protein be incorporated in the RPE plasma membrane, this affected protein may hinder wild-type bestrophin-1 function(s), for instance through either absent or abnormal oligomerization or abnormal protein binding. In addition to the disease-causing mechanism of haploinsufficiency, the abnormal bestrophin-1 protein could also exert a dominant negative effect on the wild-type protein or -if it is retained intracellularly and is not degraded- on intracellular processes.

In ARB, the RNA product of the homozygous p.Arg200X BEST1 nonsense mutation is probably subject to nonsense-mediated decay and these patients consequently may not express bestrophin-1 in the RPE plasma membrane. *In vitro* studies on the p.Arg141His and p.Pro152Ala mutations, that cause ARB in the compound heterozygous state, show that the mutant bestrophin-1 proteins are associated with a markedly reduced Cl⁻ current when expressed without wild-type bestrophin-1.⁹ However, in contrast to many BVMD mutations,^{12,23} when these ARB mutants are expressed with wild-type bestrophin-1, Cl⁻ conductance was not significantly different from that in the wild-type bestrophin-1 situation.⁹ Bearing these findings in mind, it is intriguing that the p.Arg141His mutation has also been described in a heterozygous state in BVMD, although the clinical description of these patients was incomplete and did not mention EOG findings.^{3,74} In summary, contrary to BVMD, AFVD, ADVIRC, and the MRCS syndrome, the presence of homozygous or compound heterozygous BEST1 mutations is required to cause ARB. Thus, ARB can be considered the *BEST1* null phenotype.

4.2. Genotype-phenotype correlations

Nearly all *BEST1* mutations identified in BVMD and AFVD are missense mutations. Only one nonsense mutation and two frameshift mutations have been identified, mostly in poorly documented and/or sporadic cases. A remarkable clustering of missense mutations associated with the BVMD phenotype is seen in the amino acid regions 6-30, 80-104, 221-243, and 293-312 of bestrophin-1 (Fig. 3.1). These four regions are all situated near or in the RPE plasma membrane, and may therefore be of particular importance for bestrophin-1 Cl⁻ channel function and/or Ca_v channel modulation.

There is a clear genotype-phenotype correlation for *BEST1* mutations that cause ADVIRC and MRCS syndrome. These mutations all affect splicing, leading to in-frame deletions or duplications. Although some of the *BEST1* mutations found in BVMD lie very close to the missense mutations in ADVIRC and MRCS syndrome, they do not alter splicing.⁵ Finally, the null phenotype of ARB requires homozygous or compound heterozygous nonsense or missense *BEST1* mutations.⁹

In BVMD, a markedly variable penetrance and expressivity is observed, even in patients within one family.^{83,149,196} As mentioned previously, some patients never manifest fundus changes, and a number of patients with genetically confirmed BVMD even have a normal EOG.^{99,116} Late-onset symptoms are not uncommon and these individuals with *BEST1* mutations may therefore be misdiagnosed as having AMD.^{2,67,84} Relatively mild

phenotypes may also be encountered in ADVIRC.⁵

Little is known about the exact mechanisms of such reduced penetrance and variable expression, so that (a combination of) several explanations remain possible.

In BVMD, some *BEST1* mutations may be more likely to show reduced penetrance than others. In one study, the BVMD mutations p.Ile295del and p.Asn99Lys showed reduced penetrance, since they were found in asymptomatic carriers between 11 and 42 years of age.⁹⁹ The impact of a *BEST1* mutation may depend on the presence or absence of a dominant negative effect of the mutant protein on wild-type bestrophin-1. As bestrophin-1 has been postulated to form oligomers,^{12,22,23} the pathologic effect of a bestrophin-1 mutant may also depend on the ratio of mutant protein subunits compared to wild-type protein subunits, at least in mutant proteins that do not have a dominant negative effect on wild-type bestrophin-1. Even in such dominant negative mutations, the detrimental influence of the protein may be weakened, for instance through heteromeric oligomerization with wild-type bestrophin-2,²⁰ which is also present in the RPE.¹³ Bestrophin-1 mutants such as p.Asp312Asn are unable to exert a dominant negative effect on wild-type bestrophin-2, so that upregulation of bestrophin-2 could be imagined to (partly) compensate for the deleterious effects of the bestrophin-1 mutant and its effect on the EOG.²⁰ The severity of the phenotype in ADVIRC/MRCS syndrome may depend on the different functional consequences of the different isoforms, as well as the relative proportion of the two mutated isoforms.⁸

Another plausible explanation for the decreased penetrance and variable expressivity in *BEST1*-associated phenotypes is the dependency on other genetic and environmental modifiers.^{68,98} Such factors could influence for example the level of expression of the wild-type allele. Interestingly, recent studies suggests that the transcription factors OTX2, MITF, and possibly CRX, may play a role as modifiers of *BEST1* expression.¹⁵⁻¹⁷ These transcription factors are able to bind to specific sites within the -154 to -104 base pair region of the *BEST1* gene promoter.¹⁵⁻¹⁷ In mice, this region contains positive regulatory elements that may cause a broad variation in *BEST1* promotor activity.^{15,17}

OTX and MITF play a central role in the differentiation of the RPE, photoreceptors, and bipolar cells, as well as lens and anterior segment formation.¹⁹⁷⁻¹⁹⁹ The CRX protein, which is found in the photoreceptors and RPE,^{17,200} is a target of OTX2 in photoreceptors and possibly also the RPE.²⁰¹ Human OTX2, MITF, and CRX mutations are associated with a broad range of ocular abnormalities.²⁰²⁻²⁰⁴ Heterozygous *Otx2*(+/-) mice also display a wide spectrum of ocular abnormalities that partly overlap with those seen in ADVIRC, the MRCS syndrome, and ARB, including RPE and photoreceptor abnormalities, microphthalmia, and abnormalities of the lens, cornea, and iris.²⁰⁵ Homozygous *Otx2* knockout mice die early in embryogenesis as a complete absence of *Otx2* precludes normal formation of the brain.^{205,206} Homozygously mutated *Mitf* mice show microphthalmia, RPE abnormalities and other pigmentary abnormalities related to a loss of melanocytes.^{207,208}

Therefore, as OTX2 and MITF are key players in the development and differentiation of various ocular tissues,¹⁹⁷⁻¹⁹⁹ variations in these genes may be of influence in the pathogenesis and the severity of the panretinal photoreceptor dysfunction and other

ocular developmental abnormalities in *BEST1*-related ADVIRC, the MRCS syndrome, and ARB. However, although there is a possible role for OTX2, MITF, and CRX as genetic modifiers of *BEST1*, it is currently unknown if these transcription factors actually provide an explanation for the broad clinical variability that is observed between individuals with identical *BEST1* mutations.

4.3. Pathophysiology of ocular phenotypes caused by *BEST1* mutations

It has been postulated that the accumulation of fluid and vitelliform material in BVMD is the result of the disruption of epithelial ionic transport and fluid homeostasis by the RPE.^{12,129} Such a disruption of epithelial transport could occur through the dysfunction of Cl⁻ transport in bestrophin-1 channels and/or through the disruption of Ca²⁺ signaling.³⁰ Normal apposition and functional interaction between the photoreceptors/neuroretina and the RPE requires that the RPE maintains a normal ionic microenvironment, both intracellularly and extracellularly. In this way, intracellular processes are optimized, enabling for instance the proper phagocytosis and degradation of photoreceptor outer segments,^{209,210} as well as a net transport of fluid away from the photoreceptor-RPE interface towards the choroid.²¹¹⁻²¹⁴

The accumulation of fluid between the neuroretina and photoreceptors in BVMD and AFVD separates the photoreceptors from their natural “housekeeper”, the RPE (Fig. 3.2) and K). As a consequence, normal retinoid transport between the photoreceptors and the RPE is hindered, as is the ability of the RPE cells to efficiently perform photoreceptor outer segment phagocytosis.²¹⁵ These photoreceptor outer segments are then thought to accumulate on the outer neuroretina and are also shed into the abnormal space under the detached neuroretina.¹²⁹ These shed photoreceptor outer segments contain toxic autofluorescent retinoid-derived lipofuscin precursors.¹³⁷ The toxic precursors in the subretinal space may show an increased susceptibility to oxidative damage, due to a delay in RPE phagocytosis.^{136,140,216-218} The photoreceptors may subsequently be harmed, as they are not able to get rid of their outer segments in a normal fashion, with their unphagocytosed outer segments full of potentially toxic waste still nearby. This, in turn, may induce photoreceptor cell loss, possibly even before RPE atrophy, with consequent central visual loss. Although less efficiently, due to a lack of apposition to the RPE, the damaged photoreceptor outer segments may eventually be phagocytosed by the RPE cells. These RPE cells then gradually become overload with toxic autofluorescent lipofuscin constituents.^{135,219} This lipofuscin overload is thought to disturb normal RPE function, which could eventually lead to atrophy of the RPE.

As mentioned above, bestrophin-1 Cl⁻ channel activity and intracellular Ca²⁺ channel activity, modulated by bestrophin-1, appear to be mutually dependent. As the numerous functions of the RPE, such as phagocytosis of photoreceptor outer segments and the secretion of paracrine factors such as vascular endothelial growth factor,^{46,51,215} depend on changes in intracellular Ca²⁺ concentrations, bestrophin-1 may be of considerable influence on these processes. A disruption of the normal anatomical relationships, in concert with altered levels of angiogenic factors such as vascular endothelial growth

factor, could predispose to CNV formation in *BEST1*-associated disease.

The abnormal light response on the EOG in BVMD patients was originally thought to arise from bestrophin-1 Cl⁻ channel dysfunction.¹² However, in rare instances, patients with a vitelliform lesion and a *BEST1* mutation (such as the p.Ala243Val and the p.Ile295del mutations) may display a normal EOG light response.^{3,81,99,116} Nevertheless, these mutations were shown to cause a considerably decreased Cl⁻ channel conductance *in vitro*.^{20,30} Part of the explanation could be that the EOG may (initially) not be sensitive enough to detect less pronounced Cl⁻ conductance changes in the RPE. Another explanation, as proposed by Marmorstein and colleagues based on experiments in mice,³⁹ may be that the EOG light response is not generated by bestrophin-1. *BEST1*(-/-) knockout mice were shown to have normal RPE-derived ERG light responses and a normal Ca²⁺-sensitive Cl⁻ conductance.³⁹ Such preservation of a normal Ca²⁺ activated Cl⁻ current could find its explanation in the recent hypothesis that other channels, such as those from the anoctamin (ANO or TMEM16) family, rather than the bestrophins, are the classical Ca²⁺-dependent Cl⁻ channels.²²⁰ The EOG light peak may therefore also be derived from ion channels other than the bestrophin-1 Cl⁻ channels, with bestrophin-1 possibly influencing the EOG only indirectly. Experiments in mice and rats suggest that bestrophin-1 mutants can have a gain-of-function, affecting the EOG through a disturbed regulation of Ca²⁺ channels rather than through an effect on Cl⁻ channel function.³⁹⁻⁴¹ Again, it is unclear to what extent studies of bestrophin-1 in *in vitro* and animal models relate to the human situation.

Histopathological studies in BVMD patients with confirmed *BEST1* mutations parallel the clinical observations. A histopathological study in a 86-year-old patient with advanced BVMD,²¹ as well as in his previously described son,²²¹ who both carried a p.Thr6Arg *BEST1* mutation, showed massive lipofuscin accumulation in the RPE, together with a more than 4-fold increase of A2E compared to controls.^{21,132} Such large amounts of RPE lipofuscin and A2E are in agreement with the increased EAF that is seen in most BVMD lesions. An area of disciform scarring was observed in the macula, with adjacent degeneration of the RPE and outer nuclear layer. Bruch's membrane was intact, and there was no evidence of CNV. The observed disciform scar in the macula may be the histopathological correlate of the scarring that is seen on ophthalmoscopy in many patients with advanced BVMD. Mullins and colleagues showed that the amount of bestrophin-1 was higher in the extramacular RPE than in the macular RPE.²¹ The authors hypothesized that BVMD, at least when caused by a p.Thr6Arg mutation, is the result of insufficient amounts of wild-type bestrophin-1 in the macula. The macular RPE would then be unable to support proper ion homeostasis. As a consequence, an accumulation of subretinal fluid between the photoreceptors and the RPE may arise, and possibly intracellular RPE changes that increase lipofuscin and A2E accumulation.

Another histopathological study described the findings in a 93-year-old patient with a late-onset atypical form of BVMD, who carried a p.Tyr227Asn mutation in *BEST1*.⁸² This patient showed small multifocal vitelliform lesions on ophthalmoscopy at the age of 77, that gradually became "gliotic" within the next decade. Again, Bruch's membrane

was completely intact, and there was no evidence of CNV. This patient also displayed midperipheral drusen-like flecks at the level of the RPE, both on ophthalmoscopy and on histopathological examination. Surprisingly, the amount of RPE lipofuscin was not significantly different from that in age-matched controls. The authors speculated that a possible explanation for the apparently normal amount of RPE lipofuscin accumulation in this patient may be the exceptionally late age at onset of 75 years, suggesting a decreased level of expression of the allele with the p.Tyr227Asn mutation in this case. The resulting lipofuscin accumulation in the RPE would therefore also be more modest. A normal age-related increase of RPE lipofuscin,^{222,223} both in this aged BVMD patient and in an age-matched control group, may mask possible disease-related differences that might have been more evident earlier in life. Cystic changes in the outer nuclear layer have been observed both in BVMD and AFVD, and may provide an explanation for the apparent predisposition to macular hole formation in both conditions.^{115,126,224-226} In several cases, a generally intact RPE underlying the advanced macular BVMD lesions was seen on histopathology.^{82,132} This indicates that the loss of visual acuity due to photoreceptor degeneration is not necessarily secondary to RPE atrophy.

Histopathological findings have also been described on the eyes of a homozygous p.Trp93Cys *BEST1* mutation carrier, who suffered from BVMD, just like several affected family members who carried the mutation heterozygously. Increased levels of A2E were found in the RPE, together with a large serous retinal detachment in the macula.¹³² These findings also reflect the clinical observations of increased FAF and hyperreflective material on OCT in the earlier stages of BVMD,^{123,126,144} corresponding to a dynamic process of accumulation of vitelliform material within the subretinal space.

Two earlier histopathological studies on ungenotyped BVMD cases have also found marked accumulation of lipofuscin and melanolipofuscin in the RPE, together with evidence of CNV.^{227,228} One of these studies analysed the contents of the subretinal space between the photoreceptors and the RPE. This subretinal space contained accumulations of photoreceptor outer segments, lipofuscin-like material, and melanin. In addition, phagocytic cells were seen in the subretinal space, possibly derived from RPE cells, which were packed with lipofuscin and melanin.²²⁸ Melanin-containing phagocytic cells were also found in the subretinal space in a genetically confirmed case of BVMD (p.Thr6Arg mutation).²¹ Again, these findings correlate with the clinical findings on FAF and OCT in BVMD patients.

In summary, the available histopathological data on BVMD correspond to the clinical observations and lend further credence to the proposed pathophysiological mechanism, involving the accumulation of shed photoreceptor outer segments in the abnormal subretinal space between the photoreceptors and the RPE.

The pathogenesis of *BEST1*-related AFVD is probably comparable to BVMD. Several histopathological studies on AFVD have been published, without information on the genetic background.^{85,87,224,229,230} A detachment of the neuroretina was observed in some cases, similar to the findings on OCT, with the subretinal vitelliform material derived from photoreceptor outer segments, as well as from lipofuscin-loaded pigmented cells.

Lipofuscin accumulation was also seen in the RPE, together with RPE clumping and atrophy. An attenuation of the overlying neuroretina with photoreceptor degeneration could precede RPE atrophy.²²⁴

The clinical characteristics of ADVIRC, the MRCS syndrome, and ARB, are quite distinct from those observed in BVMD and AFVD. The clinical features in ADVIRC, the MRCS syndrome, and ARB, are at least partially the result of an incomplete development of various ocular structures, including the RPE, photoreceptors, as well as the anterior segment of the eye. The observed clinical differences of these diseases with BVMD and AFVD may be explained by the differential functional and structural impact of *BEST1* mutations. In ADVIRC and the MRCS syndrome, the abnormally spliced *BEST1* pre-mRNA causes an in-frame alteration of bestrophin-1 that has unknown functional consequences for the protein.^{5,8} In ARB, the *BEST1* null phenotype, there is an absence of normal bestrophin-1 protein, which also probably affects its presumed influence on ocular development. As bestrophin-1 is proposed to play a role in ocular development, possibly in concert with OTX and MITF, *BEST1* mutations in ADVIRC, the MRCS syndrome, and presumably ARB, may adversely affect normal ocular development through mechanisms that are currently unclear. The photoreceptor abnormalities on the ERG that are observed in ADVIRC, the MRCS syndrome, as well as in ARB, may be either due to a primary effect of the mutant bestrophin-1 on the photoreceptors, but may also occur secondary to abnormal RPE function and/or RPE degeneration.

ADVIRC, the MRCS syndrome, as well as ARB, may thus be considered to be part of an overlapping continuum of ocular developmental abnormalities due to bestrophin-1 dysfunction.

Two histopathological studies on ungenotyped ADVIRC have been published on the eyes of a 26-year-old and an 88-year-old patient. These studies showed disorganization of the peripheral retina, with intraretinal RPE migration, which may correspond to the retinal hyperpigmentation, as well as multifocal atrophy of the RPE and photoreceptors, which may correspond to the atrophic zones on ophthalmoscopy.^{231,232} A preretinal membrane was also observed, which consisted of condensed vitreous together with marked proliferation of Müller cells.²³² These preretinal vitreous condensations are also observed clinically.

The currently available mouse and rat models are indispensable to acquire further insight in the pathophysiology of *BEST1*-related diseases, as well as in the development of treatment strategies. However, these animals do not have a macula and do not develop retinal lesions as seen in humans, which is a drawback in their use as a model for human disease. The *cmr* dog model does show juvenile-onset lesions that resemble those seen in multifocal vitelliform dystrophy.^{83,233} The disease in this dog model is caused by naturally occurring autosomal recessive homozygous *BEST1* mutations, and may serve as a model for the human null phenotype ARB. A small sample of heterozygous carrier dogs appeared unaffected, although further evaluation on these heterozygotes (for instance with EOG) is warranted.²³³ These animal models may aid in the development of future therapeutic strategies such as gene therapy in *BEST1*-related disease.

5. Future perspectives

Therapeutic intervention in BVMD and AFVD may be primarily aimed at the restoration of normal contact between the photoreceptors and the RPE. However, merely restoring such anatomical contact will most likely not solve the problem, as *BEST1* mutations may also affect intracellular processes in the RPE that could have damaging effects. Therefore, it seems preferable to eradicate the detrimental effect of the bestrophin-1 mutant, for instance by means of gene therapeutic strategies. Intervention with gene therapy in BVMD and AFVD caused by *BEST1* mutations may have a large time span in which it could be successfully implemented, as progression is rather slow in most patients.

Gene delivery of wild-type *BEST1* to the RPE may be efficient in the treatment of BVMD and AFVD haploinsufficiency phenotypes, caused by *BEST1* mutations that exclusively result in a loss of sufficient wild-type protein.^{238,242} Such *BEST1* gene delivery may be achieved through various methods, such as adeno-associated viral vectors and non-viral gene carriers such as DNA nanoparticles, administered for instance through subretinal injection.²³⁴⁻²³⁷ However, many *BEST1* mutations cause disease through a dominant negative effect. In these cases, a destruction of mutant messenger RNA would be required for treatment to be effective, rather than an increased expression of wild-type *BEST1* such as in haploinsufficiency phenotypes. Such a disruption of the dominant negative mutant gene is possible through RNA interference techniques and ribozymes.^{235,236,238-241} Among the many challenges in gene therapy are the achievement of sufficient transduction and transfection efficiency, a prolonged gene expression, as well as the safe administration of the therapeutic agent.^{238,242} Important issues in the application of gene therapy in BVMD and AFVD are the frequent occurrence of decreased penetrance and variable expressivity, as well as the sometimes relatively mild clinical symptoms. Such issues raise questions about who should be treated, and when treatment should be started. After all, gene therapy may have potentially serious side effects, especially when using viral vectors.^{243,244} The development of stringent inclusion criteria is obviously required to perform gene therapy for BVMD and AFVD in the safest and most effective way. Fortunately, the eye is relatively easily accessible for the localized administration of a therapeutic agent and for the clinical evaluation of its effects.^{238,245}

Retinal disease in ADVIRC, the MRCS syndrome, as well as ARB, could also respond to timely gene therapeutic intervention. However, treatment of these diseases would be more challenging, since the structural anterior segment abnormalities that predispose to angle-closure glaucoma are probably already present at birth. Furthermore, like in BVMD, variable expression and reduced penetrance of *BEST1* mutations have also been noted in ADVIRC,⁵ complicating decisions on the necessity and timing of treatment. A more practical prophylactic approach, applying YAG laser iridotomies in patients with narrow anterior chamber angles at risk for angle-closure glaucoma, appears a rational and feasible alternative that is currently possible in these cases.¹⁸⁴ Cataract extraction may be successfully performed in patients with disturbing lens opacities.^{5,184}

The broad clinical spectrum of ocular diseases caused by mutations in the *BEST1* gene

clearly alludes to the multifunctional role of its protein product, bestrophin-1. Such functional and phenotypic diversity underscores the challenging need for differentiated therapeutic approaches, which may be further developed using the currently available animal models.

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3.2. Clinical and molecular genetic analysis of Best vitelliform macular dystrophy

Abstract

Objectives: To describe the phenotype of Best vitelliform macular dystrophy (BVMD), and to evaluate genotype-phenotype and histopathological correlations.

Methods: Retrospective analysis of patients with BVMD who underwent an extensive ophthalmic examination, including best-corrected Snellen visual acuity, fundus examination by indirect ophthalmoscopy, fundus photography, fundus autofluorescence (FAF), optical coherence tomography (OCT), fluorescein angiography, and electro-oculography. In addition, molecular genetic analysis of the *BEST1* gene was performed in all patients.

Results: We examined 40 eyes of 20 patients with BVMD. Sixteen eyes (40%) had a well-defined BVMD stage, whereas 18 eyes displayed characteristics attributable to different stages. Six eyes had an atypical form of BVMD. FAF and OCT frequently detected abnormalities that were not visible on ophthalmoscopy and fluorescein angiography. All patients carried a mutation in the *BEST1* gene. Molecular genetic analysis identified eight different *BEST1* mutations in 15 families, including two novel mutations (p.Gly299Ala and p.Ile3Thr). Genotype-phenotype correlation was limited, as we observed a broad phenotypic range in association with a single *BEST1* mutation. However, the p.Ala243Val appears to cause a mild and relatively invariable BVMD phenotype.

Conclusions: A broad phenotypic variability may be observed in BVMD, even with a single *BEST1* mutation. FAF and OCT are valuable non-invasive imaging techniques for the phenotyping and follow-up of BVMD patients.

Introduction

Best vitelliform macular dystrophy (BVMD) is an autosomal dominant retinal disease with incomplete penetrance and a variable phenotypic expression.¹⁻³ BVMD is associated with mutations in the *BEST1* gene, formerly known as the VMD2 gene, which can be found in virtually all patients with a positive family history.⁴⁻⁶ The *BEST1* gene encodes the bestrophin-1 protein, which oligomerizes and is assumed to be a Ca²⁺-activated Cl⁻ channel, located at the basolateral plasma membrane of the RPE.⁷ Moreover, bestrophin-1 may also modulate the activity of voltage-gated L-type Ca²⁺ channels.^{7,8} In addition to BVMD, *BEST1* mutations may also cause a spectrum of other eye diseases, including adult-onset foveomacular vitelliform dystrophy (AFVD),⁴ autosomal recessive bestrophinopathy,⁸ autosomal dominant vitreoretinochoroidopathy, and the microcornea, retinal dystrophy, cataract, and posterior staphyloma (MRCS) syndrome.⁹

Patients with BVMD typically show bilateral yellow macular lesions that resemble an

egg-yolk at a certain point in time, in combination with a subnormal electro-oculogram (EOG). The full-field electroretinogram is usually normal, whereas the multifocal electroretinogram may reveal abnormalities early in the course of the disease.¹⁰⁻¹³ Gass has classified BVMD into six phenotypic stages: the previtelliform or carrier stage, the vitelliform stage, the pseudohypopyon stage, the vitelliruptive or scrambled-egg stage, the atrophic stage, and the cicatricial and choroidal neovascular stage.¹ Lesions may be multifocal.¹⁴ Controversy exists about the chronological order of these stages.¹⁵

The current classification of BVMD relies almost completely on the findings on ophthalmoscopy, despite emerging techniques such as optical coherence tomography (OCT) and short-wavelength fundus autofluorescence (FAF) imaging. OCT provides cross-sectional images of the various layers of the neuroretina as well as the retinal pigment epithelium (RPE).¹⁶ FAF imaging, on the other hand, has emerged as an important non-invasive imaging technique to visualize retinal changes related to lipofuscin accumulation.^{17,18} This is important especially in BVMD, since histopathological reports indicate that the yellow material in BVMD lesions is lipofuscin.¹⁹⁻²¹ With FAF and OCT, the phenotypic characteristics of retinal dystrophies such as BVMD can be studied in greater detail, thereby improving the clinical characterization. Before these techniques can be used in such a manner, the specific pathological findings in OCT and FAF should be linked to the familiar ophthalmoscopic and fluorescein angiographic images. Analysis of the underlying genetic defect in the *BEST1* gene may allow genotype-phenotype correlation. In this study, we describe in detail the FAF and OCT characteristics of the six phenotypic stages of BVMD. The clinical findings of these imaging modalities will be compared to the ophthalmoscopic and fluorescein angiographic features, and possible genotype-phenotype correlations are analyzed.

Methods

Clinical studies

This study followed the tenets of the Declaration of Helsinki and informed consent was obtained from all subjects. We examined 40 eyes of 20 BVMD patients (10 males and 10 females, aged 11-53 years) from 15 different families, who were referred to the Departments of Ophthalmology of the University Medical Centres in Nijmegen and Utrecht. The diagnosis BVMD was established based on the lesions visible on ophthalmoscopy, an autosomal dominant inheritance pattern, and an abnormal EOG. The EOG was recorded according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards and was considered abnormal if the Arden ratio was below 2.0. After the medical histories of the patients were obtained, an extensive clinical examination was performed, including best-corrected Snellen visual acuity and fundus examination by indirect ophthalmoscopy. Fundus photography and FAF imaging (Heidelberg Retina Angiograph (HRA) 2, Heidelberg Engineering, Dossenheim, Germany) were subsequently performed using a previously described protocol,²² as well as OCT (Stratus OCT, Carl Zeiss

Meditech, Inc., Dublin, CA, USA), using the “Macular Thickness Map” protocol provided by the manufacturer. Fundus photography, FAF, and OCT were repeated in 14 patients after a mean follow-up period of 11 months (range, 7 - 19 months). Finally, all patients underwent fundus fluorescein angiography (FFA).

Molecular genetic studies

Blood samples were collected from all patients. The genomic DNA was isolated according to a previously described protocol.²³ Molecular genetic analysis of the 10 coding exons and splice junctions of the *BEST1* gene was performed in all patients, according to a previously described protocol.²⁴ Primers and PCR-conditions are available on request.

Results

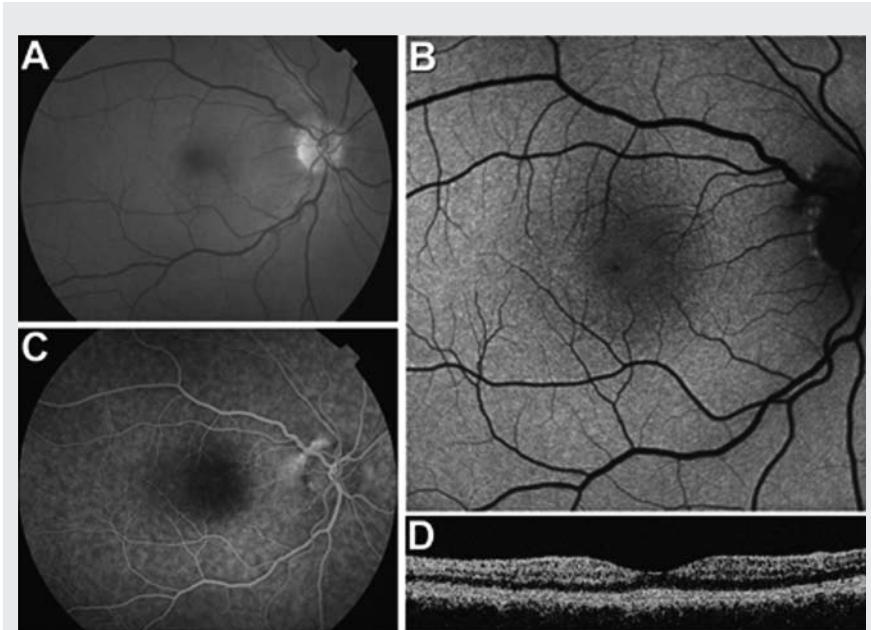
Molecular genetic findings

We identified eight different *BEST1* mutations in 20 patients of 15 different families. Patients 1 and 2 belonged to the same family (family A), as did patients 4 and 5 (family C), patients 6 and 7 (family D), and patients 16, 17, and 18 (family M). Two novel mutations were identified: p.Gly299Ala (c.895G>C, in patients 19 and 20) and p.Ile3Thr (c.8T>C, in patient 15). These mutations were not identified in 100 control alleles, and both mutations are predicted to be pathogenic. Six previously described mutations were found: p.Thr6Pro (c.16A>C, in patients 1-9),^{4,6,14,25} p.Tyr227Asn (c.679T>A, in patients 12 and 13),^{6,24,27} p.Lys194_Ala195insVal (c.583_584insTGG, in patient 10),¹⁴ p.Leu82Val (c.244C>G, in patient 11),^{25,28} p.Asp302_Asp304del (c.904-912del, in patients 16, 17, and 18),¹⁴ and p.Ala243Val (c.728C>T, in patient 14).^{4,28} Three mutations were found in more than one family. The p.Thr6Pro mutation, which has been previously described in BVMD patients from the Netherlands,⁶ was found in eight patients of five different families (Table 3.2). The p.Gly299Ala mutation was found in two unrelated patients (patients 19 and 20). The p.Tyr227Asn mutation was found in the unrelated patients 12 and 13.

Clinical findings

The clinical characteristics of the patients are summarized in Table 3.2. The Snellen visual acuity ranged from 20/200 to 20/16 (mean 20/35). During the mean follow-up period of 11 months, this mean visual acuity remained stable. Of the 40 eyes that were examined, 16 demonstrated lesions compatible with a typical stage as described by Gass: two eyes showed no macular abnormalities and thus were in the carrier stage (Fig. 3.5), one eye was in the vitelliform stage (Fig. 3.6A-E), two eyes showed a pseudohypopyon lesion (Fig. 3.6F-I), 5 eyes were in the vitelliruptive stage (Fig. 3.6J-M), 3 eyes had an atrophic lesion (Fig. 3.7A-D) and three eyes were in the cicatricial stage (Fig. 3.7E-H).

Eighteen of the 40 eyes showed macular lesions that were combinations of the BVMD stages as described by Gass. The following combinations were encountered: vitelliform/vitelliruptive stage (2 eyes), vitelliruptive/pseudohypopyon stage (1 eye), vitelliruptive/

**Figure 3.5.***

Carrier stage. **A.** Fundus photograph of patient 4 (*p.Thr6Pro* mutation in *BEST1*), the 41-year-old father of patient 5, does not show any abnormalities. **B.** Fluorescein angiography does not show any abnormalities of the macula. **C.** Fundus autofluorescence image, showing a normal autofluorescence pattern of the macula. **D.** Optical coherence tomography scan shows a normal aspect of the retinal layers.

atrophic stage (1 eye), vitelliruptive/cicatricial stage (9 eyes), vitelliruptive/pseudohypopyon/cicatricial stage (1 eye), cicatricial/atrophic stage (1 eye) and pseudohypopyon/cicatricial/atrophic stage (3 eyes). Vitelliruptive/pseudohypopyon/cicatricial lesions were differentiated from pseudohypopyon/cicatricial/atrophic lesions, as the latter lesions clearly showed profound atrophy of the RPE on ophthalmoscopy, in addition to an area of scarring. In 6 patients, the BVMD stage differed between both eyes. After a mean follow-up period of 11 months in 14 patients, 12 patients showed no or only minor changes within the lesion. Two patients displayed clear changes of lesion characteristics. After a follow-up period of 8 months in patient 7, the pseudohypopyon in the right eye evolved towards a vitelliruptive stage, whereas the left eye showed considerable enlargement of the pseudohypopyon (Fig. 3.6F-I). The lesion in the right eye of patient 3 developed a pseudohypopyon after a follow-up period of 7 months (Fig. 3.6A-E).

Six eyes of 3 patients demonstrated an atypical form of Best disease (patients 12, 14, and 20). Patient 14, who carried a *p.Ala243Val* mutation in *BEST1*, had macular lesions that simulated a pattern dystrophy (Fig. 3.8A-D). The EOG was mildly disturbed, with an

Table 3.2.
Clinical and molecular genetic characteristics of patients with Best vitelliform macular dystrophy.

Patient number	Family number	Sex	Age at onset	Age	Visual acuity	Stage	Fluorescein angiography	Fundus autofluorescence (FAF)	OCT	<i>BEST1</i> gene mutation
1	A-II.2	f	30	41	20/100	20/40	OD	OD	ODS; mixed ↓ R and ↑ R	p.Thr6Pro ^{4,6,15,26}
2	A-III.1	f	12	17	20/25	20/100	C (Fig. 3.7E)	ODS; ↑ F, passive leakage [*] into scar (Fig. 3.7G)	ODS; ↓ FAF between retina and underlying ↑ R material, OS intraretinal cystoid changes (Fig. 3.7H)	p.Thr6Pro ^{4,6,15,26}
3	B-III.1	f	10	12	20/40	20/125	V/[S] (Fig. 3.6A)	S/[C/N]	OD; subretinal ↑ R material (Fig. 3.6E)	p.Thr6Pro ^{4,6,15,26}

Table 3.2. *continued*

Patient number	Family number	Sex	Age at onset	Visual acuity OD	OD	Stage	Fluorescein angiography	Fundus autofluorescence (FAF)	OCT	BEST1 gene mutation
4	C.II.1	m	-	41	20/20	20/20 carrier (Fig. 3.5A)	carrier	ODS: normal (Fig. 3.5C)	ODS: normal (Fig. 3.5B)	ODS: normal (Fig. 3.5D) p.Thr6Pro ^{4,6,15,26}
5	C.III.1	m	<5	17	20/80	20/25	SIC	SIC	ODS: ↑ F, passive leakage	ODS: mildly ↑ FAF between retina and underlying ↑ R material p.Thr6Pro ^{4,6,15,26}
6	D.III.4	m	<5	45	20/200	20/40	P/C/A	S	OD: ↑ F due to passive leakage, possibly from scar from old, CNV OS: passive leakage and window defect	OD: marked ↑ FAF of pseudohypopyon material, ↓ R subretinal material, ↑ R of cicatrical/atrophic area, which adheres to overlying thinned retina OS: ↓ R subretinal material, some ↑ R depositions p.Thr6Pro ^{4,6,15,26}
7	D.IV.3	m	5	11	20/25	20/25	P	ODS: ↑ F of pseudohypopyon in earlier phase due to contribution of FAF, ↓ F of	ODS: marked ↑ FAF of pseudohypopyon material, mildly ↑ FAF of rest of the lesion p.Thr6Pro ^{4,6,15,26}	

Spectrum of BEST1-associated phenotypes

8	E-III.1	m	16	26	20/80	20/70	C	C/A	OD: ↑ F, passive leakage of scar, OS: passive leakage and window defect	OD: focal areas of ↑ FAF OS: ↓ FAF of cicatricial zone	OD: adhesion between retina and underlying ↑ R material, surrounded by ↓ R subretinal material OS: ↑ R of cicatricial/atrophic area	p.Thr6Pro ^{4,6,15,26}		
9	F-III.5	m	<5	17	20/25	20/100	P/S/C	S/C	ODS: ↑ F, passive leakage into scar	ODS: mildly ↑ FAF, OS: ↓ FAF in central part of scar	ODS: adhesion between retina and underlying ↑ R cicatricial material, surrounded by ↓ R subretinal material	p.Thr6Pro ^{4,6,15,26}		
10	G-II.1	m	<5	12	20/25	20/20	S	S	(Fig. 3.6f) ODS: ↑ F, passive leakage (Fig. 3.6l)	ODS: ↑ FAF of yellowish material (Fig. 3.6K)	ODS: ↓ R subretinal material, some ↑ R depositions (Fig. 3.6M)	p.Lys194-Ala195 insVal ¹⁵		
11	H-II.1	f	42	42	20/25	20/32	S/A	A	OD: ↑ F, passive leakage, OS: ↑ F, passive leakage and window	ODS: remnants of material with ↑ FAF, markedly ↓ FAF of atrophic	ODS: ↓ R subretinal material, ↑ R of atrophic areas, S	p.Leu82Val ^{26,29}		

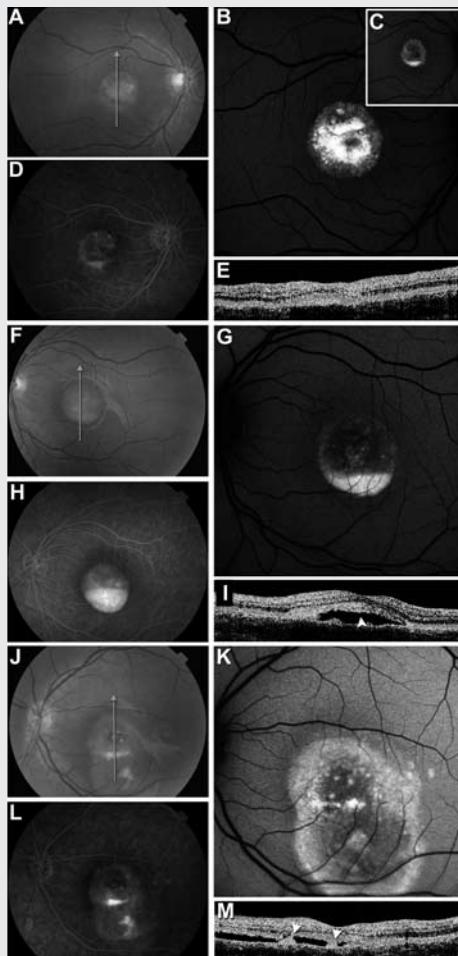
Table 3.2. continued

Patient number	Family number	Sex	Age at onset	Age	Visual acuity OD	OD	Stage OD	Fluorescein angiography	Fundus autofluorescence (FAF)	OCT	BEST1 gene mutation
							defect		areas	which adhere to overlying thinned retina	
12	I-III.2	m	<5	35	20/16	20/16	Discrete RPE changes	ODS; ↑ F, window defect	ODS; slightly ↑ FAF	ODS; discrete ↑ R in foveal area	p.Tyr227Asn 6.2528
13	J-II.2	m	36	47	20/80	20/50	P/CIA	ODS; ↑ F, passive leakage and window defect	ODS; marked ↑ FAF of pseudohypopyon material, ↓ FAF of atrophic/cicatricial part, mildly ↑ EAF of rest of the lesion	ODS; ↑ R pseudohypopyon, ↓ R subretinal material, ↑ R of cicatricial/atrophic area, which adheres to overlying thinned retina	p.Tyr227Asn 6.2528
14	K-IV.1	f	44	50	20/25	20/70	Atypical	ODS; ↑ F, passive leakage (Fig. 3.8A)	ODS; radial pattern of FAF alterations, OS; pseudohypopyon-like ↑ FAF (Fig. 3.8B)	ODS; irreflective subretinal material (Fig. 3.8D)	p.Ala243Val 4.29
15	L-III.1	f	16	24	20/25	20/100	S/C	ODS; ↑ F, passive leakage	ODS; remnants of ↑ FAF, ↓ EAF of cicatricial parts	ODS; adhesion between retina and underlying ↑ R cicatrical material,	p.Ile3Thr [†]

16	M-III.4	f	50	51	20/40	20/22	V/S	V/S	ODS; ↑ F, passive leakage	ODS; ↑ FAF in zones corresponding with yellowish material	ODS; subretinal material	surrounded by ↓ R subretinal material	p.Asp304del ₁₅
17	M-IV.3	f	12	15	20/80	20/25	S/C	S/C	OD; ↑ F, passive leakage and window defect, OS; ↑ F, passive leakage	ODS; irregular ↑ FAF, areas of ↓ FAF within cicatricial zones	OD; adhesion between retina and underlying ↑ R cicatrical material, surrounded by ↓ R subretinal material	ODS; subretinal layer with ↑ R material	p.Asp304del ₁₅
18	M-IV.4	f	2	17	20/25	20/20	P/S	S/C	ODS; ↑ F, passive leakage	ODS; ↑ FAF in zones corresponding with yellowish material	ODS; ↓ R subretinal material with some ↑ R deposits	ODS; ↓ R material	p.Asp304del ₁₅
19	N-III.1	f	36	44	20/70	20/33	A (Fig. 3.7A)	A	ODS; ↑ F, window defect (Fig. 3.7C)	ODS; ↓ FAF surrounded by ring of ↑ FAF (Fig. 3.7B)	ODS; thinning of “outer red line” with underlying ↑ R	ODS; ↓ R material	p.Gly299Ala [†]
20	O-II.3	m	40	53	20/80	20/200	S/C/A (multifocal)	S/C/A (multifocal)	ODS; ↑ F, passive leakage and window defect [‡] (Fig. 3.8E)	ODS; multifocal lesions with ↑ FAF of yellowish depositions at outer border, ↓ FAF centrally (Fig. 3.8F)	ODS; elevating retina, OD intraretinal cystoid changes (Fig. 3.8H)	ODS; ↓ R material	p.Gly299Ala [†]

Passive leakage is defined as the apparent leakage of fluorescein into the lesion during later stages of the fluorescein angiogram, without signs of choroidal neovascularization.¹ A window defect is defined as hyperfluorescence on the fluorescein angiogram due to an increased transmission of choroidal fluorescence as a result of atrophy of the overlying retinal pigment epithelium.² Mutation that has not been previously described in literature.

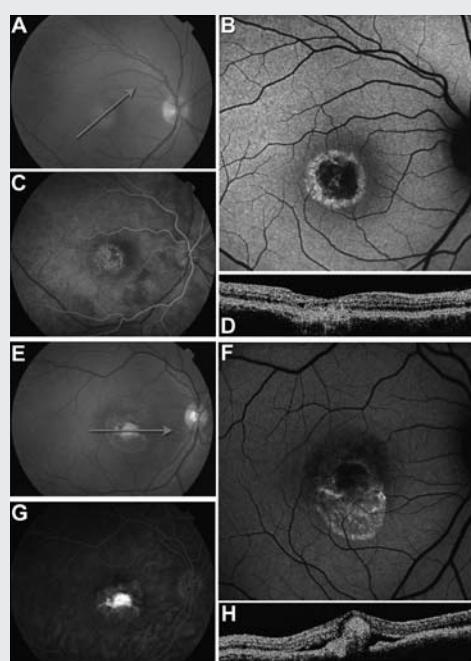
Abbreviations: V, vitelliform; P, pseudohypopyon; S, vitelliruptive/scrambled-egg; A, atrophic; C, cictricial; N, neovascular; CNV, choroidal neovascularization. OCT, Optical Coherence Tomography; ↑ R, hyperreflective hyperreflectivity; ↓ R, hyporeflective (hyporeflectivity); BYMD, Best vitelliform macular dystrophy.

**Figure 3.6.***

Vitelliform stage. **A.** Fundus photograph of patient 3 (*p.Thr6Pro* mutation in *BEST1*), showing a yellowish lesion with some fibrosis and pigmentation at the boundaries of the lesion. **B.** Fundus autofluorescence (FAF) of the yellowish parts of the lesion is significantly increased, but becomes less intense towards the lesions margins. Zones of increased FAF are beginning to cluster within the lesion. **C.** Seven months later, the ophthalmoscopic and FAF picture evolved to a lesion with combined characteristics of the scrambled-egg and pseudohypopyon stage. **D.** At this point in time, fundus fluorescein angiography (FFA) was performed, showing patchy hyperfluorescence in the superior part of the lesion, and a hyperfluorescent pseudohypopyon. **E.** A vertical optical coherence tomography (OCT) scan (arrow in panel A) shows a hyperreflective subretinal lesion in the vitelliform stage. Pseudo-hypopyon stage. **F.** Fundus photograph of patient 7, who also carried a *p.Thr6Pro* mutation, showing yellowish material inferiorly with transparent fluid in the superior part of the lesion. **G.** The yellowish material of the pseudohypopyon shows

substantially increased FAF, with only some small spots of increased FAF in the superior part of the lesion. **H.** The pseudohypopyon is intensely hyperfluorescent in the mid-late phase of FFA.

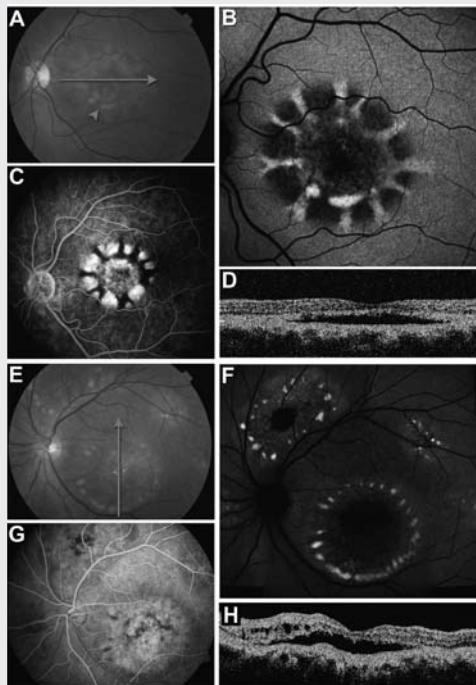
I. The pseudohypopyon corresponds to subretinal material of increased reflectivity on a vertical OCT section (arrow in panel F). Additional hyperreflective material is attached to the outer retina and to the bottom of the subretinal space (arrowhead). Vitelliruptive/Scrambled-egg stage. **J.** Fundus photograph of patient 10 (*p.Lys194_Ala195insVal* mutation in the *BEST1*), showing a large lesion with scattered clusters of yellowish material within transparent fluid. **K.** In addition to the locations of clinically apparent yellowish material, increased FAF can be observed at the borders of the lesion where yellowish subretinal material cannot readily be visualized by ophthalmoscopy. **L.** On FFA, the lesion shows patchy hyperfluorescence. **M.** OCT scan taken vertically (arrow in panel J), showing elevation of the retina by subretinal fluid that has no reflectance. The clusters of yellow material that were seen on the fundus photograph appear as prominent hyperreflective structures (arrowheads).

**Figure 3.7.***

Atrophic stage. **A.** Fundus photograph of patient 19 (p.Gly299Ala mutation in BEST1) showing an atrophic lesion. **B.** The fundus autofluorescence (FAF) image shows markedly decreased FAF of the atrophic area, surrounded by a ring-shaped area of increased FAF. **C.** Fundus fluorescein angiography (FFA) shows a well-circumscribed, hyperfluorescent window defect due to atrophy of the retinal pigment epithelium. **D.** This atrophic area corresponds with retinal thinning with underlying hyperreflectivity on the optical coherence tomography (OCT) scan (arrow in panel A). Small intraretinal cystoid changes can also be discerned. Cicatricial stage. **E.** The fundus photograph of patient 2 (p.Thr6Pro mutation in BEST1) shows a prominent yellow-white subretinal scar with pigmented borders, surrounded by a serous retinal detachment. The visual acuity (20/25) was remarkably preserved. **F.** FAF imaging reveals significantly decreased FAF of the central scar, surrounded by small amounts of material of increased FAF intensity scattered throughout the rest of the lesion. This material is not evident on the fundus photograph. **G.** In the late phase of FFA, the scar is intensely hyperfluorescent due to passive leakage of fluorescein into the scar, whereas the surrounding part of the lesion shows more discrete, patchy hyperfluorescence. **H.** OCT (arrow in panel E) shows a prominent and hyperreflective structure, in contact with the overlying retina. This structure is surrounded by elevated retina with underlying spots of increased reflectivity.

Arden ratio of 1.7 in the left eye and 1.8 in the right eye. Despite these atypical clinical characteristics, evidence of an autosomal dominant inheritance pattern and discrete vitelliform lesion aspects on ophthalmoscopy prompted a molecular genetic evaluation of the *BEST1* gene. Patient 20, who carried a p.Gly299Ala mutation in *BEST1*, displayed the phenotype of multifocal vitelliform macular dystrophy with an absent light peak on EOG (Fig. 3.8E-H). Patient 12, a 35-year-old male, showed discrete RPE changes in the fovea that had not changed since the first examination at the age of 6 years, with no light rise on EOG (Arden ratio of 1.0) in both eyes. This phenotype is not described in the classification of Gass, but corresponds to stage I in the classification of Mohler and Fine.¹⁵ Analysis of the *BEST1* gene in this patient, performed because of the absent light rise on the EOG, revealed a p.Tyr227Asn mutation.

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**Figure 3.8.***

Atypical forms of Best vitelliform macular dystrophy. **A.** Fundus photograph of patient 14 (*p.Ala243Val* mutation in *BEST1*). A small pseudohypopyon (arrowhead) and some scattered yellow deposits are combined with a spoke-like pattern of subretinal yellowish changes, resembling a pattern dystrophy. **B.** The lesion shows increased fundus autofluorescence (FAF) of the small pseudohypopyon and a radial pattern of spoke-like extensions with increased FAF on a background of markedly decreased FAF. **C.** The spokes of increased FAF are hypofluorescent on fundus fluorescein angiography (FFA), whereas the spokes of decreased FAF are hyperfluorescent. **D.** A horizontal optical coherence tomography (OCT) scan (arrow in panel A) shows an elevated retina with

irreflective subretinal material. **E.** Fundus photograph of patient 20 (*p.Gly299Ala* mutation in *BEST1*), who presented with multifocal vitelliform lesions. The different lesions all have characteristics of the vitelliruptive stage, with circular deposition of yellowish material along the edges of the lesions. The lesions show some degree of fibrosis and atrophy as well. **F.** FAF shows that the yellowish deposits are intensely autofluorescent, whereas areas with atrophy and fibrosis show absent FAF. **G.** On FFA, lesions are predominantly hyperfluorescent. **H.** A vertical OCT scan (arrow in panel E) through the central lesion reveals a serous retinal elevation with intraretinal cystoid changes. The underlying layer shows increased reflectivity, probably corresponding with the fibrosis seen on ophthalmoscopy.

Fundus autofluorescence, optical coherence tomography, and fluorescein angiography

In the carrier stage, no abnormalities were detectable on FAF, OCT, and FFA (Fig. 3.5). In the vitelliform stage, however, the yellowish material of the “egg-yolk” lesion corresponded with an intense FAF signal. This material was hyperreflective on OCT (Fig. 3.6A-E). In the early phase of FFA, the vitelliform material blocked fluorescence and thus appeared hypofluorescent. The vitelliform material subsequently became hyperfluorescent, and in the late stages became hypofluorescent again. Increased FAF was seen inferiorly in the pseudohypopyon lesions, where a fluid level with relatively transparent fluid is formed in the superior part of the lesion (Fig. 3.6F-I). This fluid did not have an increased FAF

signal and showed no reflectivity on OCT. On FFA, the lower part of the lesion, which contained vitelliform material, had a similar aspect as in vitelliform lesions. The upper part showed patchy hyperfluorescence, that sometimes increased in intensity towards the later phases of the angiogram (Fig. 3.6H). The vitelliruptive/scrambled-egg stage showed clusters of vitelliform material scattered throughout the lesion. These lesions showed increased FAF and increased reflectivity on OCT, and were generally hyperfluorescent on FFA (Fig. 3.6J-M). In the atrophic stage, lesions displayed a predominantly decreased FAF signal, corresponding with an irregular posterior thickening of the highly reflective external band on OCT, due to a deeper penetration of the light to the choroid through the atrophic retina (Fig. 3.7A-D). Areas of RPE atrophy showed well-circumscribed hyperfluorescence on the fluorescein angiogram. Finally, in the cicatricial stage, areas with discrete scarring showed slightly increased FAF, whereas FAF in areas with advanced cicatrization was decreased compared to normal FAF. These areas with sub- and intraretinal fibrosis generally appeared as prominent hyperreflective structures on OCT, and were intensely hyperfluorescent on the FFA (Fig. 3.7E-H). Nineteen of the 40 eyes (47,5%) showed evidence of subretinal yellow-white fibrous tissue and scarring. Fourteen eyes (35%) showed adhesions between the retina and subretinal hyperreflective structures on OCT (Fig. 3H). In five eyes (12,5%) of four different patients, intraretinal cystoid changes overlying the lesion were observed (Fig. 3.7D and 3.8H). One patient (patient 3) had evidence of previous choroidal neovascularization in one eye on FFA.

Discussion

Phenotype

Less than half (40%) of the BVMD patients in our study showed a typical lesion stage as originally described by Gass. A substantial number (45%) of the BVMD lesions simultaneously displayed abnormalities that were attributable to two or even more different BVMD stages. The remaining 15% of the eyes showed an atypical form of BVMD. Gass assumed a chronological order in the various stages, but the patients in our study show that this sequence is not always followed. In three eyes, a pseudohypopyon together with evidence of scarring and atrophy was observed, resulting in a visual acuity that did not exceed 20/50. Gass placed the atrophic stage before the cicatricial stage, but many lesions in the present study showed signs of subretinal fibrous tissue before marked atrophy was evident on ophthalmoscopy, FAF and OCT. Our results indicate that a strict classification in stages is too rigid, since almost half of the BVMD lesions show aspects of different stages. Most BVMD lesions remain stationary after a considerable follow-up period,^{15,29} but our study also illustrates that a minority of lesions may show notable stage changes within less than a year. The variable amount of vitelliform material and the sometimes alternating pseudohypopyon and vitelliruptive stages have been described previously.²⁹ This phenomenon may find its origin in variations in photoreceptor outer segment disc shedding, RPE phagocytotic activity, as well as variable physical activity of the patient.³⁰

In accordance with previous studies on BVMD,^{15,29,31} our study indicates that choroidal neovascularization (CNV) is rare in this type of retinal dystrophy. In some BVMD patients, lesions with signs of scarring and/or discrete signs of fluorescein leakage on the fluorescein angiogram might suggest a mild form of CNV. However, Bruch's membrane was completely intact in several histopathological studies,^{27,32,33} also in areas with overlying scars or atrophy. This shows that scars and atrophy are not necessarily indicative of previous CNV. Staining of these lesions in the late phases of FFA may be caused by passive leakage and subretinal accumulation of fluorescein due to abnormal RPE structure and function. CNV should still be suspected when subretinal hemorrhages and exudation are seen within the BVMD lesion, in combination with visual loss. In these cases, FFA and possibly indocyanine green angiography may be performed to confirm the presence of CNV. After all, identification of CNV may have therapeutic consequences, as treatment of CNV with a form of anti-angiogenic therapy may be effective.^{34,35} It should be emphasized, however, that the majority of BVMD patients with subretinal hemorrhage do not have evident signs of CNV on the fluorescein angiogram and generally show spontaneous and significant improvement of visual acuity.³¹

Comparison of fundus autofluorescence, optical coherence tomography, and fluorescein angiography

We observed increased FAF in the earlier stages of BVMD (except for the carrier stage), corresponding with the yellowish vitelliform material on ophthalmoscopy. FAF imaging was also able to visualize small amounts of vitelliform material of increased FAF that were not seen on ophthalmoscopy, OCT, and FFA. In later stages, the amount of this material decreased in favor of relatively transparent subretinal fluid that lacked the increased FAF signal. Scars and atrophic regions in advanced BVMD lesions were characterized by decreased FAF. Often, FAF more clearly shows the extent of the lesion. As mentioned previously, it remains unclear why this yellowish material with increased FAF, which is initially abundant, may alternately disappear and reappear in some cases. It is also unknown how the transparent fluid without increased FAF appears. This fluid may possibly find its origin in the disturbed ion and fluid transport by the RPE in BVMD patients, associated with bestrophin-1 dysfunction.^{5,36}

OCT imaging also provides further insight into the composition of BVMD lesions. Both the yellowish vitelliform and the subretinal fibrosis appeared hyperreflective with this technique. OCT was able to detect abnormalities that were not seen on ophthalmoscopy, FAF, and FFA, such as an adhesion between retinal tissue and subretinal hyperreflective structures, as well as hyporeflective intraretinal cystoid changes, a finding which has been described previously in BVMD.³⁷ OCT is able to visualize the antero-posterior dimensions of a BVMD lesion and thus adds another dimension to the insight into the lesional constitution. In addition, OCT may better delineate the size and shape of a subretinal scar and its position relative to the overlying retina. Atrophic areas, accumulation of hyperreflective material, and the location and amount of sub- and intraretinal fluid may also be easily visualized with OCT. The OCT findings may

correlate well with findings with other imaging methods. For instance, the areas of accumulation of subretinal material of increased FAF seem to correspond largely with the hyperreflective material within BVMD lesions on OCT (Fig. 3.6G and I). The lack of apposition between the photoreceptors and the RPE, presumably caused by subretinal fluid accumulation due to bestrophin-1 dysfunction, results in inefficient phagocytosis of the photoreceptor outer segments by the RPE.³⁰ The hyperreflective material adjacent to the outer surface of the neuroretina in BVMD on OCT (Fig. 3.6I) could therefore correspond to accumulations of shed photoreceptor outer segments, that are filled with A2E-precursor fluorophores responsible for high FAF.³⁰ As these shed photoreceptor outer segments further accumulate, they may eventually be distributed throughout the lesion, corresponding with an egg-yolk appearance on ophthalmoscopy, high FAF of the entire lesion, and a completely hyperreflective lesion on OCT (Fig. 3.6A, B, and E). With time, this material gravitates to the inferior part of the lesion, causing a pseudohypopyon, with a displacement of high FAF and hyperreflectivity on OCT predominantly to this part of the lesion (Fig. 3.6C, F, G, H, and I). Our results show that the anatomical level of such accumulations (adjacent to the retina, the RPE, or both) is more readily identified with OCT as compared to ophthalmoscopy, FAF, and FFA. We used the Stratus OCT, but the use of higher resolution spectral domain OCT imaging techniques may further increase our insight in the composition and pathophysiology of BVMD lesions.

The OCT and FAF findings in our study are in accordance with a previous study combining fundus camera-based FAF and OCT in nine BVMD patients, without information on the underlying molecular genetic defect.³⁸ Our OCT observations also conform to a recent study in BVMD patients using high-definition OCT.³⁹

Genotype-phenotype correlation

Many specific *BEST1* mutations are associated with a striking range of BVMD disease expression. The p.Thr6Pro mutation, for instance, may cause a spectrum of phenotypes ranging from an abnormal EOG without ophthalmoscopic abnormalities to multifocal vitelliform dystrophy.¹⁴ Certain mutations in the *BEST1* gene may lead to a more severely affected RPE, sometimes resulting in the formation of multifocal vitelliform lesions that extend beyond the macula.^{27,40,41} Presently unknown additional genetic and environmental modifying factors obviously exert their influence on the phenotypic outcome. The high phenotypic variability is not unique to *BEST1*-related disease and may also be seen in other autosomal dominantly inherited retinal diseases, such as those caused by *peripherin/RDS* mutations.⁴²

There appears to be a certain degree of genotype-phenotype correlation in association with the p.Ala243Val mutation in *BEST1*. Patient 14, who carried this p.Ala243Val mutation, displayed an atypical phenotype of BVMD with a radial, spoke-like configuration of deposits on ophthalmoscopy and FAF, together with a near-normal EOG. This phenotype may be easily confused with pattern dystrophy of the retina. A small pseudohypopyon lesion in the centre of the lesion and the finding of a p.Ala243Val mutation in *BEST1* supported the diagnosis of BVMD. The p.Ala243Val mutation has been reported previously

in association with both BVMD and AFVD, but generally seems to cause a mild phenotype with a normal or near-normal EOG, just like in our study.^{4,13} A study by Yu and colleagues indicates that this mutation alters the Cl⁻ channel function of human bestrophin-1 and that the absence of a dominant negative effect of the mutation might be the cause of the milder symptoms, such as the normal or near-normal EOG.³⁶ BVMD and AFVD may share clinical features, and in a number of AFVD cases, mutations in the *BEST1* gene have been identified.^{4,43,44} Compared to BVMD, AFVD shows smaller lesions, with a later age at onset and often a normal EOG. However, lesions in BVMD may also be small and the mean age at onset in BVMD is higher than initially described.¹³ An Arden ratio of the EOG below 2.0 has been rather arbitrarily chosen as a cut-off point to consider the EOG abnormal. Therefore, we propose that *BEST1*-associated AFVD may be regarded as a mildly expressed form of BVMD, in which the RPE has preserved sufficient overall function to result in an EOG within the normal range.

Histopathological correlations

The histopathological correlate of one of the most frequent *BEST1* mutations in our study, p.Tyr227Asn (patients 12 and 13), has been described. This study described the findings in the eye of a patient with a late-onset atypical, multifocal form of BVMD, who carried a p.Tyr227Asn mutation in *BEST1*.²⁷ Somewhat surprisingly, the amount of RPE lipofuscin was not significantly different from that in age-matched controls. Patients 12 and 13 (both also carrying a p.Tyr227Asn mutation) in our study, however, clearly showed abnormally increased FAF, consistent with an abnormally high level of lipofuscin-related fluorophores in the RPE and/or under the neuroretina. This difference with the published histopathological findings may be explained by differences in age and age at onset, due to possible modifying factors.

In addition, histopathological studies have been carried out on two BVMD patients who carried a p.Thr6Arg mutation in *BEST1*, an amino acid substitution on the same site as in patients 1-9 in our study, who carried a p.Thr6Pro missense mutation. In a patient with advanced BVMD,³³ as well as in his previously described son,²¹ massive lipofuscin accumulation was seen in the RPE, together with a more than 4-fold increase of A2E, the major fluorophore of lipofuscin, compared to normal controls.^{32,33} These markedly elevated amounts of RPE lipofuscin and A2E are in agreement with the finding of increased FAF in most BVMD stages in our study. In the case of the p.Thr6Arg mutation, the amount of bestrophin-1 was higher in the extramacular RPE than in the macular RPE.³³ The authors hypothesized that BVMD, at least when caused by a p.Thr6Arg mutation, is a result of insufficient amounts of wild-type bestrophin-1 in the macula. As a consequence, the macular RPE is unable to support proper ion homeostasis, resulting in the accumulation of subretinal fluid, a detachment of the photoreceptors from the RPE, and possibly intracellular RPE changes that increase lipofuscin and A2E accumulation. In a homozygous p.Trp93Cys *BEST1* mutation carrier, who was known with BVMD just like several affected family members who carried the mutation heterozygously, increased A2E levels were also found in the RPE, together with a large serous retinal detachment

in the macula.³² Again, these findings parallel our clinical observations of increased FAF and hyperreflective material under the neuroretina on OCT in the earlier stages of BVMD, corresponding to a dynamic process of accumulation of vitelliform material within a subretinal space. In all of the histopathological cases mentioned above, gliotic scar tissue was found in the macula, which could correspond to our findings in the patients with lesions in the cicatricial stage (Fig. 3E-H). A central scar was observed in these lesions, that showed decreased FAF and a hyperreflective prominence on OCT.

Two earlier histopathologic studies on ungenotyped BVMD cases, performed before the era of molecular genetic confirmation, have also found marked accumulation of lipofuscin and melanolipofuscin in the RPE, besides evidence of choroidal neovascularization.^{19,20} One of these studies analysed the contents of the subretinal space between the photoreceptors and the RPE. An accumulation of photoreceptor outer segments, lipofuscin-like material, and melanin was found. This again corresponds to our FAF and OCT findings and lends further credence to the proposed pathophysiological mechanism, involving the accumulation of shed photoreceptor outer segments between the detached neuroretina and the RPE. In addition, phagocytic cells were seen in the subretinal space, possibly derived from RPE cells, which were packed with lipofuscin and melanin.²⁰ Such phagocytic cells containing melanin were also found in the subretinal space in a genetically confirmed case of BVMD (p.Thr6Arg mutation).³³

Conclusion

Many BVMD patients simultaneously display characteristics of the different BVMD stages described by Gass. A broad phenotypic variability may be observed in association with a single BVMD mutation. The p.Ala243Val mutation, however, shows a considerable degree of genotype-phenotype correlation. FAF and OCT appear to be excellent imaging methods for phenotyping and follow-up of BVMD patients, and provide valuable insight into the pathogenesis of BVMD. Both FAF and OCT are non-invasive and relatively patient-friendly. In our opinion, adequate phenotyping and follow-up of BVMD patients should therefore include OCT and FAF imaging, in addition to ophthalmoscopy and EOG. These techniques may be able to replace FFA as the imaging method of choice for further evaluation and follow-up of BVMD lesions, in addition to ophthalmoscopy. However, FFA remains indicated in BVMD cases where CNV is suspected. In every patient suspected of BVMD, a molecular genetic analysis of the *BEST1* gene should be performed.

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3.3. Clinical and genetic heterogeneity in multifocal vitelliform dystrophy

Abstract

Objective: To describe the clinical and genetic findings in 15 patients with multifocal vitelliform lesions.

Methods: All patients and, if possible, affected family members underwent an extensive ophthalmic examination. In addition, their genomic DNA was analyzed for mutations in the *BEST1* gene. Patients who did not have a mutation in *BEST1* were screened for mutations in the *peripherin/RDS* gene.

Results: The age at onset of the disease was highly variable, ranging from 5 to 59 years. The peripheral lesions varied in number, size and overall appearance, but showed similar characteristics on fundus autofluorescence imaging and optical coherence tomography, when compared to the central vitelliform lesion. Mutations in the *BEST1* gene were identified in nine of the 15 patients. One patient without a *BEST1* mutation carried a sequence variant in the 5' untranslated region of the *peripherin/RDS* gene.

Conclusions: Multifocal vitelliform dystrophy is a clinically and genetically heterogeneous retinal disease, which can be caused by mutations in the *BEST1* gene. Other genes associated with this phenotype remain to be identified.

Introduction

Multifocal vitelliform lesions have been described in several reports.¹⁻¹⁶ Lesions typically present as sharply demarcated yellowish cysts in the macula, near the retinal vascular arcades and around the optic disk. A variation in size and number (up to 20) of these lesions can be observed. The lesions may grow and merge with neighboring lesions. The evolution of the peripheral lesions may parallel the evolution of the central lesion, but their evolution may also show a different time course.^{1,7,9,10,12} The previously reported cases of multifocal vitelliform lesions were either familial or sporadic. The majority of lesions showed at least partial staining on fluorescein angiography.^{3,8,10} Although the electro-oculogram (EOG) was often reduced, some patients displayed normal EOG responses.^{5,8} Electroretinographic (ERG) results were normal in most patients with multifocal vitelliform lesions.⁸ Some authors consider this phenotype an atypical variant of Best vitelliform macular dystrophy (BVMD), especially in combination with an autosomal dominant mode of inheritance and a disturbed EOG. The BVMD phenotype is associated with mutations in the *BEST1* gene, which can be found in virtually all patients with a positive family history, as well as in 28% to 69% of the sporadic cases.¹⁷⁻¹⁹ Typically, the EOG is moderately to severely disturbed, although normal EOGs have been reported in patients with BVMD who carry a mutation in *BEST1*.^{5,17,20,21}

In adult-onset foveomacular vitelliform dystrophy (AFVD), vitelliform lesions are generally unifocal and located in the macula, but on rare occasions a multifocal distribution may be encountered.²² Contrary to typical BVMD, the lesions in AFVD are usually one-third to one disk diameter in size, with a central pigmented spot, although lesions may also mimic those seen in BVMD.²³ If symptomatic, the age at onset of visual disturbance in AFVD is usually between 30 and 50 years. The EOG results in AFVD are normal or only slightly reduced. AFVD shows genetic heterogeneity: mutations in the *peripherin/RDS* gene were found in up to 18% of AFVD patients, whereas in up to 25% of the cases mutations in the *BEST1* gene were identified.^{17,24,25}

To date, two studies have been published describing a total of two patients with multifocal vitelliform lesions carrying mutations in the *BEST1* gene.^{11,14} The purpose of this study is to clinically characterize 15 patients with multifocal vitelliform lesions and to investigate the possible role of the *BEST1* gene and the *peripherin/RDS* gene in the pathogenesis of this peculiar phenotype.

Methods

Clinical studies

This study conformed to the tenets of the Declaration of Helsinki and was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Centre (Nijmegen). Informed consent was obtained from all participants. Fifteen probands who were diagnosed with multifocal vitelliform dystrophy, based on the ophthalmoscopic appearance, were examined. When possible, the affected family members and their parents were examined. Their medical histories were obtained, and subsequent clinical examination included best-corrected Snellen visual acuity, Amsler grid testing, fundus examination by indirect ophthalmoscopy, and fundus photography. In addition, fundus autofluorescence (FAF) imaging (Heidelberg Retina Angiograph, HRA 2, Heidelberg Engineering, Dossenheim, Germany) and optical coherence tomography (Stratus OCT, OCT 3, Carl Zeiss Meditech, Inc., Dublin, CA, USA) were performed. Fluorescein angiography was performed previously in 11 of 15 patients. All patients underwent an EOG. Eight of these patients were tested according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards. In seven patients, the EOGs were recorded prior to ISCEV regulations, according to an older protocol described by Thijssen and colleagues.²⁶ Six patients gave permission to perform full-field electroretinography (ERG), which was carried out following the ISCEV guidelines.

Molecular genetic studies

Blood samples were obtained from all probands and, when possible, their parents and other affected family members. The genomic DNA was isolated according to previously described protocols.²⁷ Sequence analysis of the 10 coding exons and splice junctions of the *BEST1* gene was performed in all patients.²⁸ In the patients who did not have mutations in

BEST1, the three exons and splice junctions of the *peripherin/RDS* gene were amplified by the polymerase chain reaction (PCR) and analyzed by direct sequencing.²⁹

Results

Fifteen patients with multifocal vitelliform dystrophy were included in this study. Six patients were male and nine were female, ranging from 5 to 59 years of age (mean, 40.6 ± 18.1 ; median, 49.0) at the time of their first visit. Nine patients reported additional family members with comparable complaints of visual disturbances. All patients experienced a reduction of visual acuity as the initial symptom.

Analysis of the *BEST1* and *peripherin/RDS* genes in patients with multifocal vitelliform dystrophy

Sequence analysis of the *BEST1* gene revealed mutations in nine of the 15 patients (60%), whereas no mutations in *BEST1* were detected in the remaining six patients. *BEST1* mutations in these nine patients included five different missense mutations, one in-frame deletion and one in-frame insertion. Four mutations (p.Asn296Lys, p.Lys194-Ala195insVal, p.Asp302_Asp304del, p.Ser16Tyr) are novel, whereas three mutations (p.Ala195Val, p.Thr6Pro, p.Phe298Ser) have been described previously in BVMD and AFVD.^{17-19,30,31} The four novel mutations were not identified in 185 control individuals. The missense mutation p.Ala195Val was found in two unrelated probands (patients 1 and 8). Two other patients with multifocal vitelliform lesions (patients 3 and 9), who were not related, both carried the missense mutation p.Thr6Pro. In patient 1, two amino acid changes were detected: p.Ala195Val and p.Leu134Val. Segregation analysis in the patient's family members indicated that both changes reside on the same allele. The p.Ala195Val mutation has been described previously in patients with BVMD,^{18,31} whereas the pathogenic nature of p.Leu134Val is unknown. This p.Leu134Val variant was not identified in 185 control individuals. Sequence analysis of the *peripherin/RDS* gene in the remaining six patients identified one sequence variant (c.-11A>C) in the 5' untranslated region in patient 15. This change is not a known polymorphism, is not present in the SNP databases, and was not found in 92 control individuals. The variant is not predicted to affect regulatory elements or transcription factor binding sites. However, pathogenicity of this variant cannot be excluded.

Clinical features of patients with multifocal vitelliform dystrophy and *BEST1* mutations

Three male and six female patients carried a mutation in the *BEST1* gene. The age at onset was highly variable (mean 29.8 ± 17.7 , median 33), as was the visual acuity (Table 3.3). The visual acuity ranged from 20/20 to 20/800. The aspect of the central lesions varied from typical vitelliform round-to-oval lesions (Fig. 3.9B), to large and diffuse lesions with multiple yellowish deposits of vitelliform material (Fig. 3.10A). Peripheral lesions did

not only vary in number, but also in size and aspect, from small and well-circumscribed yellow-white spots to large and diffuse lesions with irregular yellow-white deposits, comparable to the central lesion. These eccentric lesions were located superior and nasal to the optic disc, and adjacent to the temporal vascular arcades. Like the central lesions, the lesions outside the macula varied in aspect, size and number during a follow-up period of several years (Fig. 3.9A and B).

On FAF imaging, areas of highly increased FAF within lesions corresponded with the yellow-white material as seen on ophthalmoscopy (Fig. 3.9C and 3.10B). Fluorescein angiographic characteristics were variable, but all lesions had a predominantly hyperfluorescent aspect in the early and late phases. OCT imaging of the central lesion showed hyporeflective material under the macula, which elevated the central retina to a variable degree. In a number of cases, areas of increased reflectivity could be seen within this hyporeflective space, corresponding with the yellowish material seen on ophthalmoscopy. Areas within the lesion with a cicatricial aspect on ophthalmoscopy were prominent and hyperreflective on OCT, and were often in contact with the overlying retina. The OCT characteristics of the larger extramacular lesions were similar to those of the central lesions (Fig. 3.9D and E). The size of the extramacular lesions ranged from smaller than one optic disc area (25 of 38 lesions) to larger than 5 optic disc areas (7 of 38 lesions). The larger extramacular lesions were found superior to the optic disc. Demarcation of individual lesions was better possible with FAF imaging (Fig. 3.10) and OCT.

The EOG was abnormal in all cases. ERG testing was done in three patients and revealed normal cone and rod responses. Eight patients reported family members with similar visual complaints. Mutation screening in nine of these family members revealed the same mutation in the *BEST1* gene as the proband. Ophthalmic examination was performed in all of these nine family members, and revealed multifocal lesions in five cases and a unifocal lesion in four cases. Three of the four family members who had unifocal BVMD were 15 years of age or younger. The 44-year-old father of patient 4, who carried a p.Ala195_Trp196insVal mutation in the *BEST1* gene like his daughter, displayed no abnormalities on elaborate eye examination, and had a normal EOG (Arden ratio OD 2.0, OS 2.2).

Clinical features of patients with multifocal vitelliform lesions without *BEST1* mutations

This group, consisting of two males and four females, also displayed a variable age at onset (mean 37.8 ± 14.0 , median 38.5) and visual acuity (Table 3.4). A decreased visual acuity was the presenting symptom in all patients. One patient had a positive family history for similar visual disturbances. Funduscopic, FAF and fluorescein angiographic characteristics in this group were comparable to those described in the group of patients who carried a mutation in the *BEST1* gene (Fig. 3.11A and B and 3.12A and B). The extramacular lesions also had the same aspect on OCT as the central lesions, which resembled the OCT aspect of the lesions seen in the group of patients with multifocal

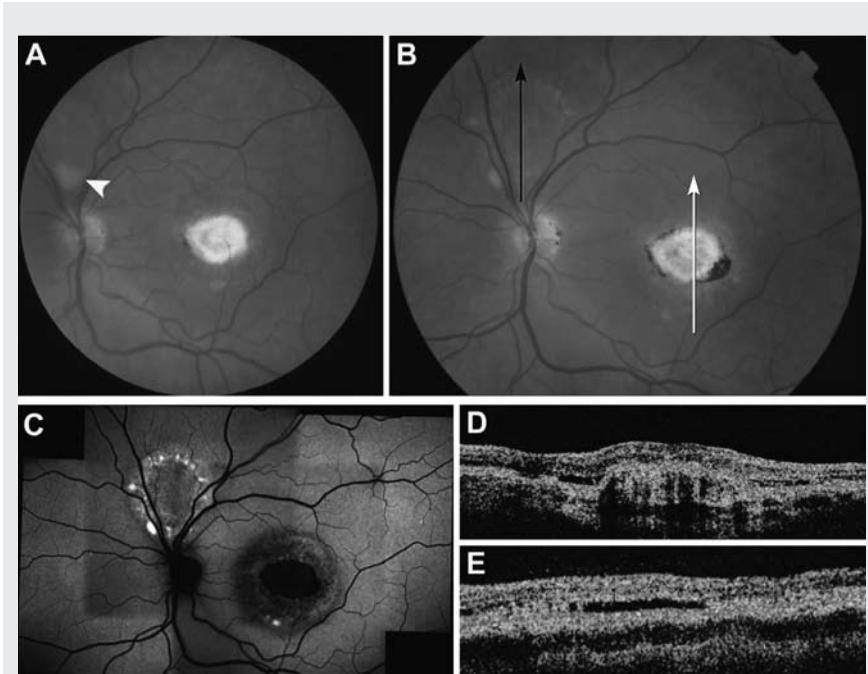
Table 3.3.
Clinical and molecular genetic findings in patients with BEST1 mutations.

Patient	Gender	Family history	Age at onset (years)	VA OD	VA OS	Number of lesions OD / OS	EOG ^{#§} OD / OS	ERG	BEST1 mutation	Effect
1	f	+	21	49	20/70	20/400	3 / 2	1.0 / 1.0	-	c.[584C>T; 400C>G] p[Ala195Val; Leu134Val]
2	f	-	50	72	20/40	20/50	3 / 3	1.0 / 1.0*	nl	c.891C>A p.Asn296Lys
3	m	+	33	63	20/800	20/200	2 / 5	1.6 / 1.4*	-	c.16A>C p.Thr6Pro
4	f	+	5	15	20/20	20/100	2 / 2	1.0 / 1.0	nl	c.583_584insTGG p.Lys194_Ala195insVal
5	f	+	14	44	20/100	20/100	2 / 2	1.2 / 1.1*	-	c.904_912delGATGATGAT p.Asp302_Asp304del
6	f	+	10	55	20/125	20/100	6 / 2	1.0 / 1.0	nl	c.47C>A p.Ser16Iyr
7	f	+	39	67	20/70	20/50	2 / 2	1.1 / 1.2*	-	c.893T>C p.Phe298Ser
8	m	+	46	61	20/100	20/40	2 / 3	1.3 / 1.3*	-	c.584C>T p.Ala195Val
9	m	+	50	55	20/250	20/20	7 / 6	1.0 / 1.0	-	c.16A>C p.Thr6Pro

[#]EOG results marked with an asterisk were recorded prior to the ISCEV standards and have been performed according to a previously described protocol.²⁶

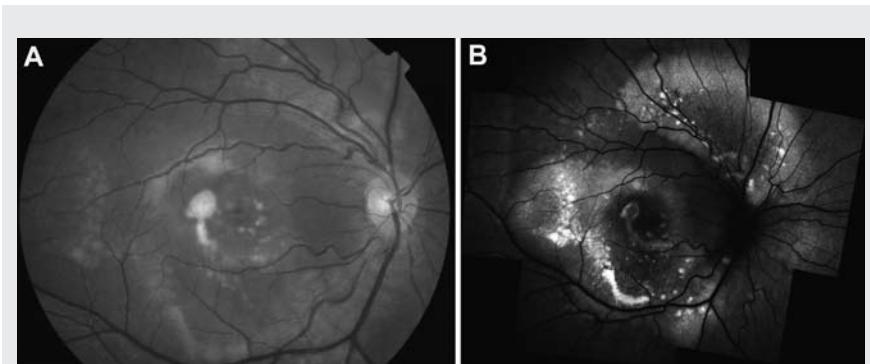
[§]Values are Arden ratios: ISCEV: normal if ≥ 2.0, non-ISCEV: normal if ≥ 1.8.

Abbreviations: VA, visual acuity; EOG, electro-oculogram; ERG, electroretinogram; nl, normal.

**Figure 3.9.***

A. Fundus image of the left eye of patient 5 (*p.Asp302_Asp304del* mutation) at the age of 18 years. Besides a central cicatricial lesion, a lesion superior to the optic disc with characteristics of a pseudohypopyon is evident (arrowhead). At the age of 44, the lesion superior to the optic disc shows a “scrambled-egg” aspect (**B**). On the fundus autofluorescence (FAF) image (**C**), the central lesion shows markedly decreased FAF of the area that corresponds with the scar on ophthalmoscopy, surrounded by a zone of mottled alterations of FAF intensity. The suprapapillary lesion shows spots of increased FAF at the edges of the lesion, which correspond with the remnants of vitelliform material on ophthalmoscopy. **D.** A vertical optical coherence tomography (OCT) scan through the central lesion (white arrow in Fig. 3.9B) reveals a prominent hyperreflective structure, corresponding with the central scar seen on ophthalmoscopy. This structure is surrounded by a hyporeflective subretinal space, suggesting subretinal fluid. **E.** A vertical OCT section through the suprapapillary lesion (black arrow in Fig. 3.9B) shows a dome-shaped structure with a optically clear centre and a hyperreflective band above it, which possibly corresponds to the elevated photoreceptor layer.

vitelliform dystrophy carrying *BEST1* mutations (Fig. 3.11C and D). The number of lesions was higher in the group of patients without *BEST1* mutations. All lesions in this group were smaller than one optic disc area, except for the lesions in patient 15. The EOG findings were abnormal in two of the six patients. The ERG, recorded in three patients,

**Figure 3.10.***

Fundus photograph and autofluorescence image of the right eye of patient 4, carrying a p.Lys194_Alai95insVal mutation in the *BEST1* gene (A and B, respectively). **A.** The fundus image shows extensive lesions with scattered yellow-white deposits. **B.** These deposits are intensely autofluorescent. The lesions show a generally increased autofluorescence signal, which enables a better appreciation of the size and extent of the lesions.

was normal. The father of patient 10 had multifocal pigmentary changes in both eyes in combination with a normal EOG, when he was examined at the age of 65 years. The 64-year-old brother of patient 10 had nonspecific multifocal pigmentary changes in both eyes. His EOG findings were within the normal range. Patient 15 experienced a further loss of visual acuity at the age of 65, when she developed choroidal neovascularization in the fovea of both eyes.

Discussion

Multifocal vitelliform dystrophy shows characteristic lesions that are also seen in BVMD and AFVD. In multifocal vitelliform dystrophy, multiple yellow-white lesions are encountered, that are often located in the posterior pole and adjacent to the vascular arcades. This phenotype should be differentiated from several other disorders, including acute exudative polymorphous vitelliform maculopathy syndrome, central serous chorioretinopathy, white dot syndromes such as birdshot chorioretinopathy, multifocal serous detachments of the retinal pigment epithelium (RPE), and ocular presentations of large cell non-Hodgkin lymphoma.^{32,33}

We identified seven different *BEST1* mutations in nine out of 15 unrelated patients with multifocal vitelliform dystrophy.^{11,14} A comparison of the clinical data between the group of patients with and without confirmed mutations in the *BEST1* gene revealed differences in the age at onset, visual acuity and electrophysiological findings. The age at onset was highly variable, but appears to be lower in the group of patients with a *BEST1*

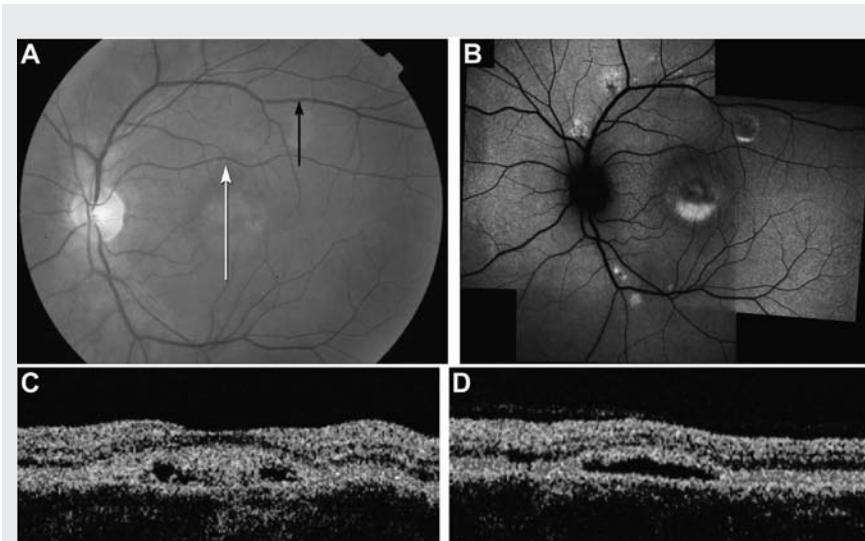
Table 3.4.
Clinical findings in patients without *BEST1* mutations

Patient	Gender	Family history	Age at onset (years)	Age (years)	VA OD	VA OS	Number of lesions OD / OS	EOG ^{#§} OD / OS	ERG
10	f	+	20	50	20/40	20/25	6 / 9	2.9 / 3.1	-
11	m	-	47	54	20/25	20/25	13 / 9	2.6 / 2.4	nl
12	f	-	37	39	20/40	20/40	12 / 8	2.6 / 2.1	nl
13	f	-	25	33	20/20	20/20	9 / 11	2.3 / 2.0	nl
14	m	-	40	76	20/20	20/100	6 / 10	1.3 / 1.1*	-
15	f	-	58	65	20/20	20/40	3 / 4	1.4 / 1.5*	-

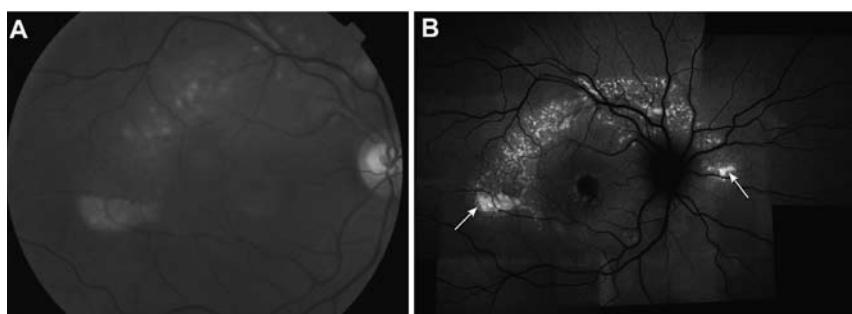
#EOG EOG results marked with an asterisk were recorded in our research laboratory prior to ISCEV regulations, according to a previously described protocol.²⁶

§Values are Arden ratios; ISCEV: normal if ≥ 2.0 , non-ISCEV: normal if ≥ 1.8 .

Abbreviations: VA, visual acuity; EOG, electro-oculogram; ERG, electroretinogram; nl, normal.

**Figure 3.11.***

A. Fundus image of the left eye of patient 12, who did not carry a mutation in BEST1. Multiple vitelliform lesions are visible, which can be more clearly delineated with autofluorescence imaging (**B**). Both the central and superior temporal lesion show a pseudo-hypopyon-like distribution of the material of increased autofluorescence. Vertical optical coherence tomography images of the central lesion (**C**, white arrow in Fig. 3.11A) and the lesion temporal and superior to the central lesion (**D**, black arrow in Fig. 3.11A) are highly similar, although the central lesion shows more subfoveal material of increased reflectivity.

**Figure 3.12.***

A. Fundus image of the right eye of patient 15 (no BEST1 mutation), showing a small central vitelliform lesion and a large arcuate lesion, below the superior temporal vascular arcade. **B.** A composition of fundus autofluorescence (FAF) images shows the large eccentric lesion with scattered areas of increased FAF. At the inferior edges of this lesion, pseudohypopyon-like deposits of material of increased FAF can be observed (arrows).

mutation, with a mean age at onset of 29.8 versus 37.8 in the group of patients without *BEST1* mutations. The loss of visual acuity was more pronounced in the patients who carried mutations in *BEST1*. All patients with *BEST1* mutations showed an abnormal EOG, compared to two of the six patients without *BEST1* mutations. The widespread dysfunction of the RPE in patients with *BEST1* mutations, as indicated by the abnormal EOG, is not unexpected since the bestrophin-1 protein encoded by the *BEST1* gene is located in the basolateral membrane of the RPE, where it is assumed to function as a Ca²⁺-sensitive Cl⁻ channel and a regulator of regulation of voltage-dependent Ca²⁺ channels.³⁵

The aspect of the extramacular lesions varied in both groups, ranging from small vitelliform spots to large and diffuse lesions. These extramacular lesions seemed to have the same characteristics as the central lesion on ophthalmoscopy, FAF, and OCT, although the stages between the lesions could differ.³⁶ The aspect on fluorescein angiography varied, but lesions were usually hyperfluorescent in both the early and late stages, probably depending on the amount of vitelliform material and the degree of atrophy in the lesions.

BVMD generally causes central lesions in the posterior pole. However, on indocyanine green angiography, small peripheral hyperfluorescent spots may be observed.³⁷ Histopathological reports have also shown abnormalities of the RPE, Bruch's membrane and the choroid throughout the fundus.^{11,38-40} It is possible that certain mutations in the *BEST1* gene may lead to a more severely affected RPE, resulting in the formation of multifocal, extramacular vitelliform lesions. This is supported by the finding of five affected family members in our study who also had multifocal lesions. Four affected family members had a unifocal central lesion. Three of these four patients were younger than fifteen years of age. Possibly, extramacular lesions are more likely to develop with older age. Contradictory to this hypothesis is the finding that the father of patient 4 had the same mutation as his daughter, despite having a normal fundus appearance and even a normal EOG, which is a rare exception on the rule that heterozygous carriers of a *BEST1* mutation have an abnormal EOG.^{17,20,41} Additional genetic and environmental factors may therefore also influence the development of multifocal vitelliform lesions in patients with mutations in the *BEST1* gene.

Analysis of the *BEST1* and *peripherin/RDS* gene did not reveal a mutation in six patients. A disease-related alteration in these genes cannot be ruled out completely, since heterozygous deletions of one or more exons are missed by PCR-based mutation analysis, and the introns and promotor have not been analysed for mutations. An alternative explanation could be the involvement of a thus far unknown gene in the pathogenesis of the multifocal vitelliform phenotype in these patients. The EOG was normal in four of these six patients without a mutation in the *BEST1* gene, indicating that there was no generalized disturbance of the electrical activity of the RPE in this subgroup. In patient 15, an alteration in the 5' untranslated region of the *peripherin/RDS* gene was found, of which the pathogenic nature is unclear.

The clinical and genetic heterogeneity of retinal disorders with multifocal vitelliform lesions suggests that the clinical diagnosis is inaccurate and overlap between the

phenotypes may occur. In our opinion, a patient that presents with the combination of multifocal vitelliform lesions, an autosomal dominant inheritance pattern, an abnormal EOG and a mutation in the BEST1 gene, should receive the diagnosis of multifocal Best vitelliform macular dystrophy.

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Chapter 4

Clinical and molecular genetic analysis of phenotypes associated with mutations in the *peripherin/RDS* gene

Section 4.1 is adapted from:

The spectrum of retinal dystrophies caused by mutations in the *peripherin/RDS* gene. **C.J.F. Boon**, A.I. den Hollander, C.B. Hoyng, F.P.M. Cremers, B.J. Klevering, J.E.E. Keunen.
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Central areolar choroidal dystrophy. **C.J.F. Boon**, B.J. Klevering, F.P.M. Cremers, M.N. Zonneveld-Vrieling, T. Theelen, A.I. den Hollander, C.B. Hoyng.
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Mutations in the *peripherin/RDS* gene are an important cause of multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. **C.J.F. Boon**, M.J. van Schooneveld, A.I. den Hollander, J.J.C. van Lith-Verhoeven, M.N. Zonneveld-Vrieling, T. Theelen, F.P.M. Cremers, C.B. Hoyng, B.J. Klevering.

Br J Ophthalmol 2007;91:1504-1511.

4.1. The spectrum of retinal dystrophies caused by mutations in the *peripherin/RDS* gene

Abstract

Peripherin/rds is an integral membrane glycoprotein, mainly located in the rod and cone outer segments. The relevance of this protein to photoreceptor outer segment morphology was first demonstrated in *retinal degeneration slow* (*rds*) mice. Thus far, over 90 human *peripherin/RDS* gene mutations have been identified. These mutations have been associated with a variety of retinal dystrophies, in which there is a remarkable inter- and intrafamilial variation of the retinal phenotype. In this paper, we discuss the characteristics of the *peripherin/RDS* gene and its protein product. An overview is presented of the broad spectrum of clinical phenotypes caused by human *peripherin/RDS* gene mutations, ranging from various macular dystrophies to widespread forms of retinal dystrophy such as retinitis pigmentosa. Finally, we review the proposed genotype-phenotype correlation and the pathophysiological mechanisms underlying this group of retinal dystrophies.

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Abbreviations: adRP, autosomal dominant retinitis pigmentosa; AFVD, adult-onset foveomacular vitelliform dystrophy; AMD, age-related macular degeneration; CACD, central areolar choroidal dystrophy; DGGE, denaturating gradient gel electrophoresis; EOG, electro-oculogram; ERG, electroretinography; FAF, fundus autofluorescence; OSs, outer segments; rds, rds, retinal degeneration slow; RP, retinitis pigmentosa; RPA, retinitis punctata albescens; RPE, retinal pigment epithelium; SSCA, single strand conformational analysis; STGD1, autosomal recessive Stargardt disease.

1. Introduction

The retinal degeneration slow (*rds*) mouse is one of the oldest animal models of retinal degeneration. The abnormal photoreceptor outer segment formation in the *rds* mouse is caused by mutations in the *rds* gene and results in a slowly progressive photoreceptor degeneration.^{1,4}

The role of mutations in the human homologue of *rds*, the *peripherin/RDS* gene, in human retinal dystrophies was first recognized in autosomal dominant retinitis pigmentosa in two simultaneous reports.^{5,6} Subsequent reports identified *peripherin/RDS* mutations not only in autosomal dominant retinitis pigmentosa, but also in a variety of macular dystrophies, such as adult-onset foveomacular vitelliform dystrophy and butterfly-shaped pigment dystrophy of the fovea.^{7,8} Other retinal dystrophies that have been found in association with *peripherin/RDS* mutations are central areolar choroidal dystrophy,^{9,10} retinitis punctata albescens,¹¹ cone-rod dystrophy,¹² multifocal pattern dystrophy simulating Stargardt disease (STGD1)/fundus flavimaculatus,¹³ age-related macular degeneration (AMD)-like late-onset maculopathy,¹⁴ and other unspecified autosomal dominant macular dystrophies.^{15,16} The combination of a specific mutation in *peripherin/RDS* (p.Leu185Pro) and a mutation in the *ROM1* gene causes a digenic form of retinitis pigmentosa.^{17,18}

Several studies showed that a striking inter- and intrafamilial phenotypic variation may be observed in association with *peripherin/RDS* mutations.¹⁹⁻²¹ Mutations in this gene not only account for a broad spectrum of retinal disorders, but causative *peripherin/RDS* mutations are also relatively frequent in patients with retinal dystrophies. *Peripherin/RDS* mutations are present in up to 25% of the central retinal dystrophies, depending on the inclusion criteria, methods of mutation analysis, and ethnic background of the patient population.²²⁻²⁷ Furthermore, *peripherin/RDS* is one of the most frequently involved genes in patients with autosomal dominant retinitis pigmentosa.^{28,29}

This paper aims to review the characteristics of the *peripherin/RDS* gene, as well as the broad spectrum of retinal dystrophies caused by mutations in this gene. In addition, *in vitro* studies and transgenic animal models are discussed, which add to the insight in the pathogenesis of *peripherin/RDS*-related retinal dystrophies and improve the genotype-phenotype correlations. Finally, future therapeutic perspectives are discussed.

2. Molecular biology of the human *peripherin/RDS* gene

2.1. The *peripherin/RDS* gene

Two years prior to the identification of the human *peripherin/RDS* gene, the murine ortholog was found.⁴ Since abnormalities in this gene result in a slowly progressive degeneration of photoreceptors, the gene was named Retinal Degeneration Slow (*rds*).² The phenotype in *rds* mice is due to haploinsufficiency and is dose-dependent: retinal degeneration is faster and more pronounced in *rds*(-/-) mice compared to *rds*(+/-) mice.^{1,30}

There has been some variation in nomenclature of the *peripherin/RDS* gene, as other authors have used *RDS/peripherin*, *peripherin-2* and *rd2* as the name of this gene. The official gene symbol approved by the Human Gene Nomenclature Committee is *PRPH2* or *peripherin-2*. We will, however, maintain the name *peripherin/RDS*, as this name is widely used and accepted by the vision research community (the abbreviation *RDS* has been used for nearly 30 years). The *peripherin/RDS* gene localizes to chromosome 6p21.2, spans 26 kilobases of genomic DNA and contains 3 exons.

2.2. The *peripherin/rds* protein

Rod and cone photoreceptor outer segments (OSs) contain hundreds of orderly arranged flat membranous structures, appropriately named discs (in rod OSs) and lamellae (in cone OSs), in which phototransduction is initiated. The human *peripherin/RDS* cDNA encodes the peripherin/rds protein, which plays a pivotal role in the formation, maintenance and renewal of these photoreceptor OSs.^{4,31-34} Peripherin/rds encompasses an open reading frame of 346 amino acids, with a polypeptide weight of 39.3 kilodalton.⁴ Most of the knowledge about the function of peripherin/rds originates from *in vitro* studies with murine and bovine peripherin/rds, as well as mouse models.^{35,36}

Murine and bovine peripherin/rds polypeptides both are 91% identical to their human ortholog.^{4,37} Peripherin/rds is an integral membrane glycoprotein with four helical transmembrane domains,^{37,38} and is predominantly localized to the rim regions of rod and cone OS discs and lamellae.^{39,40} Its N- and C-termini are located in the extradiscal space (within the cytoplasm), whereas two extracellular loops (D1 and D2, also termed EC1 and EC2) are located in the intradiscal space (Fig. 4.1).^{37,38} A single N-glycosylation site is present at p.Asn229, which is not required for normal peripherin/rds biosynthesis or function.^{38,41}

Peripherin/rds functions as an oligomer, composed of dimer subunits that form strong noncovalently bound tetramers, mediated by the large D2 loop.^{42,43} Besides homotetramers, peripherin/rds also forms heterotetrameric complexes with the homologous protein rom1, through a specific region in the D2 loop.⁴⁴⁻⁴⁹ Rom1 is likely to function as a regulator of photoreceptor OS disc morphogenesis.⁵⁰⁻⁵² The D2 loop contains 7 cysteine residues (Fig. 4.1). Six of these cysteines are important for protein structure through the formation of intramolecular disulfide bonds, whereas one specific cysteine (p.Cys150) mediates polymerization of tetramers.^{48,53} Tetrameric assembly of peripherin/rds dimers and peripherin/rds - rom1 dimers is required for proper targeting to and

incorporation into newly formed rod OS disc membranes.⁵⁴ Tetramerization also enables interaction with intracellular and extracellular structures through disulfide-mediated polymerization.^{43,45,48,55-57} Furthermore, peripherin/rds may also be required for the proper interaction between cone OSs and the extracellular “cone matrix sheath”.^{31,58-60}

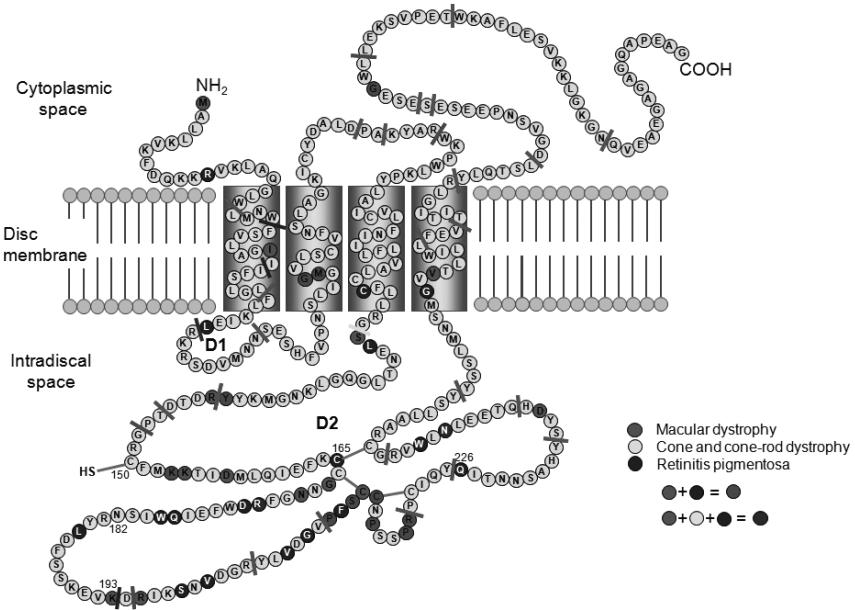
The C-terminus is a structurally flexible region that probably has multiple functional activities.^{32,61,62} A highly conserved region within the C-terminal domain of peripherin/rds plays a critical role in membrane fusion, which is important for disc morphogenesis and disc shedding.^{35,56,63-68} The C-terminal region also functions as a signal sequence for the targeting and localization of peripherin/rds to the rod OS.⁶⁹ Calcium-dependent association of calmodulin with the C-terminal domain modulates peripherin/rds function.⁷⁰ An intramembrane glutamic acid residue (p.Glu276) also acts as an important regulator of peripherin/rds functional activity.⁵¹ Finally, peripherin/rds may also influence or even participate in the visual cycle.⁷¹

The proposed functional properties of peripherin/rds, as described above, clearly indicate that peripherin/rds is a multifunctional protein, important in disc morphogenesis, maintenance of proper disc alignment and disc shedding.^{32,72} Interestingly, peripherin/rds appears to play a different role in rod and cone OS morphogenesis. In mice, absence of peripherin/rds leads to rods without OSs that eventually undergo apoptosis.^{30,73-75} The cones, on the other hand, develop atypical OSs that not completely preclude cone functionality.^{30,31,73-75}

2.3. Human *peripherin/RDS* gene mutations

2.3.1. Macular dystrophies

The involvement of the *peripherin/RDS* gene in macular dystrophies was first recognized in 1993, when different *peripherin/RDS* mutations were identified in a variety of macular phenotypes.^{78,20,76,77} Mutations in the *peripherin/RDS* gene are a frequent cause of autosomal dominant macular dystrophies. Prevalence numbers vary considerably, due to differences in mutation analysis, interpretation of the retinal phenotype as well as ethnic background. Kohl and colleagues analysed the *peripherin/RDS* gene in a group of patients from continental Europe, with a predominantly central retinal dystrophy and intrafamilial variability of disease expression.²⁶ They also included patients with phenotypes similar to those previously reported with *peripherin/RDS* mutations. In this patient group, *peripherin/RDS* mutations were identified in approximately 11% of patients. Mutation detection in this study consisted of combined single strand conformational analysis (SSCA) and heteroduplex analysis under two different gel conditions. In a Spanish population of patients with autosomal dominant macular dystrophies, a *peripherin/RDS* mutation frequency of 23% was found with denaturing gradient gel electrophoresis (DGGE) and direct genomic sequencing.²⁵ *Peripherin/RDS* mutations were identified in approximately 7% of British patients with various dominantly inherited macular dystrophies, using heteroduplex analysis and direct sequencing of polymerase chain reaction-amplified coding exons.⁷⁸ A p.Arg172Trp mutation (located in the

**Figure 4.1.***

Protein model of *peripherin/rds* (adapted from Connell and Molday,³⁷ Travis et al.,³⁸ and Goldberg et al.⁵³). The mutations associated with retinal dystrophies are indicated. Colored residue: missense mutation or in-frame deletion. Colored bar: nonsense or frameshift mutation.

important D2 loop of the protein was found in a notably high frequency in this study, accounting for 50% of identified mutations, and was shown to be the result of a founder effect. Mutations in the *peripherin/RDS* gene appear to be rare in Japanese patients with macular dystrophies.^{22,24} In a specific form of macular pattern dystrophy, adult-onset foveomacular vitelliform dystrophy, the mutation detection rate is 2-18% in European and Caucasian-American patients.^{23,79,80}

The majority of mutations in macular dystrophies such as central areolar choroidal dystrophy and pattern dystrophies are missense mutations and are located in the intradiscal D2 loop (Fig. 4.1), but mutations may also be found in other parts of the *peripherin/rds* protein.⁸¹⁻⁸⁴ An exception is multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus, which appears to be caused mainly by frameshift mutations.^{13,85} An overview of *peripherin/RDS* mutations and the associated phenotypes can be found on the Retina International website (http://www.retina-international.org/sci-news/rds_mut.htm) and in the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk/ac/index.php>).

2.3.2. Cone and cone-rod dystrophy

Six *peripherin/RDS* mutations have been reported in well-documented cases of cone and cone-rod dystrophy: p.Arg172Trp,⁸⁶⁻⁸⁸ p.Tyr184Ser,⁸⁹ p.Lys197Glu,²⁶ p.Val200Glu,⁹⁰ p.Asn244His,^{12,89} and likely p.Ser27Phe.⁹¹ The p.Tyr184Ser, p.Asn244His and p.Val200Glu mutations have all been reported in Japanese families. Except for p.Ser27Phe, which is associated with cone dystrophy, all of these mutations are located in the D2 loop of *peripherin/rds*. *Peripherin/RDS* mutations probably account for a small number of cone and cone-rod dystrophy cases, but firm data are lacking on this subject.^{24,28}

2.3.3. Retinitis pigmentosa

Retinitis pigmentosa (RP) shows extreme genetic heterogeneity. Over 45 genes have been identified, with autosomal dominantly inherited RP (adRP) accounting for 30-40% of the cases.⁹² *Peripherin/RDS* mutations were found in 3-9% of adRP patients of mostly European ancestry in extensive genetic surveys using SSCA,^{17,28,29} and in 8% of Swedish adRP cases using DGGE.⁹³ These results indicate that mutations in the *peripherin/RDS* gene are among the most prevalent genetic causes of adRP in the United States of America and Northern Europe. *Peripherin/RDS* mutations appear to be rare in Southern European adRP patients (0-1.4%),^{94,95} as well as in Japanese RP patients (0-1.9%).^{22,24,96}

To date, 34 different *peripherin/RDS* mutations have been implicated in adRP, including 24 missense mutations, five frameshift mutations leading to premature termination, four in-frame deletions and one stop mutation. The majority of these mutations is located in the D2 loop, although mutations have also been described in parts of the *peripherin/rds* that are predicted to be situated in transmembrane domains 2-4 and the cytoplasmic N- and C-terminal ends.

2.3.4. Digenic retinitis pigmentosa

A specific *peripherin/RDS* missense mutation, p.Leu185Pro, causes RP only in patients who also carry a mutation in the *ROM1* gene, which is located on chromosome 11.¹⁸ To date, two *ROM1* mutations have been described to cause digenic RP when co-inherited with the p.Leu185Pro mutation in *peripherin/RDS*: a null mutation and a p.Gly113Glu missense mutation.^{17,18} This digenic inheritance of double heterozygous *peripherin/RDS* and *ROM1* mutations is relatively rare, accounting for 0.5-3% of cases of presumed adRP in a population of predominantly European origin.^{17,29} The p.Leu185 residue is situated in the large D2 loop and is critical for tetramer formation.⁹⁷ Two reports have described a heterozygous *ROM1* variant without detectable mutations in the *peripherin/RDS* gene.^{98,99} However, none of these variants has been shown to be conclusively pathogenic.¹⁷

3. Ocular findings in patients with *peripherin/RDS* gene mutations

3.1. Introduction

The group of retinal dystrophies is genetically and phenotypically heterogeneous.^{100,101} Clinical classifications encounter difficulties as there is an ongoing debate about the parameters that should be used for categorization. Both cone and rod photoreceptors are compromised in the majority of retinal dystrophies, although the extent to which each of the photoreceptor systems is affected varies greatly. Detection of electrophysiological abnormalities in retinal dystrophies depends on the sensitivity of the detection technique.^{31,100-102} In addition, electrophysiological results may vary over the course of the disorder. In *peripherin/RDS*-related retinal disease, as with many types of retinal dystrophies, phenotypes are encountered that are not easily classified in the current clinical classification system.^{13,19,84} An evolution from one clinical phenotype to another, for instance from a macular dystrophy towards a cone-rod or rod-cone dystrophy, is not uncommon.^{13,16,88}

The alternative, a classification based solely on underlying molecular genetic abnormalities, is not practical in view of the high clinical heterogeneity associated with mutations in genes such as *peripherin/RDS* and *ABCA4*.^{13,20,103-105} The next best option therefore remains a classification based on the clinical findings, supplemented by knowledge of the underlying molecular genetic defect.^{100,101,106,107}

To enable categorization of the phenotypes associated with *peripherin/RDS* mutations, it is inevitable to formulate convenient defining features, as incomplete as they might be. In this review, we classified the broad phenotypic range according to the ophthalmoscopically observed retinal abnormalities, in combination with full-field electroretinographic (ERG) findings. This pragmatic approach allows us to classify most *peripherin/RDS*-related phenotypes.

We define macular dystrophy broadly, as a retinal phenotype that is largely confined to the central fundus at ophthalmoscopy, with a generally normal photopic and scotopic full-field ERG earlier in the course of the disease. One must bear in mind, however, that many “macular” dystrophies may probably be considered panretinal disorders at the molecular level. This is illustrated by the observation that some phenotypes, though initially confined to the macula, eventually will extend beyond the retinal vascular arcades, with cone and/or rod dysfunction on the full-field ERG. This has been shown not only in *peripherin/RDS*-related dystrophies, but also in macular dystrophies associated with genes such as *ABCA4*, *TIMP3* and *BEST1*.^{13,20,108,109} Patients with macular dystrophies often report central visual loss, and display fairly symmetrical macular abnormalities at ophthalmoscopy. The peripheral retina and peripheral visual fields are typically normal. In macular dystrophies with a normal cone and rod function on full-field ERG, the multifocal and pattern ERG often do show photoreceptor dysfunction.^{15,110}

The clinically and genetically heterogeneous group of progressive cone and cone-rod dystrophies is characterized by progressive visual loss, color vision abnormalities, visual

field loss, a variable degree of photophobia, and sometimes nystagmus.¹¹¹ In cone-rod dystrophy, cones and rods both show panretinal abnormalities. However, cone function is affected earlier and more severely than rod function on the full-field ERG, and symptoms associated with cone dysfunction are generally more pronounced.

Patients with RP, also termed rod-cone dystrophy, a clinically variable and genetically heterogeneous retinal disorder, typically experience night blindness and constriction of the peripheral visual field early in the course of the disease.^{92,105,112} The rod-driven full-field ERG is predominantly affected in RP, which is in contrast to cone-rod dystrophy. In later stages of both RP and cone-rod dystrophy, however, photopic and scotopic responses on full-field ERG may become nonrecordable and the clinical characteristics may coalesce.

3.2. The clinical spectrum of retinal dystrophies associated with mutations in the *peripherin/RDS* gene

3.2.1. Macular dystrophies

Mutations in the *peripherin/RDS* gene are associated with a broad spectrum of macular dystrophies: pattern dystrophies, such as butterfly-shaped pigment dystrophy and adult-onset foveomacular vitelliform dystrophy, as well as central areolar choroidal dystrophy, and AMD-like late-onset macular dystrophy.

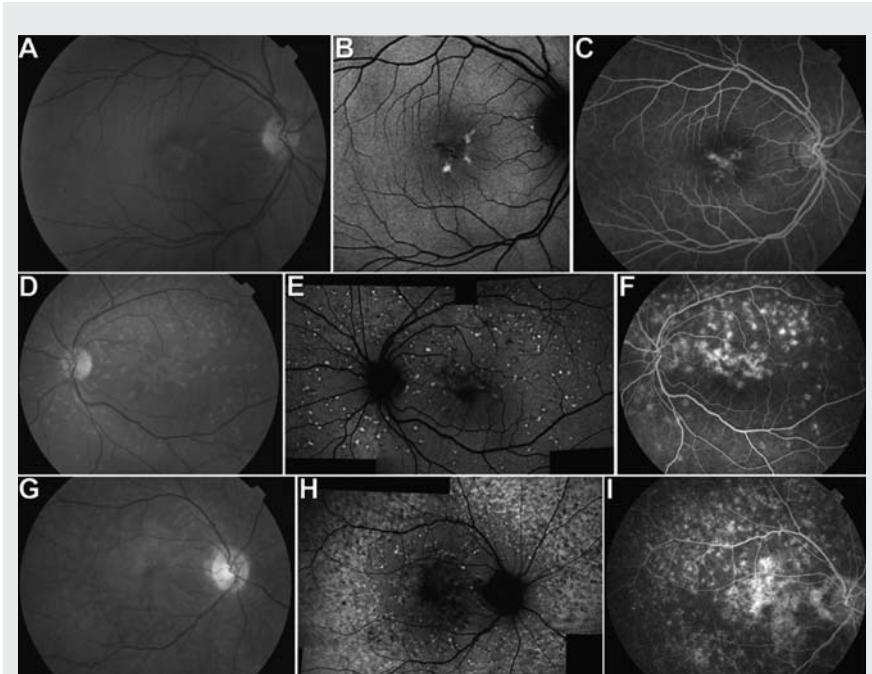
The pattern dystrophies constitute a group of retinal disorders characterized by a variety of deposits of yellow, orange or grey pigment, predominantly in the macular area.¹¹³⁻¹¹⁵ According to the classification of Gass, five main categories of pattern dystrophies are discriminated on the basis of the pattern of pigment distribution: adult-onset foveomacular vitelliform dystrophy, butterfly-shaped pigment dystrophy, reticular dystrophy of the pigment epithelium, multifocal pattern dystrophy simulating fundus flavimaculatus, and fundus pulverulentus.¹¹³ Mutations in the *peripherin/RDS* gene appear to be a relatively frequent cause of pattern dystrophies, and have been described in adult-onset foveomacular vitelliform dystrophy, butterfly-shaped pigment dystrophy, as well as multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus.^{7,13,23,26,82,101,103,116-118} In 2-18% of patients with adult-onset foveomacular vitelliform dystrophy, *peripherin/RDS* mutations are found.^{23,79,80} Thus far, the remaining two types of pattern dystrophy, reticular dystrophy of the pigment epithelium and fundus pulverulentus, have not been linked to mutations in the *peripherin/RDS* gene. The age at onset in pattern dystrophy is highly variable, but patients tend to remain asymptomatic until the 5th decade and some patients may remain asymptomatic. In general, mild disturbances of central vision are first noticed in midlife. The course of the disease is relatively benign, although it should be emphasized that severe vision loss occurs in up to 50% of the affected individuals after the age of 70, due to chorioretinal atrophy and/or the development of choroidal neovascularization.^{13,82,103,119,120} Fluorescein angiography and fundus autofluorescence (FAF) imaging may aid to discriminate between the different types of pattern dystrophy.^{13,121}

Adult-onset foveomacular vitelliform dystrophy (AFVD) is clinically and genetically heterogeneous.^{23,79,122} Besides the *peripherin/RDS* gene, AFVD is associated with mutations

in the *BEST1* gene, which accounts for up to 25% of cases.^{122,123} The majority of underlying genetic causes remain to be identified.⁸⁰ Ophthalmoscopy typically shows symmetrical, solitary, yellow-white, round to oval, slightly elevated subretinal lesions, often with a central pigmented spot.^{79,113} These lesions are usually one-third to one disc diameter in size, but may occasionally be larger and even multifocal, and may thus be confused with Best vitelliform macular dystrophy.^{23,113,124} Subretinal neovascularization seldomly occurs.^{79,120,125} Fluorescein angiography typically shows a central nonfluorescent spot, corresponding with the vitelliform lesion, surrounded by a small irregular hyperfluorescent ring. Most patients show increased FAF of the vitelliform lesion, which is in accordance with the histopathologic finding of increased lipofuscin quantities in AFVD lesions.^{126,127} Vitelliform lesions in AFVD can also be clearly delineated with optical coherence tomography.^{128,129} Contrary to Best vitelliform macular dystrophy, the electro-oculogram (EOG) in AFVD is usually normal.^{23,79} In general, the full-field ERG in AFVD caused by *peripherin/RDS* mutations is also normal.^{80,84,128}

In butterfly-shaped pigment dystrophy of the fovea, a spoke-like pigment pattern resembling the shape of a butterfly is observed in the central macula, surrounded by a zone of depigmentation (Fig. 4.2A).¹³⁰ Butterfly-shaped pigment dystrophy is genetically heterogeneous: apart from *peripherin/RDS* mutations,^{7,103} a locus on 5q21.2-q33.2 is also associated with autosomal dominant butterfly-shaped pigment dystrophy.^{131,132} On FAF, lesions in butterfly-shaped pigment dystrophy may show increased as well as decreased FAF, corresponding with changes in lipofuscin (Fig. 4.2B). On fluorescein angiography, pigmented regions within the lesion correspond with hypofluorescence, whereas depigmented zones corresponding with chorioretinal atrophy are hyperfluorescent (Fig. 4.2C).^{81,103,121} Rarely, butterfly-shaped pigment dystrophy may be complicated by subretinal neovascularization.¹³³ Full-field ERG and dark adaptometry are generally normal, but patients may exhibit abnormal color vision and EOG results.^{7,81,114,134}

Multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus is characterized by irregular yellow-white flecks scattered throughout the posterior pole, often extending beyond the retinal vascular arcades (Fig. 4.2D and G).^{13,113} Besides these remarkable changes, patients display macular abnormalities, ranging from various patterns of yellow or greyish deposits to well-demarcated lesions of marked chorioretinal atrophy.¹³ Visual loss is usually first experienced in the 5th decade, but patients may remain asymptomatic. The flecks seen in multifocal pattern dystrophy resemble those encountered in STGD1/fundus flavimaculatus, an autosomal recessive retinal dystrophy caused by mutations in the *ABCA4* gene. The resemblance can be observed on ophthalmoscopy, as well as on FAF (Fig. 4.2E and H) and optical coherence tomography.^{13,135} Flecks are usually hyperfluorescent in the early and late phase of the fluorescein angiogram, sometimes with a central hypofluorescent spot (Fig. 4.2F and I). Contrary to most STGD1 cases,¹³⁶ the fluorescein angiogram in multifocal pattern dystrophy generally does not show a so-called “dark choroid”,¹³ although an atypical patient (carrying a 4-base pair insertion at codon 140 of *peripherin/RDS*) has been described with a multifocal pattern dystrophy phenotype in combination with a dark choroid and choroidal neovascularization.¹³⁷

**Figure 4.2.**

Pattern dystrophies caused by mutations in the peripherin/RDS gene. (A-C) Butterfly-shaped pigment dystrophy of the fovea. A. Color fundus photograph of a 62-year-old patient carrying a p.Arg220fsX34 mutation in peripherin/RDS. A butterfly-shaped lesion is observed in the fovea, containing areas of increased and decreased pigmentation. On a fundus autofluorescence (FAF) image (B), some parts of the lesion display increased FAF, whereas FAF is decreased in other parts. C. Fluorescein angiogram of the same patient. Hyperfluorescent areas correspond with the depigmented areas at ophthalmoscopy, whereas hypofluorescent parts largely coincide with the pigmented parts of the lesion. The visual acuity in this patient was 20/20.

(D-I) Multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus.

D. Color fundus photograph of a 57-year-old patient carrying a p.Pro147fsX4 mutation in the peripherin/RDS gene. An irregularly shaped yellow-white lesion is observed in the central macula, surrounded by irregular yellow-white STGD1-like flecks scattered throughout the posterior pole. **E.** These lesions can be more clearly delineated on FAF. In general, lesions show markedly increased FAF, sometimes bordered by zones of decreased FAF.

F. Fluorescein angiogram of the same patient, showing many hyperfluorescent lesions.

Note the normal choroidal background fluorescence. This patient had a visual acuity of 20/20. **G.** Color fundus photograph of advanced multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. In this 56-year-old patient, who carried a p.Asn54fsX9 mutation in peripherin/RDS, numerous, confluent, mildly atrophic lesions are seen in the

posterior pole. **H.** The FAF image shows a large, ring-shaped zone of increased FAF, surrounding the macula and optic disc. Granular zones of decreased FAF may be observed within this area, corresponding with atrophy of the retinal pigment epithelium (RPE). The macula seems relatively unaffected on FAF, which is reflected in the visual acuity of 20/16. On the fluorescein angiogram (**I**), however, a markedly hyperfluorescent macular lesion is observed, consistent with RPE atrophy. This macular lesion is surrounded by many irregular, predominantly hyperfluorescent lesions.

Central macular lesions may remain fairly discrete, thus causing little or no visual loss, but they may also evolve towards well-defined chorioretinal atrophy. The STGD1-like flecks may show confluence with advancing age (Fig. 4.2G and H), and extensive atrophy of the posterior pole tends to occur in patients over 60 years of age.¹³ Contrary to the previously described pattern dystrophies, this phenotype frequently shows signs of panretinal degeneration, especially later in the course of the disease. In the majority of patients, a mild to marked constriction of the peripheral visual field is seen.^{13,16,20} Night blindness in combination with RP-like pigmentary changes and chorioretinal atrophy in the (mid-)peripheral retina may also be observed. Most patients show an abnormal EOG.¹³ Full-field ERG findings are often normal in early multifocal pattern dystrophy simulating STGD1. With advancing disease, cone and rod function become compromised on the panretinal level, which is reflected by the appearance of full-field ERG abnormalities. In general, the decrease of cone and rod responses on the full-field ERG is relatively equal, but results may show a slight preponderance of either cone or rod dysfunction, probably depending on the type of mutation.^{13,13,16,19,21,85,138,139} Like many so-called macular dystrophies, multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus should therefore be regarded as a disorder that involves the entire retina. However, the multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus phenotype may be preceded or even accompanied by butterfly-shaped lesions and adult-onset foveomacular vitelliform lesions.¹³ Clinical findings that may aid to distinguish between multifocal pattern dystrophy and STGD1 are the autosomal dominant pattern of inheritance, the relatively late age at onset, the comparatively good and stable visual acuity and the absence of a “dark choroid” in most patients.¹³

Different types of pattern dystrophy may be observed in a single family with individuals carrying an identical peripherin/RDS mutation. Occasionally, RP-like changes are seen in combination with these pattern dystrophies.^{13,19-21,140} An evolution from one type of pattern dystrophy to another may be observed within a single patient, and the type of pattern dystrophy may even be different between the two eyes of a patient.^{13,120,137} Age-dependent progression of a phenotype may be seen, evolving from localized patterns of macular abnormalities to more widespread retinal disease and atrophy of the retinal pigment epithelium (RPE)-photoreceptor complex.^{13,82,141}

Central areolar choroidal dystrophy (CACD) is a macular dystrophy that affects the

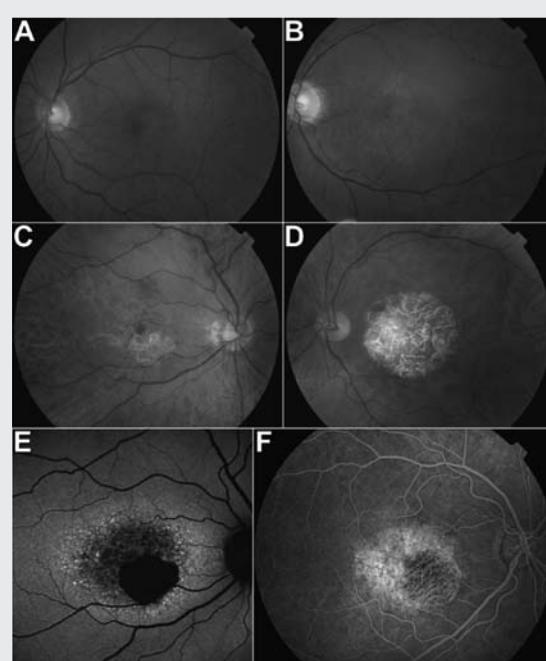
retina, RPE, and choriocapillaris.¹⁴²⁻¹⁴⁴ To date, 5 *peripherin/RDS* mutations have been reported in CACD or CACD-like phenotypes: p.Arg142Trp,^{9,145} p.Arg172Trp,^{8,15,26,78,86-88,146-148} p.Arg172Gln,^{8,15,88} p.Arg195Leu,^{110,149} and p.Leu307fsX83, the last mutation causing a somewhat atypical late-onset CACD.¹⁵⁰ Missense variants at codon 172 appear to be the most frequent *peripherin/RDS* mutations in macular dystrophy and have been reported to cause a relatively constant CACD phenotype.^{15,78,87} The p.Arg172Trp mutation, however, may be associated with incomplete penetrance, intrafamilial variability, cone-rod dystrophy and adRP.^{86,147}

A CACD patient usually presents with mild central visual loss and metamorphopsia, sometimes accompanied by mild photophobia. Nyctalopia is reported rarely in CACD, and seems to be associated with pigmentary changes of the peripheral retina.⁸⁶ The course of the disease is divided into four stages that can be observed on ophthalmoscopy.^{87,144} In stage I, slight parafoveal pigmentary changes of the RPE are seen, usually in the adolescent patient (Fig. 4.3A). Stage II is characterized by a round to oval, mildly atrophic hypopigmented area of 1.5 to several disc diameters (Fig. 4.3B). In stage III, one or more areas of well-circumscribed atrophy of the RPE and choriocapillaris appear outside the fovea (Fig. 4.3C). When stage III evolves to stage IV, visual acuity often decreases severely. In this stage, an area of well-circumscribed chorioretinal atrophy affects the fovea (Fig. 4.3D). End-stage CACD appears as a round to oval area of chorioretinal atrophy in the posterior pole, sometimes also surrounding the optic disc. The appearance of the macular lesions in *peripherin/RDS*-associated CACD is fairly constant, although with a variable age at onset and rate of progression.^{9,15,110,144} CACD has also been described in association with drusen-like changes and dominant drusen.^{10,87} Rarely, the macula in CACD may have a bull's eye aspect.⁸⁶

The mean age at onset of visual disturbances in CACD varies with the specific mutation, ranging from the 3rd to 4th decade in the phenotype caused by the p.Arg172Trp and p.Arg195Leu mutation, to the 5th to 6th decade in the phenotype associated with the p.Arg142Trp mutation. A severe decrease in visual acuity to 20/200 or less in one or both eyes generally occurs in the 5th to 8th decade, also depending on the type of mutation. Both fluorescein angiography and FAF imaging are excellent means to detect early macular changes that are difficult to discern with ophthalmoscopy alone.^{15,110,144}

FAF imaging, which visualizes changes in RPE lipofuscin, appears to be a convenient non-invasive method aiding the diagnosis and follow-up of *peripherin/RDS*-related retinal dystrophies such as CACD.^{13,15,110,151,152} In early CACD, a speckled increase of FAF predominates, whereas sharply demarcated areas of severely decreased to absent FAF appear in stage III and IV CACD (Fig. 4.3E).

The first fluorescein angiographic findings, in stage I CACD, are small parafoveal window defects.¹⁴⁴ As the lesion enlarges towards a round-to-oval area of RPE atrophy, the area of hyperfluorescence also becomes progressively larger (Fig. 4.3F). Full-field ERG testing in CACD is often normal, but may display abnormalities in a cone or cone-rod pattern. Full-field ERG abnormalities tend to occur in the later stages and in association with the p.Arg172Trp mutation.^{15,26,86,110,144,146} Tests of the macular function, such as the pattern ERG

**Figure 4.3.**

Central areolar choroidal dystrophy (CACD) caused by a p.Arg142Trp mutation in the peripherin/RDS gene. **(A-D)** Color fundus photographs of CACD. **A.** Stage I CACD in a 30-year-old patient, showing slight hypopigmentation in the temporal parafoveal area. The visual acuity was 20/20. In stage II CACD (**B**), an oval, mildly atrophic area is observed in the macula. Note the small yellow-white dots at the border of the lesion. This 55-year-old patient had a visual acuity of 20/32. **C.** Stage III CACD, showing a parafoveal area of

well-circumscribed chorioretinal atrophy. This patient was 51 years of age, and the visual acuity was 20/20. **D.** In stage IV CACD, the well-circumscribed area of profound chorioretinal atrophy affects the fovea, resulting in a visual acuity of 20/1000 in this 59-year-old patient. **E.** Fundus autofluorescence (FAF) image of stage III CACD, of the same patient as in panel (C). The severely atrophic part of the lesion corresponds with a well-demarcated absence of FAF, surrounded by speckled areas of decreased and increased FAF in the remaining part of the lesion. **F.** Fluorescein angiogram of stage III CACD. A well-demarcated round patch with increased visibility of the underlying choroidal vessels is seen in the nasal part of the lesion, adjacent to the fovea, consistent with profound chorioretinal atrophy. The remaining parts of the lesion show mottled hyperfluorescence, corresponding with mild chorioretinal atrophy on ophthalmoscopy.

and multifocal ERG, as well as results from color vision testing, may be abnormal early in the course of the peripherin/RDS-related retinal dystrophies, such as CACD and the pattern dystrophies.^{15,86,88,110,128,144,153} The EOG is usually normal or slightly subnormal.^{15,87,144}

A peculiar macular dystrophy has been described in a single large family with 17 affected family members.¹⁴ Bearing a striking resemblance to AMD, this macular dystrophy was termed AMD-like late-onset maculopathy. In this macular dystrophy, which is caused by a p.Tyr141Cys missense mutation in peripherin/RDS, vision loss usually manifests in the 5th to 7th decade and is relatively mild in the majority of patients. A wide spectrum of AMD-like exudative and non-exudative macular changes are observed

on ophthalmoscopy, such as drusen-like deposits, pigmentary changes, geographic chorioretinal atrophy, choroidal neovascularization and disciform scars.¹⁴ On fluorescein angiography, leakage of fluorescein dye as a result of choroidal neovascularization may be seen, and a dark choroid may be present. Full-field ERG and EOG testing indicates that overall retinal function remains largely intact.¹⁴

Macular dystrophies caused by *peripherin/RDS* mutations may share several hallmark features of AMD, the most common cause of blindness at older age in the Western world.¹⁵⁴ *Peripherin/RDS* phenotypes with a relatively late age at onset may therefore be confused with AMD. The presence of drusen and geographic atrophy may also falsely lead to the diagnosis of an atrophic form of AMD,^{10,13,14,21,82,87} whereas *peripherin/RDS*-related retinal dystrophies with choroidal neovascularization, exudative changes and disciform scars may be confused with neovascular AMD.^{14,26,120,125,137,155} The specific characteristics of the *peripherin/RDS*-related phenotypes, in combination with the autosomal dominant inheritance pattern, as well as molecular genetic analysis in selected cases, can assist the clinician in establishing the correct diagnosis. Contrary to other genes that are relatively frequent causes of macular dystrophies, such as *ABCA4* and *BEST1*, the possible role of the *peripherin/RDS* gene in AMD has not been thoroughly evaluated to date.^{156,157}

3.2.2. Cone and cone-rod dystrophy

In cone-rod dystrophy caused by *peripherin/RDS* mutations, loss of visual acuity starts in the 2nd to 4th decade, sometimes accompanied by mild photophobia and/or night blindness. Macular abnormalities are frequently observed on ophthalmoscopy and may appear similar to lesions in CACD, ranging from granular, mildly atrophic RPE changes to areas of profound, well-circumscribed chorioretinal atrophy. Some individuals may show bull's eye maculopathy and/or (mid-)peripheral areas of RPE atrophy and pigmentary changes.^{86,89,90} Color vision testing is often severely abnormal, and the peripheral visual field may gradually become constricted. FAF imaging tends to show lesions with speckled areas of increased and decreased FAF, similar to the observations in CACD.^{15,86} The full-field ERG shows a severely diminished to non-recordable cone response and variable abnormalities in the rod-derived responses.^{19,86,89,90,138,139} In asymptomatic mutation carriers, a reduced cone response on the photopic ERG may be the first detectable clinical feature.⁹⁰ The pattern ERG, as a marker of macular dysfunction, is markedly abnormal. Interestingly, the p.Arg172Trp mutation may lead to CACD as well as cone-rod dystrophy, the latter apparently evolving from a localized, central cone dystrophy to a more generalized cone and/or cone-rod dystrophy.^{15,86-88} The p.Ser27Phe variant, presumably a pathologic mutation, has been described in a family with late-onset cone dystrophy.⁹¹ This phenotype was characterized by photophobia and central visual loss, together with impaired color vision, mild non-specific foveal changes and subnormal macular and full-field cone ERG responses.

3.2.3. Retinitis pigmentosa

In adRP caused by *peripherin/RDS* mutations, the onset and severity of the phenotype

depends on the type of mutation. A relatively early age at onset, between the 1st and 3rd decade, has been associated with the p.Asp173Val and p.Asn244Lys mutations, as well as a three-base pair deletion of codon 118 or 119, residing in the 3rd transmembrane domain.^{5,89,158} The majority of adRP patients with other *peripherin/RDS* mutations have an age at onset in the 3rd to 5th decade.^{140,158-161} These patients usually first experience night blindness, followed by a progressive loss of the peripheral visual field. In most cases, a decrease in visual acuity does not occur before the age of 50. In general, an earlier age at onset leads to earlier and more profound visual impairment. On ophthalmoscopy, the classic features of RP are encountered: bone spicule pigmentation, retinal arteriolar attenuation and a waxy pale optic disc (Fig. 4.4). The full-field ERG reveals the absence of a rod response in the early teens, often accompanied by a mildly to severely decreased and delayed cone response. Dark adaptometry shows an abnormally delayed recovery of rod sensitivity, together with a rod threshold elevation of at least 1.0 log units compared to normal individuals.^{162,163} The EOG may also be abnormal.¹⁴⁰ Color vision testing is normal, except in cases with macular involvement.^{160,163} Kinetic visual field examination shows a progressive constriction of the peripheral visual field.^{5,20,140,160,163}

The adRP phenotype may be associated with macular dystrophy.^{140,164,165} The relatively severe adRP phenotype caused by a p.Asn244Lys mutation in *peripherin/RDS*, described in a Japanese family, is associated with bull's eye maculopathy.^{163,166} These macular lesions may evolve towards profound chorioretinal atrophy, resulting in a low visual acuity of counting fingers or even hand movements after the 5th decade. In this family, both the scotopic and photopic ERG were often extinguished in the early 20s.

A very typical form of autosomal dominant rod-cone dystrophy is retinitis punctata albescens (RPA). This retinal dystrophy is characterized by night blindness, innumerable round white deposits in the retina, progressive attenuation of the retinal arterioles, abnormal fundus pigmentation, progressive restriction of the visual fields, and non-detectable or severely reduced full-field ERG responses.¹⁶⁷ An autosomal dominant RPA-like phenotype has also been described in families with *peripherin/RDS* mutations.^{11,19,85} The full-field ERG in *peripherin/RDS*-related RPA shows a relatively equal loss of rod and cone function, which is in contrast to RPA related to mutations in the *RLBP1* and *rhodopsin* genes, where the rod system is generally affected more severely.¹⁶⁷⁻¹⁶⁹ The RPA phenotype caused by *peripherin/RDS* mutations may be difficult to differentiate from multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. Both are characterized by multiple yellowish subretinal lesions and both may also be combined with night blindness and adRP-like changes such as bone spicule-like pigmentation.^{11,13,19} However, lesions in RPA appear to be less irregular, more punctate, higher in number, and distributed throughout the entire retina.

The scarce clinical data on patients with digenic RP suggest that it has an onset of night blindness before the 4th decade and that it may be associated with a loss of visual acuity in the later stages.^{6,17,18,170} Other clinical features, such as the ophthalmoscopic picture and the preferential rod involvement on the full-field ERG, appear to be comparable to *peripherin/RDS*-related adRP. The *peripherin/RDS* and *ROM1* genes are located



Figure 4.4.

Color fundus photograph of autosomal dominant retinitis pigmentosa in a 60-year-old patient, caused by a p.Pro147fsX4 mutation in peripherin/RDS. The typical features of retinitis pigmentosa are evident: bone spicule pigmentation, narrow retinal arterioles, and a waxy pale optic disc. Extensive chorioretinal atrophy is seen outside the macular area. The visual acuity was 20/100. He experienced night blindness since early childhood and was diagnosed with retinitis pigmentosa at the age of 12 years. A decrease in visual acuity and photophobia were first experienced around 40 years of age. This patient was a cousin of the patient with multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus, described in Fig. 4.2D-F, who carried the same mutation. Analysis of the ROM1 gene in these patients did not reveal any mutations, thus making ROM1 unlikely to be the modifying genetic factor in these family members.

on different chromosomes. An affected individual from unrelated and unaffected parents has a 25% chance of transmitting both mutations to the offspring.^{17,18,105} This is the cause for the pseudodominant transmission of the phenotype in the case of digenic RP. The possibility of digenic RP should therefore be kept in mind in RP families with a specific segregation pattern, although most digenic RP patients are isolated cases.

4. From gene mutation to disease

4.1. *Peripherin/RDS* mutations and protein structure

Phenotypes associated with *peripherin/RDS* mutations do not show a straightforward dependence on the position of the mutation in the primary protein sequence or the type of mutation.³² Moreover, a single mutation may cause a spectrum of phenotypes ranging from a number of different macular dystrophies to adRP.^{13,19,20,86,87,147} Clearly, this remarkable phenotypic heterogeneity makes the interpretation of animal models of human disease more complex. As with other retinal disorders, the obvious anatomical differences between human and mouse retina also complicate the interpretation of these animal models.^{32,33,171} Despite these reservations, *in vitro* and animal models have provided valuable insights into peripherin/rds function, because of the high degree of identity of peripherin/rds sequence between human, bovine and murine orthologs.³⁶

The region between p.Cys165 and p.Asn182 in the D2 loop (Fig. 4.1) is required for peripherin/rds - rom1 interaction.⁴⁵ Mutations within this region have been described in macular dystrophies as well as adRP. These mutations may lead to a disturbed peripherin/rds - rom1 interaction, but different mutations in this region apparently affect rods and cones in a different way. The p.Arg172Trp mutation, located in the same region of peripherin/rds, may cause a spectrum of phenotypes, ranging from macular dystrophy to cone-rod dystrophy and adRP.^{86,87,93} Several functional studies, however, report a preferential cone involvement.^{86,88,138,139} The p.Arg172Trp mutation also causes cone and cone-rod dystrophy in a transgenic mouse model.¹⁷² In these mice, the p.Arg172Trp mutant protein localized properly to the rod and cone OSs and showed normal complex formation and association with rom1. A direct correlation was seen between transgene expression levels and the onset and severity of the phenotype. The results indicated that the p.Arg172Trp phenotype in mice is caused by a dose-dependent dominant negative effect that preferentially affects cones. In this mouse model, genetic supplementation of wild-type peripherin/rds provided partial, long-term rescue of rod function, but no permanent rescue of cone function.¹⁷³

A relatively large number of mutations associated with adRP have been found in a specific region within the large D2 loop, ranging from p.Lys193 to p.Glu226. This region contains two adjacent cysteine residues (p.Cys213 and p.Cys214), which form two of the three intramolecular disulfide bonds that are present in the D2 loop of peripherin/rds (Fig. 4.1). These cysteines appear to be essential for peripherin/rds protein folding and subunit assembly.³² Most of the mutations in the p.Lys193 to p.Glu226 region are missense mutations, the majority leading to changes in peripherin/rds that may affect rods more than cones. However, some mutations in this region, including p.Cys213Arg, p.Cys213Tyr and p.Cys214Tyr, may also cause different types of macular dystrophy, as well as autosomal dominant cone-rod dystrophy.^{26,103,110,149}

Two adRP-causing mutations in the D2 loop have been studied in animal models and *in vitro* experiments: p.Cys214Ser and p.Pro216Leu.^{6,28,161} The p.Cys214Ser mutant protein, which causes a late-onset adRP phenotype in humans,¹⁶¹ is retained in the rod inner

segments, as this grossly misfolded protein does not pass certain quality control mechanisms in the rod photoreceptor cell.^{54,174} The resultant lack of wild-type peripherin/rds and tetramers causes a haploinsufficiency/loss-of-function phenotype.^{54,72,175} In contrast, the adRP phenotype caused by the p.Pro216Leu mutation is probably due to a dominant negative effect on rod OS structure.^{54,72,176,177} The p.Pro216Leu mutant protein does not preclude tetramer formation, but the formed tetramers are dysfunctional, presumably as a result of hyperglycosylation of the protein.^{54,177} Another late-onset slowly progressive adRP is caused by a C-terminal 1 base pair deletion at codon 307 of *peripherin/RDS*, which is predicted to result in a completely altered and shorter C-terminal domain.¹⁹ This abnormal C-terminus may lead to an absent localization signal and other C-terminal dysfunction.^{32,56,62,63,65,69} A murine model of the same mutation indicates that the phenotype is caused by a dominant negative effect, in conjunction with haploinsufficiency.¹⁷⁸

Digenic RP is caused by the p.Leu185Pro *peripherin/RDS* mutation, when it is co-inherited double heterozygously with a *ROM1* mutation.¹⁸ *In vitro* studies on COS-1 cells have found that these mutations prevent sufficient forming of homotetramers and higher order disulfide-linked oligomers,^{97,179} and suggested that functional asymmetry between peripherin/rds and rom1 plays a role in digenic RP.⁵⁰ A transgenic mouse model, *Xenopus* immunofluorescence, as well as COS-1 cell studies pointed out that digenic RP is a haploinsufficiency phenotype.^{54,72,180} Like in the p.Cys214Ser mutation phenotype, insufficient amounts of tetramers are formed to allow proper OS disc morphogenesis. No photoreceptor degeneration was detected in *rom1(+/-)/rds(+/+)* mice, but *rom1(-/-)/rds(+/+)* mice showed a very mild photoreceptor degeneration.^{17,50,180}

Six *peripherin/RDS* mutations lead to a change in cysteine residues. Cysteine residues are important for peripherin/rds protein structure through the formation of intramolecular disulfide bonds. The replacement of a cysteine by another amino acid, or the appearance of an extra cysteine may therefore alter protein structure. Three of the six *peripherin/RDS* mutations that affect a cysteine residue have been described in adRP: p.Tyr141Cys,²⁸ p.Cys165Tyr,¹⁸¹ and p.Cys214Ser.¹⁶¹ The remaining three mutations (p.Cys213Arg, p.Cys213Tyr and p.Cys214Tyr), on the other hand, are associated with various macular dystrophies.^{78,103,145} Using a specific computer prediction programme of secondary protein structure, a significant correlation was found between the predicted molecular alteration and clinical severity.^{149,182} However, the computer-predicted changes in secondary structure were not able to predict the clinical subtype of retinal degeneration.

In summary, mouse models and *in vitro* experiments suggest that both haploinsufficiency and dominant negative mechanisms play a role in human retinal dystrophies caused by mutations in the *peripherin/RDS* gene.

Rods and cones differ in several important aspects.^{183,184} Some studies in *rds(+/+)* mice indicate that haploinsufficiency has a greater impact on rods than on cones, and that the defect is more detrimental to L (“red”) and M (“green”) cones than to S (“blue”) cones.^{185,186} Other mouse and human studies report that *rds(+/-)* haploinsufficiency phenotypes result in a relatively equal rod and cone cell loss.^{85,138,139,187,188}

4.2. Genotype-phenotype correlations

The phenotypic spectrum caused by a specific *peripherin/RDS* mutation, even in family members with identical mutations, may range from no visible abnormalities to various macular dystrophies, cone-rod dystrophy, as well as adRP.^{13,19,20,86} Incomplete penetrance of *peripherin/RDS* mutations has been described in several families.^{13,86,116,137} Differences in age and therefore disease progression alone are insufficient to explain the variability of the phenotypic outcomes. This makes it difficult to recognize consistent genotype-phenotype correlations.^{32,117,182,189}

Most missense mutations and in-frame deletions (75%) are located in the D2 loop (Fig. 4.1). More than half (52%) of the mutations in the group of macular dystrophies are nonsense or frameshift mutations, whereas missense mutations and in-frame deletions account for the majority (81%) of the mutations in adRP patients (Fig. 4.1). Some mutations lead to specific phenotypes with relative consistency. The p.Arg142Trp and p.Arg172Trp mutations, for instance, generally result in a localized macular dystrophy or in cone-rod dystrophy.^{9,15,87,144} Missense mutations in the p.Pro210 to p.Pro216 region of the D2 loop in the peripherin/rds protein, as well as the p.Asn244Iys mutation, are more likely to cause adRP, also with a fairly uniform phenotype.^{6,159,161,163} The p.Tyr141Cys mutation, on the other hand, is associated with AMD-like macular dystrophy,¹⁴ pattern dystrophies,¹²⁰ and adRP.²⁸

To date, no satisfactory explanation has been reported to explain this remarkable phenotypic variability and incomplete penetrance associated with *peripherin/RDS* mutations. Other factors, such as the genetic background, modifying genes and/or environmental factors, obviously exert their influence on the phenotypic outcome.¹⁹⁰ The phenotype may depend on the amount of residual wild-type and mutant peripherin/rds protein, especially in haploinsufficiency phenotypes.^{54,180} In the case of the p.Arg172Trp mutation, mouse studies indicate that differences in expression level of the mutant protein may aid in explaining the differences in onset and severity of the disease in animals of the same age.¹⁷²

The phenotypic expression may also be influenced by the genetic background and by modifying genes.¹⁹⁰ In the search for such factors, the *ROM1* gene seemed a likely candidate. It has been shown, however, that variations in this gene do not constitute an important modulating factor.^{14,20,191} One of the many possible modifying genes may be *RPE65*, through the modulation of rhodopsin regeneration kinetics and light-damage susceptibility.¹⁹² Environmental factors may also influence phenotypes. Experimental evidence from animal models indicates that light exposure can modify the progression of several forms of retinal degeneration.¹⁹³ In *rds*(-/-) and *rds*(+/-) mice, constant light exposure accelerates photoreceptor cell loss compared to normal mice. This retinal cell loss progresses from the central retina to the periphery and appears to affect rods earlier than cones.¹⁹⁴ Other animal models in which retinal degeneration is accelerated by light exposure include animal models of adRP caused by *rhodopsin* mutations,^{138,195,196} animal models of Oguchi disease caused by *rhodopsin kinase* or *arrestin* mutations,^{197,198} and in the *abca4*(-/-) mouse model for STGD1.^{199,200} Dietary habits and nutritional components, such

as the intake of vitamin A, lutein, zeaxanthin, and omega-3 polyunsaturated fatty acids, may also be of influence on the clinical course of retinal degenerations.²⁰¹⁻²⁰⁶ The role of smoking as a possible modifying factor in retinal dystrophies is unclear, although smoking is known to be strongly associated with AMD.²⁰⁷

4.3. Pathophysiology of retinal dystrophies caused by *peripherin/RDS* gene mutations

A histopathological study in a donor eye of a patient with advanced butterfly-shaped pattern dystrophy, caused by a p.Cys213Tyr mutation in *peripherin/RDS*, revealed a central area of complete photoreceptor and RPE cell loss, with an intact choriocapillaris, and lipofuscin-containing cells in the subretinal space.¹⁰³ In the surrounding region, RPE cells were distorted and greatly distended by lipofuscin, whereas the photoreceptors were partly atrophic.

The differential effect of *peripherin/RDS* mutations on cone and rod photoreceptors has been discussed in paragraph 4.1. Phenotypes dominated by rod photoreceptor loss may be caused by both a haploinsufficiency and/or a dominant negative effect. Phenotypes with predominantly cone dysfunction appear to be the result of a dose-dependent dominant negative effect.¹⁸⁶ The multifocality of lesions in phenotypes related to *peripherin/RDS* mutations may be explained by local differences in retinal cell interactions, distribution, morphology and metabolism.²⁰⁸

The exact mechanisms through which *peripherin/RDS* mutations cause photoreceptor and RPE cell death are as yet unclear. Animal studies indicate that photoreceptors containing mutant peripherin/rds may lead to a shortening of average OS length, whorls of OS membranes in the subretinal space, and alterations in the normal OS-RPE interface and disc phagocytosis.^{32,209} Changes secondary to *peripherin/RDS* mutations may lead to abnormalities in photoreceptor energy metabolism, physiology and intercellular interactions.^{183,210-212} These changes may lead to an accumulation of autofluorescent waste products like lipofuscin and toxic lipofuscin components like A2E in the photoreceptors and RPE.²¹³⁻²¹⁵ The rate of lipofuscin accumulation may be influenced by environmental factors such as light exposure and vitamin A intake.^{193,216-218} Finally, a mutation in peripherin/rds may initiate photoreceptor cell death through apoptosis.^{219,220} In *peripherin/RDS* mutations that preferentially affect rods, cone cell loss at a later stage may be caused by a primary detrimental effect of the mutation on cone photoreceptors. An alternative or additional explanation may be that cone degeneration occurs secondary to rod loss, for instance due to a decrease in rod-derived cone viability factor.^{60,100,139,221-223}

5. Future perspectives

A better understanding of the underlying mechanisms of retinal dystrophies, such as the *peripherin/RDS*-related disorders in this review, is necessary for the future development of effective therapeutic strategies. Information from animal model research is

indispensable to demonstrate the efficacy and safety of such treatments.^{36,186,224} Detailed studies on retinal dystrophies caused by *peripherin/RDS* mutations are mandatory, not only to provide the patient with accurate information about his or her disease and its prognosis, but also to be able to obtain proper outcome parameters in future therapeutic trials.^{106,107,225,226} This may be facilitated by the employment of new functional and imaging techniques.^{129,227,228}

The eye is a unique target organ for therapeutic intervention, as the therapeutic agents may be precisely administered at the required location. Equally important, the eye is readily accessible to evaluate the effects of this intervention in great detail.^{188,229,230} Currently, gene therapy appears to be the most promising approach in *peripherin/RDS*-related disease. In animal studies of haploinsufficiency phenotypes, a dose-dependent rescue effect on photoreceptors was observed through the gene delivery of wild-type *rds*.^{186,188,231} In mice homozygous for a *rds* null mutation, subretinal injection of adenovirus-associated viral vectors carrying *rds* cDNA resulted in significant improvement on the ultrastructural and functional level.^{232,234} However, limited transduction efficiency was seen in these studies, and the effect was partial and of limited duration.^{229,233} Moreover, concerns remain about toxic side effects that may be caused by the AAV vector.^{235,236} A promising and possibly safer alternative for the use of viral vectors in gene therapy is the use of DNA nanoparticles.²³⁷ Unfortunately, this approach may also be complicated by a low transfection efficiency, as well as a short duration of gene expression.^{186,237} In the case of dominant negative mutations, the phenotype may not only result from a lack of normal *peripherin/rds*, but also from a deleterious effect of the mutant protein on photoreceptors. Disruption of the mutant gene, for instance through RNA interference techniques and ribozymes, may be necessary in these cases besides an increased expression of wild-type *peripherin/RDS*.^{31,54,229,238-240} The delivery of trophic factors may also constitute a therapeutic approach.²⁴¹⁻²⁴⁶ Delivery of ciliary neurotrophic factor (CNTF) via an AAV vector, in *rds(+/-)* mice carrying the dominant negative p.Pro216Leu mutation, resulted in a partial, dose-dependent, but long-term rescue of photoreceptors after subretinal administration.^{241,242,247} Prolonging rod survival may also promote survival of cones, for instance through the influence of rod-derived cone viability factor.^{221,231,248,249} The use of rod-derived cone viability factor may therefore be an interesting approach to preserve cones in retinal dystrophies such as RP.²⁴⁴

The aforementioned studies also illustrate that, before the treatment of patients using these strategies may actually start, a considerable number of challenges have to be overcome. Despite all these promising experimental studies, there are currently no specific treatment options for retinal dystrophies caused by *peripherin/RDS* mutations. A reduction in light exposure may mildly influence the rate of disease progression through a reduction of A2E accumulation.^{170,193,218,250,251} Data on the impact of dietary influences on the outcome of retinal dystrophies are relatively scarce.^{201,206} It is likely that the nature of the disease-causing genes and mutation(s) may have a considerable influence on the effect of nutritional factors and supplements. The role of vitamin A supplementation is still subject for debate, but large studies indicate that at least a subset of RP patients

may benefit from this therapy, possibly in combination with docosahexaenoic acid, an omega-3 polyunsaturated fatty acid.^{202,216,217,252-254} However, these studies did not take information on the underlying genetic defects into account. Studies on *abca4(-/-)* mice showed that a reduction in serum vitamin A may reduce the accumulation of A2E and lipofuscin in the RPE.²⁰⁵ In AMD, a higher dietary intake of nutritional components such as lutein/zeaxanthin and omega-3 polyunsaturated fatty acids may promote photoreceptor survival through numerous mechanisms, including a decrease in oxidative stress.²⁵⁵⁻²⁶⁰ In autosomal dominant Stargardt-like retinal dystrophy (STGD3), caused by mutations in the *ELOVL4* gene, supplementation with specific omega-3 polyunsaturated fatty acids may reduce the severity of the macular phenotype.^{204,258} Patients with X-linked RP may also benefit from supplementation with docosahexaenoic acid.²⁰³ However, the effect of the aforementioned nutritional factors has, to the best of our knowledge, not been studied in retinal dystrophies associated with *peripherin/RDS*. Therefore, it seems reasonable to practice reservation with regard to the use of nutritional supplements in *peripherin/RDS*-related disease.

Reports in single patients or small patient groups indicate that choroidal neovascularization in retinal dystrophies may respond to various treatments, such as photodynamic therapy,^{261,262} and intravitreal or systemic bevacizumab.^{263,264} In general, choroidal neovascularization is a rare event in inherited retinal dystrophies, and may have a relatively favorable prognosis compared to choroidal neovascularization in AMD.²⁶⁵ Cystoid macular edema in RP patients, although never reported in *peripherin/RDS*-related adRP, may respond to topical treatment with dorzolamide.²⁶⁶

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4.2. Central areolar choroidal dystrophy

Abstract

Objectives: To describe the clinical characteristics, follow-up data and molecular genetic background in a large group of patients with central areolar choroidal dystrophy (CACD).

Methods: One hundred and three patients with CACD from the Netherlands. Ophthalmological examination, including color vision testing, fundus photography, fluorescein angiography, fundus autofluorescence (FAF) imaging, optical coherence tomography, full-field electroretinography (ERG), multifocal ERG, and electro-oculography (EOG). Blood samples were obtained for DNA extraction and subsequent analysis of the *peripherin/RDS* gene, as well as haplotype analysis.

Results: The mean age at onset of visual loss was 46 years, with a subsequent gradual deterioration in visual acuity. Ninety-eight patients carried a p.Arg142Trp mutation in *peripherin/RDS*, whereas five affected members of a CACD family carried a p.Arg172Gln *peripherin/RDS* mutation. A remarkable variation in disease severity was observed, and non-penetrance was seen up to the age of 64 years, in up to 21% of mutation carriers. However, the great majority of macular lesions in mutation carriers conformed to a typical stage of CACD. Substantial changes were seen on FAF imaging after a mean follow-up period of 11 months. Electrophysiological data were consistent with a central cone dystrophy. The age at onset and phenotypic characteristics of CACD show a considerable overlap with atrophic age-related macular degeneration (AMD). The great majority of p.Arg142Trp-carrying CACD patients originated from the southeast region of the Netherlands. Haplotype analysis strongly suggested a common founder mutation in these patients.

Conclusions: CACD caused by a p.Arg142Trp mutation in the *peripherin/RDS* gene is a central cone dystrophy phenotype. This mutation, which most likely originates from a common founder in most patients, is associated with a significant degree of non-penetrance. In the elderly patient, CACD may be confused with atrophic AMD, especially in cases with decreased penetrance.

Introduction

Central areolar choroidal dystrophy (CACD) is a hereditary retinal disorder that principally affects the macula, often resulting in a well-defined area of atrophy of the retinal pigment epithelium (RPE) and choriocapillaris in the centre of the macula.¹⁻⁴ A dysfunction of the macular photoreceptors usually leads to a decrease in visual acuity, which generally occurs between the age of 30 and 60 years.⁴⁻⁶

In most cases, CACD is inherited as an autosomal dominant trait,⁷ although autosomal

recessive cases have been reported.⁸ Autosomal dominant CACD is genetically heterogeneous,^{9,10} but mutations in the *peripherin/RDS* gene (official Human Gene Nomenclature Committee gene symbol: *PRPH2* or *peripherin-2*) appear to be the most frequent cause.^{11,12} To date, five different mutations in *peripherin/RDS* have been described in autosomal dominant CACD: p.Arg142Trp,⁹ p.Arg172Trp,^{5,13,14} p.Arg172Gln,^{5,15} p.Arg195Leu¹⁶ and p.Leu307fsX83.¹⁷ The latter mutation causes an atypical variant of CACD. In the case of the mutations at codon 172, full penetrance and uniform symptoms and signs were reported initially,^{5,14,15} but a more recent study described a marked intrafamilial variation and non-penetrance associated with the p.Arg172Trp mutation.¹⁸ Another study indicated that the higher frequency of the p.Arg172Trp mutation in the British population was the result of a founder effect.¹²

Full penetrance and a highly uniform age-related clinical presentation have also been reported in patients with CACD caused by the p.Arg142Trp mutation in *peripherin/RDS*, which is mainly found in CACD patients originating from a specific region in the south-eastern part of the Netherlands.^{4,9} We report the clinical characteristics and follow-up data of a group of 103 patients with CACD caused by two specific *peripherin/RDS* mutations, and compare them to CACD phenotypes caused by other mutations in the *peripherin/RDS* gene. We describe three large families that show evidence of non-penetrance of the p.Arg142Trp mutation. In addition, we provide evidence showing that the frequent finding of the p.Arg142Trp mutation in Dutch CACD patients is the result of a founder effect. We propose a pathophysiological disease sequence of CACD. Finally, the close resemblance and differential diagnosis of CACD with atrophic age-related macular degeneration (AMD) is discussed.

Methods

Clinical studies

We included 103 patients (204 eyes, excluding one amblyopic eye and one eye with previous trauma) with CACD, who were all examined at the Institute of Ophthalmology of the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands), between June 1972 and December 2007. This department of ophthalmology serves as a tertiary referral centre for patients from the north-east, middle-east, and south-east regions of the Netherlands.

Patients received the diagnosis of CACD based on the aspect of the lesions on ophthalmoscopy, as described by Hoyng et al.⁴ In selected cases, the results of *peripherin/RDS* mutation analysis helped in differentiating CACD from other macular disorders. Four stages were discerned, according to a previously described classification.^{4,14} In stage I, slight parafoveal pigmentary changes of the RPE are seen, usually in the adolescent patient. Stage II is characterized by a round to oval, mildly atrophic hypopigmented area of 1.5 to several disc diameters. In stage III, one or more areas of well-circumscribed atrophy of the RPE and choriocapillaris appear outside the fovea. In stage IV, this area

of well-defined chorioretinal atrophy affects the fovea. When the area of profound chorioretinal atrophy has reached the centre of the macula and is about to involve central foveal function, with a still fairly good visual acuity, lesions were categorized as stage III-IV. The diagnosis of CACD was supported by mutation analysis of the *peripherin/RDS* gene: 98 of the 103 patients carried a p.Arg142Trp mutation in *peripherin/RDS*, which has been previously described as a common finding in CACD patients that originate from the south-east of the Netherlands.⁹ The remaining five patients were found to carry a p.Arg172Gln mutation in *peripherin/RDS*, which has also been implicated in the CACD phenotype.¹⁹

After the medical histories of the patients were obtained, an extensive clinical examination was performed, including best-corrected Snellen visual acuity testing, Amsler grid testing, fundus examination by indirect ophthalmoscopy, and fundus photography.

Fundus autofluorescence (FAF) imaging (Heidelberg Retina Angiograph (HRA) 2, Heidelberg Engineering, Heidelberg, Germany) was performed in 58 patients using a previously described protocol.²⁰ FAF was used to detect RPE atrophy (dark macular regions) and lipofuscin increase (bright macular spots). Follow-up FAF images were available in 11 patients and were analysed using a standard protocol, based on previously published methods.²¹⁻²³ These methods of FAF analysis are published online (see online supplemental material on <http://aaojournal.org>).

Optical coherence tomography (Stratus OCT, Carl Zeiss Meditech, Inc., Dublin, CA, USA) was done in 58 patients, using the “Macular Thickness Map” protocol provided by the manufacturer.

Full-field electroretinography (ERG) was performed in 55 patients. Twenty-three of these patients underwent full-field ERG according to the standards of the International Society for Clinical Electrophysiology of Vision (ISCEV).²⁴ In 32 patients, a full-field ERG was recorded prior to the introduction of ISCEV standards, according to a previously described protocol.²⁵ In 17 patients, multifocal ERG testing was performed according to the ISCEV guidelines, using 103 hexagons.²⁶ Electro-oculography (EOG) was done in 49 patients: 16 of these patients underwent EOG according to the ISCEV standards,²⁷ whereas the EOG in the other 33 patients was performed prior to ISCEV regulations, according to a protocol by Thijssen et al.²⁵

Color vision testing was performed in 36 patients, using a standard protocol. The Tokyo Medical College (TMC) test was used as a screening test for color vision. When the TMC testing result was normal, the Ishihara test (1970 edition) was performed. When a patient failed the TMC test, the Hardy Rand and Rittler (HRR) test (second edition) was used. After this test, the Standard Pseudoisochromatic Plates test was performed, followed by the New Color Test (NCT, box 4/8). After these tests, the patients underwent a desaturated Panel D-15 test and, if possible, the Farnsworth-Munsell 100 Hue test.

This study adhered to the tenets of the Declaration of Helsinki and was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Centre (Nijmegen). Informed consent was received from all subjects.

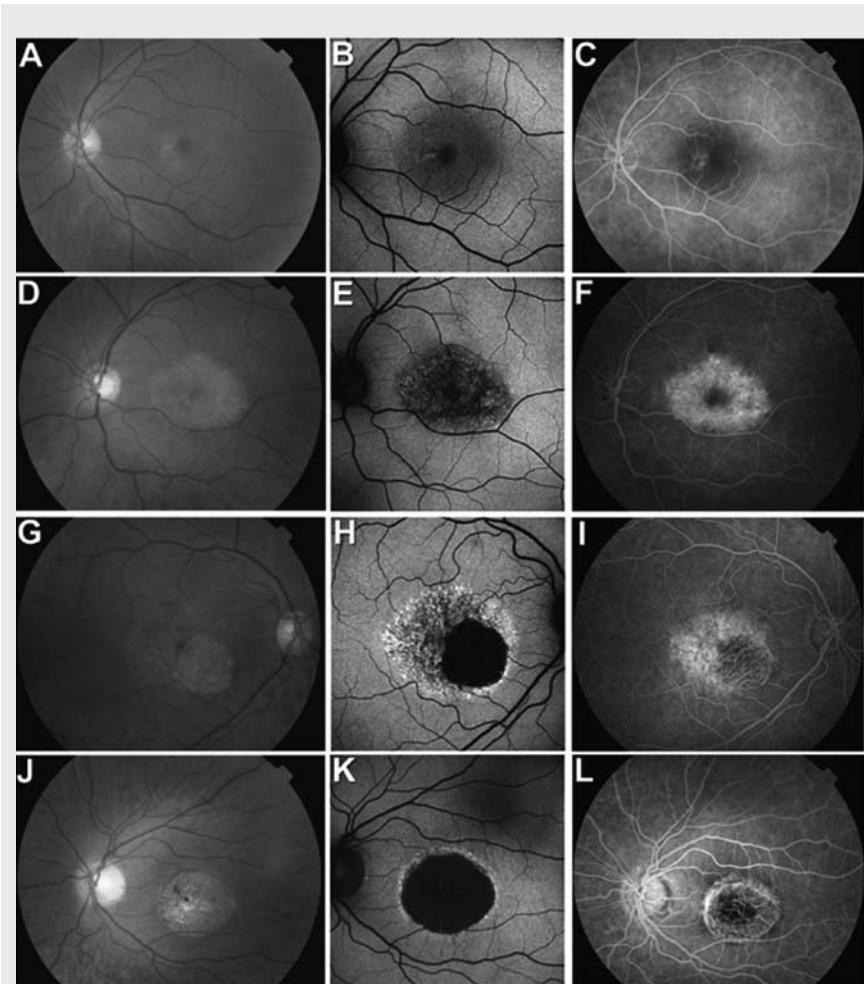


Figure 4.5.*

Clinical stages of central areolar choroidal dystrophy (CACD). **A.** Color fundus photograph of stage I CACD, showing slight parafoveal hypopigmentation. **B.** This area of hypopigmentation on ophthalmoscopy corresponds with an area increased fundus autofluorescence (FAF). **C.** Fluorescein angiography shows hyperfluorescent parafoveal changes in stage I CACD. **D.** In stage II CACD, a round to oval area of hypopigmentation is seen in the macula. **E.** FAF in stage II shows a corresponding area of speckled changes of increased and decreased FAF. Initially, increased FAF may predominate, but with time, as the lesion enlarges and atrophy of the retinal pigment epithelium (RPE) progresses, decreased FAF may become more prominent. **F.** The fluorescein angiogram in stage II displays a speckled hyperfluorescence of the lesion, corresponding with partial atrophy of the RPE. **G.** Stage III CACD shows one or more patches of well-circumscribed chorioretinal atrophy, appearing outside the central fovea, within the area of slight

hypopigmentation. **H**. These areas of chorioretinal atrophy correspond to severely decreased to absent FAF. **I**. Fluorescein angiography in stage III CACD clearly visualizes the remaining choroidal vessels in the area of chorioretinal atrophy. In the later phase of the angiogram, discrete leakage of fluorescein could be observed at the edge of the lesion, corresponding with incomplete atrophy of the choriocapillaris. **J**. In stage IV CACD, the well-defined area of chorioretinal atrophy involves the fovea, with a corresponding severe decrease in visual acuity. **K**. This area corresponds with a round to oval zone of absent FAF involving the fovea in late stage IV CACD, bordered by a small residual band of increased FAF. **L**. End-stage CACD also shows a well-demarcated area of chorioretinal atrophy on the fluorescein angiogram, with enhanced visibility of the residual underlying choroidal vessels.

Mutation screening and haplotype analysis

Peripheral venous blood samples were obtained from all patients for analysis of the *peripherin/RDS* gene. The genomic DNA was isolated as described elsewhere.²⁸ The three coding exons and splice junctions of the *peripherin/RDS* gene were amplified by the polymerase chain reaction (PCR), and these fragments were then subjected to sequence analysis to detect mutations as described previously.²⁹ Subsequently, in three patients carrying the p.Arg142Trp mutation who were members of large families, the p.Arg142Trp mutation was also screened in a total of 28 clinically affected and unaffected family members. Likewise, 15 additional family members of a patient carrying a p.Arg172Gln mutation were also screened for this mutation in codon 172. Family members who were found to carry the p.Arg142Trp or the p.Arg172Gln mutation underwent an ophthalmic examination as described above.

Haplotype analysis was performed with microsatellite markers and single nucleotide polymorphisms (SNPs) surrounding the *peripherin/RDS* gene. Microsatellites D6S1552 and D6S1582 were amplified with primers containing a M13-forward or -reverse tail. A second PCR was performed with a fluorescently labelled M13-forward primer and an unlabelled M13-reverse primer. PCR products were mixed with a fluorescent size marker (Applied Biosystems), and samples were analyzed on a 3100 or 3730 DNA Analyzer (Applied Biosystems). Fragment lengths were analyzed with GeneMapper software (Applied Biosystems). SNPs with a high heterozygote frequency in European descendants were selected from the International HapMap Project. Selected SNPs (rs2281462, rs9349227, rs9471874, rs373341, rs3846893, rs9381218, rs6941837) were analyzed by PCR amplification and direct sequencing.

Control Group

The p.Arg142Trp variant has been previously screened in 200 chromosomes from 100 normal individuals from the Netherlands, and was found in none of these patients.⁹ In addition, we screened a group of 57 persons originating from the same region in the

south-east of the Netherlands for the p.Arg142Trp variant. These 57 control subjects were over 70 years of age, did not experience visual disturbances, and did not have a family history of macular disease. The p.Arg172Gln mutation has also been previously tested in a control group.⁵

Results

Clinical characteristics

The 103 CACD patients originated from 46 different families. Of the 98 patients with a p.Arg142Trp mutation, 96 patients (98%) originated from the south-east region of the Netherlands, despite the considerably wider area of tertiary referral. Three patients with CACD caused by a p.Arg142Trp mutation were identified after analysis of the *peripherin/RDS* gene in a group of 21 patients of whom blood was sent to the Human Genetics department with the diagnosis of “early-onset AMD” (age < 55 years). Information about the age at onset of visual loss was available in 88 patients. The mean age at onset of visual loss was 46 years (range, 12-63 years) in a group of 59 CACD patients with the p.Arg142Trp mutation. Twenty-nine of the 88 CACD patients (33%) did not experience visual loss. In these patients, the phenotype was discovered by coincidence or as a result of ophthalmological screening because of a positive family history. Twenty-one patients (20%) did not have a family history of visual loss compatible with macular disease. The visual acuity varied widely, generally depending on the disease stage. Table 4.1 shows the mean age, the mean lesion size, and the visual acuities differentiated by CACD stage, as well as the number of patients with visual disturbances in each CACD stage.

Follow-up data were available in 86 eyes of 43 patients, and are depicted in Table 4.2. In the group of patients with a follow-up time of more than five years, 36 of 64 eyes (56%) showed progression to another stage.

Of the 103 CACD patients, 99 (96%) patients showed a CACD stage on ophthalmoscopy that largely corresponded to the typical stages as described by Hoyng and colleagues (Fig. 4.5).⁴ The four patients with an atypical phenotype showed either atypical parafoveal pigmentary changes (3 patients), or lesions resembling pattern dystrophy in a butterfly-shaped configuration (1 patient). Asymmetry of stages between both eyes was observed in 26 patients (25%). When comparing the observed CACD phenotypes in our study to the stages described by Hoyng and co-workers, mild variation of the CACD phenotype was common. For instance, in 20 patients (19%), the macular lesion was not confined to the central macula but reached the retina adjacent to the temporal part of the optic disc or completely surrounded the optic disc (Fig 4.6A and B). In one patient, the lesions extended beyond the temporal retinal vascular arcades. Small drusen-like lesions at the edge of the macular lesion were seen in 11 patients (11%), whereas two patients belonging to the same family had larger drusen in the centre of the lesion. One patient had extensive macular and peripheral basal laminar drusen in combination with the macular CACD lesion. Macular lesions had a “bull’s eye” aspect in 4 patients (4%) from 3

different families. Two of the patients with a bull's eye appearance of the CACD lesion on ophthalmoscopy, who received a full-field ERG evaluation, had normal photopic and scotopic full-field ERGs. Relative foveal sparing in advanced CACD, with a correspondingly good visual acuity, was seen in four patients. One patient with high myopia developed choroidal neovascularization.

The characteristics on fluorescein angiography are depicted in Figure 4.5. Discrete abnormalities in stage I CACD could be easily visualized with fluorescein angiography. Except in some cases of early stage I CACD, CACD lesions were easily identified with FAF imaging (Fig. 4.5). Fluorescein angiography appeared most sensitive to identify some cases of early stage I CACD, as it was able to visualize even minuscule areas of discrete RPE atrophy that were not readily detected on ophthalmoscopy or FAF. The analysis of follow-up FAF images is depicted in Table 4.3. Individual examples of increasing lesion size and progression of chorioretinal atrophy on FAF are given in Figure 4.6, and are shown in online videos 1 and 2 (available at <http://aojournal.org>).

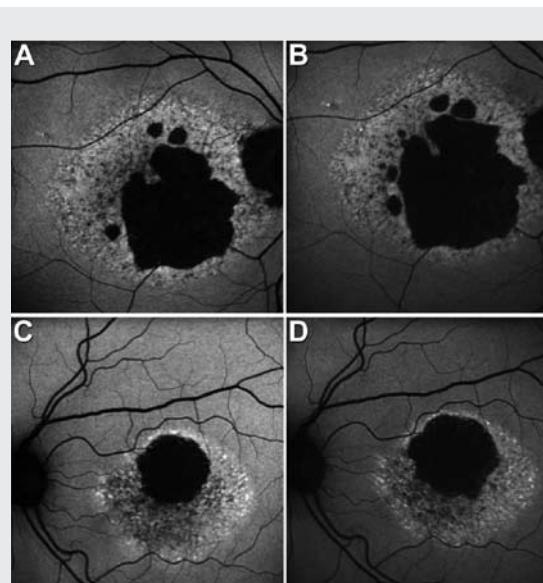


Figure 4.6.

Follow-up of patients with fundus autofluorescence (FAF, also see Table 4.3 and online supplemental movies). **A and B.** After 19 months of follow-up in this patient with stage III central areolar choroidal dystrophy (CACD), the total area of FAF abnormalities changed from 22.95 mm^2 to 24.20 mm^2 (an increase of 1.25 mm^2). The area of absent FAF, corresponding with atrophy of the retinal pigment epithelium, increased from 7.61 mm^2 to 9.27 mm^2 . **C and D.** In this example of another patient with stage III CACD, the total area of FAF changes increased from 9.46 mm^2 to 10.14 mm^2 , an increase of 0.68 mm^2 after a follow-up period of 30 months. The area of absent FAF increased from 2.18 mm^2 to 3.24 mm^2 (a 1.06 mm^2 increase).

4

On optical coherence tomography, increased reflectivity of the “outer red line” could be observed,³⁰ which was proportional to the degree of chorioretinal atrophy observed on ophthalmoscopy and FAF.

Table 4.1.
Visual acuity and visual disturbances in central areolar choroidal dystrophy (CACD).

CACD stage	Number of patients (Patients with symmetrical stage)	Mean age (years) [†]	Number of eyes	Mean lesion size (in disc diameters) [‡]	Mean VA (range) [§]	Number of patients with visual disturbances (%)	Principal visual complaints
I	10 (8)*	45 (22 - 73)	18	-	20/20 (20/33 - 20/16)	0/8 (0%)	-
II	52 (31) [#]	42 (5 - 77)	83	2.6 (range, 1.5 - 4.5)	20/23 (20/80 - 20/16)	13/31 (42%)	VA: 7/13, central scotoma: 3/13, metamorphopsia: 3/13, nyctalopia: 2/13
III	38 (20)**	49 (26 - 65)	59	3.3 (range, 1.5 - 5.0)	20/27 (20/100 - 20/16)	15/20 (75%)	VA: 14/15, metamorphopsia: 5/15, central scotoma: 3/15, color vision abnormalities: 1/15, diplopia: 1/15, micropsia: 1/15, nyctalopia: 1/15
III-IV	15 (3) [¶]	58 (42 - 75)	18	-	20/38 (20/125 - 20/25)	13/15 (87%)	VA: 11/13, metamorphopsia: 2/13, central scotoma: 2/13
IV	14 (2) [%]	56 (40 - 81)	15	3.4 (range, 1.5 - 5.0)	20/182° (20/2400 - 20/33)	14/14 (100%)	VA: 14/14

*Of the 2 patients with unilateral stage I CACD, the fellow eye showed stage III CACD. In stage I and II, the mean age of patients with identical stages was calculated. In stage III, III-IV and IV, the mean age was calculated including patients who had stage III, III-IV, or stage IV as the highest stage. The fellow eye was in the same or a lower stage. [†]The mean age was estimated on fundus autofluorescence or fundus angiography or fundus fluorescence. [‡]Of the 30 CACD patients over 60 years of age, 8 patients (27%) had a visual acuity below 20/60 in the better eye, which is compatible with the World Health Organization definition (1992) of low vision or visual impairment. In the 19 patients over 70 years of age, 11 patients (58%) corresponded to this definition of low vision. ^{||}In stages I - III, only patients with a symmetrical stage are included in this count, as visual disturbances caused by the fellow eye in a higher stage may dominate in patients with a symmetrical stages. [#]Twenty-one patients had unilateral stage II CACD, the other eye showing either stage III (14/21 eyes), stage III-IV (4/21 eyes), or stage IV CACD (3/21 eyes). ^{**}Of the 19 patients with unilateral stage III-IV CACD, the fellow eye showed stage II (15/19 eyes), stage III-IV (1-19 eyes), or stage IV CACD (3/19 eyes). [¶]Twelve patients had unilateral stage III-IV CACD, the other eye showing either stage II (4/12 eyes), stage III (1/12 eyes), or stage IV CACD (7/12). [%]In the 11 patients with unilateral stage IV CACD, the fellow eye showed stage II (2/11 eyes), stage III (3/11 eyes), or stage III-IV CACD (7/11 eyes). ^{*}Of the 14 patients with stage IV CACD, 11 patients (79%) had a visual acuity below 20/60 in the better eye, whereas the remaining 3 patients were slightly above this limit. Abbreviation: VA, visual acuity.

Table 4.2.

Follow-up data in central areolar choroidal dystrophy (CACD).

Change of CACD stage	Number of eyes*	Mean time (years) between change of CACD stage (range)
Stage I → Stage II	5	18.2 (13 - 20)
Stage I → Stage III	1	13
Stage II → Stage III	12	11.8 (5 - 17)
Stage II → Stage IV	7	19.6 (13 - 31)
Stage III → Stage IV	16	6.4 (2 - 16)

*From a total of 86 eyes on which follow-up information was available.

Table 4.3.

Follow-up of fundus autofluorescence (FAF) imaging in central areolar choroidal dystrophy patients.

	Initial visit	Follow-up*
Mean surface area of FAF changes (in mm ² , n = 22 eyes)	10.6 (range, 0.3 – 23.0)	11.2 (range, 0.7 – 24.2)
Mean area of absent FAF [†] (in mm ² , n = 22 eyes)	1.69 (range, 0 – 7.61)	2.11 (range, 0 – 9.27)
Mean area of absent FAF in patients with area of absent FAF at initial visit (in mm ² , n = 15 eyes)	2.48 (range, 0.05 – 7.61)	3.09 (range, 0.12 – 9.27)

*Mean follow-up time: 18 months (range, 11 – 30 months).

†The area of absent FAF corresponds to profound chorioretinal atrophy on ophthalmoscopy.

Table 4.4.

Results from psychophysical testing in patients with central areolar choroidal dystrophy (CACD).

		Normal	Abnormal
ISCEV Full-field ERG (n = 23)	Photopic	20	3*
	Scotopic	23	0
Non-ISCEV Full-field ERG	Photopic	29	3
	Scotopic	31	1

Table 4.4. continued

Multifocal ERG (n = 17)	2 [†]	15 [‡]
ISCEV EOG (n = 16)	15	1 [§]
Non-ISCEV EOG (n = 33)	25	8 ["]
Color vision testing (n = 36)	9	27 [#] (75%)

ISCEV = International Society for Clinical Electrophysiology of Vision; ERG = electroretinography; EOG = electro-oculography.

*One patient with bilateral stage II CACD had an abnormal photopic ERG (defined as a response below 1% of the normal range: < 69 µV). Two patients, one with bilateral stage II CACD and one with bilateral stage III, had a unilaterally abnormal photopic full-field ERG. Of the remaining majority of patients with a normal photopic ERG, 22 eyes were in stage III or IV CACD.

[†]Patients with stage I and early stage II CACD.

[‡]Six patients were in stage II CACD. Severely decreased multifocal ERG responses were observed, especially in the parafoveal area. The most prominent decrease in central retinal response amplitude was seen in stage III and IV CACD. Three patients showed relative foveal sparing of the response amplitude.

[§]An abnormal ISCEV EOG is defined as having an Arden ratio below 2.0 in at least one eye.

["]An abnormal non-ISCEV EOG is defined as having an Arden ratio below 1.8 in at least one eye.

Six of these patients showed stage IV CACD and 2 patients showed stage III CACD.

[#]In this group, 14 patients showed a blue-yellow defect, 4 patients showed a red-green defect, and 9 patients showed a combined red-green/blue-yellow defect. All patients with a combined red-green/blue-yellow defect had stage III or IV CACD, whereas either a blue-yellow or a red-green defect could be observed in stage II, III, or IV.

Abbreviations: ISCEV, International Society for Clinical Electrophysiology of Vision; ERG, electroretinography; EOG, electro-oculography.

The results from full-field ERG, multifocal ERG, EOG and color vision testing are summarized in Table 4.4.

Mutation screening, clinical examination and haplotype analysis in four large families of CACD patients

We examined three large families with CACD patients with the p.Arg142Trp mutation (Families A-C). In addition, we examined the family of a patient carrying a p.Arg172Gln mutation (Family D), which has been previously described in patients with CACD-like macular dystrophy.^{5,15,31} Two of these four families did not have a positive family history for visual symptoms attributable to macular disease. The pedigrees of families A-D are depicted in Figure 4.7A and C. A summary of the clinical characteristics of the affected family members is given in Tables 4.5 and 4.6.

A striking variability of disease severity was observed (Fig. 4.8). The frequent occurrence of the p.Arg142Trp mutation in the patient cohort of this study, and the finding that the great majority of mutations carriers (96 of 98 patients, 98%) originated from the south-

Table 4.5.
Clinical characteristics of the patients carrying the p.Arg142Trp mutation in families A-C.

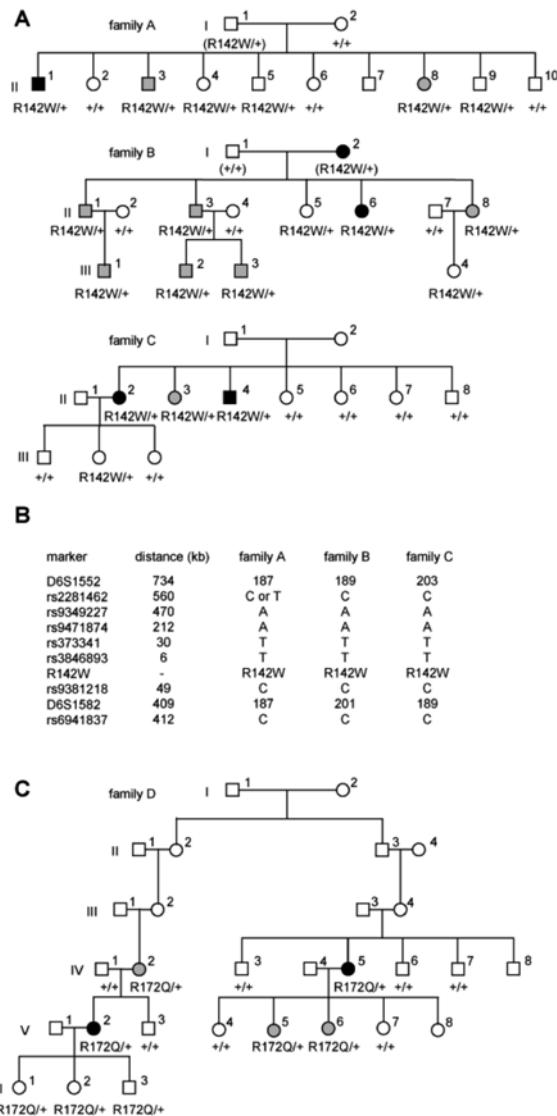
Patient	Age at onset (years)	Age at examination (years)	OD	Visual acuity OS	OD	Fundus	OS
A-II.1	44	59	0.8	0.025	1.0	Stage III	Stage IV (Fig 4.8A)
A-II.3	-	56	1.0		1.0	Stage II (Fig 4.8B)	Stage II
A-II.4	-	55	0.8		1.0	Normal	Normal
A-II.5	-	53	1.0		1.0	Stage I	Stage I
A-II.8	-	49	1.0		1.0	Stage II	Stage II
A-II.9	-	47	1.0		1.0	Normal	Normal
B-II.1	-	60	0.025 (trauma)	0.8	Extensive atrophy	Stage II	Stage II
B-II.3	-	58	0.8	0.8	Stage II	Stage II	Stage II
B-II.5	-	64	1.0	1.0	Normal (Fig 4.8C)	Normal	Normal
B-II.6	48	53	1.0	1.0	1.0	Stage II	Stage II
B-II.8	-	48	1.0	0.8	Stage II	Stage III	Stage III
B-III.1	-	32	1.0	1.0	Stage I	Stage I	Stage I
B-III.2	-	32	1.0	1.0	Stage II	Stage II	Stage II
B-III.3	-	30	1.0	1.0	Stage II	Stage II	Stage II
B-III.4	-	39	1.0	1.0	Normal	Normal	Normal
C-II.2	39	75	0.0167	0.1	Stage IV	Stage IV	Stage IV
C-II.3	-	73	0.8	0.9	Small atypical lesions (Fig 4.8D)	Small atypical lesions	Small atypical lesions
C-II.4	45	71	0.9	0.6	Stage III	Stage III	Stage III
C-II.2	-	50	1.0	1.0	Normal	Normal	Normal

Abbreviations: OD, right eye; OS, left eye.

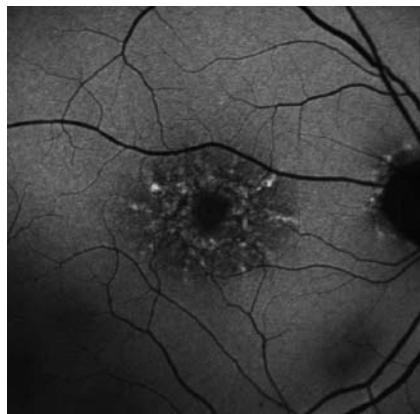
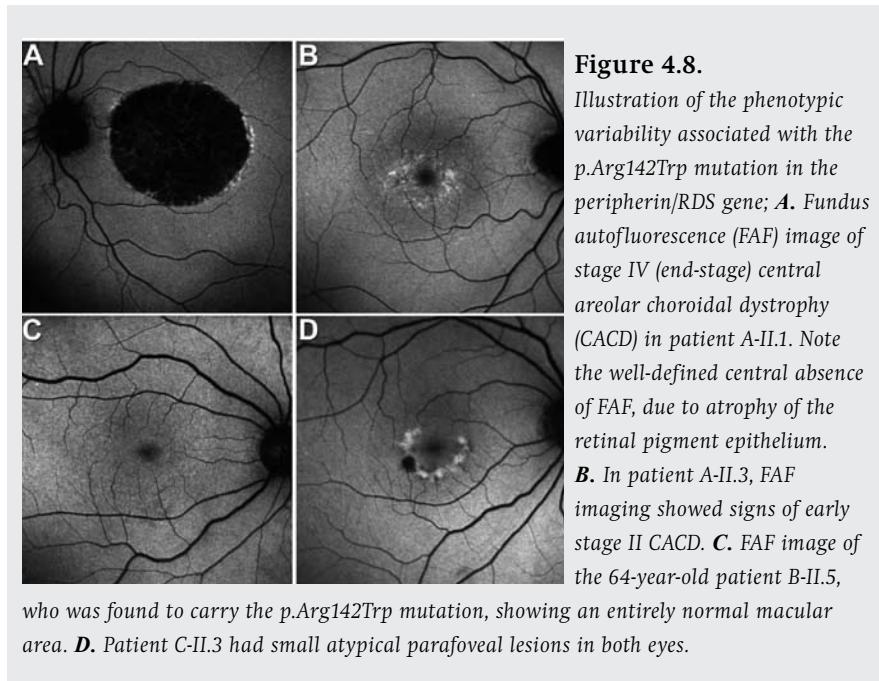
Table 4.6.
Clinical characteristics of patients carrying the p.Arg172Gln mutation in family D.

Patient	Age at onset (years)	Age at examination (years)	OD	Visual acuity OS	OD	Fundus	OS
D-IV.2	-	71	0.9	0.9	Normal		Small parafoveal pigmentary abnormalities
D-IV.2	36	45	0.6	0.6	Stage II		Stage II
D-IV.5	50	69	0.05 (macular hole)	0.0167 (amblyopia)	Stage III		Stage III
D-V.1	-	21	0.8	1.0	normal		Normal
D-V.2	-	19	1.0	1.0	normal		Normal
D-V.3	-	17	1.0	1.0	normal		Normal
D-V.5	-	35	1.0	1.0	Stage III		Stage III
D-V.6	-	36	0.8	1.0	Stage II		Stage II

Abbreviations: OD, right eye; OS, left eye.

**Figure 4.7.**

Molecular genetic analysis of the peripherin/RDS gene in four large families. **A.** Pedigrees of three families with the p.Arg142Trp mutation. **B.** Haplotype surrounding the p.Arg142Trp mutation in families A, B and C. The distance of each marker to the p.Arg142Gln mutation is shown in kilobases (kb). **C.** Pedigree of a family with the p.Arg172Gln mutation. Black symbols denote individuals with central areolar choroidal dystrophy (CACD) who noted visual disturbances. Grey symbols denote individuals who did not note visual disturbances, but showed macular abnormalities compatible with CACD on ophthalmic examination. Mutation carriers who did not note visual disturbances, and did not show macular abnormalities, are denoted with white symbols.



eastern part of the Netherlands, suggest that it is a founder mutation. Employing selected CA markers and SNPs near *peripherin/RDS*, we analysed several individuals of families A, B, and C (data not shown). The deduced at-risk haplotypes of three mutation carriers are depicted in Figure 4.7B. All three families share the same chromosomal segment of 519 kilobases encompassing the *peripherin/RDS* gene, which is highly suggestive of a common founder mutation.

The carrier frequency of the p.Arg142Trp mutation in this population was analyzed in 57 asymptomatic control individuals (of at least 70 years of age) from the same south-eastern region of the Netherlands. The mutation was identified in 1 of 57 (1.8 %) controls. This mutation carrier was a 76-year-old male, who did not have any visual disturbances, and neither did his parents nor his 11 siblings. Ophthalmoscopy and FAF imaging, however, clearly demonstrated early stage II CACD (Fig. 4.9).

Discussion

Phenotype

In CACD caused by an p.Arg142Trp mutation in the *peripherin/RDS* gene, our study found a mean age at onset of 46 years, which is consistent with previous studies on this mutation.^{4,9} The course of CACD caused by this mutation is variable: some patients never reach stage IV, III or even stage II CACD. In most CACD patients in our study, it takes more than a decade to progress from stage II to stage III (Table 4.2). The progression from stage III to stage IV lesions - when severe visual loss occurs - is somewhat faster, with a mean of 6.4 years (Table 4.2). The multifocal ERG shows abnormalities that are consistent with central cone dysfunction, especially in the parafoveal area. Such abnormalities on the multifocal ERG have also been noted in CACD caused by other *peripherin/RDS* mutations.⁶ Even in patients with advanced (stage IV) disease caused by the p.Arg142Trp mutation, the photopic and scotopic full-field ERG generally remain within the limits of normality. In contrast with p.Arg172Trp-related CACD (see below), the CACD phenotype associated with the p.Arg142Trp mutation appears to be largely confined to the macula, both ophthalmoscopically and electrophysiologically. This type of CACD may therefore be categorized as a central cone dystrophy. FAF imaging appears to be a useful tool in the diagnosis and follow-up of CACD. Small, subclinical lesions may be easily identified on FAF, and enlargement of the lesions and progression of chorioretinal atrophy can be closely monitored. Substantial changes within the same CACD stage may be noted on FAF imaging within 11 months of follow-up. In lesions with an area of profound chorioretinal atrophy (geographic atrophy), the mean enlargement of this area after a mean follow-up time of 18 months was 0.61 mm².

Genotype

The great majority of CACD patients with a p.Arg142Trp mutation in *peripherin/RDS* originated from the same southeastern region of the Netherlands. Analysis of CA markers and SNPs near the *peripherin/RDS* gene in three families carrying the p.Arg142Trp mutation revealed a shared chromosomal segment of at least 519 kb, strongly suggesting that this mutation represents a founder mutation. A founder effect was also found in a study on the p.Arg172Trp mutation, the most frequent *peripherin/RDS* mutation in British patients with autosomal dominant macular dystrophies.¹²

In our study, a striking variation in severity of the phenotype, as well as non-penetrance, were observed in several families in association with both the p.Arg142Trp

and p.Arg172Gln *peripherin/RDS* mutations. These variations could not be explained solely by the age of the patient and the stage of the macular dystrophy. Non-penetrance, defined as an absence of macular abnormalities on ophthalmoscopy, FAF, as well as on fluorescein angiography, in mutation carriers was observed up to 64 years of age, and this non-penetrance was seen in up to 21% of patients (4 out of 19 patients in Families A-C). Our study indicates that a considerable number of p.Arg142Trp mutation carriers may remain asymptomatic up to 76 years of age. Due to this non-penetrance and variable expression of mutations such as p.Arg142Trp, cases may appear sporadic or the inheritance pattern of CACD may appear autosomal recessive in some families. Molecular genetic analysis of the *peripherin/RDS* gene may facilitate genetic counseling in these cases. The p.Arg142Trp variant was found in one individual out of a group of 57 control persons beyond 70 years of age from the south-eastern region of the Netherlands. This 76-year-old asymptomatic person was shown to have early stage II CACD. This indicates that carriership of p.Arg142Trp may be quite frequent in this region, and that non-penetrance and/or a decreased penetrance of this mutation is even more frequent than the 21% estimated above.

Genotype-phenotype correlation

There are insufficient data to explain the broad phenotypic variation that is encountered in association with identical mutations in the *peripherin/RDS* gene.³²⁻³⁴ A study by Samardzija and colleagues indicates that sequence variants in the RPE65 gene may act as a modifier of light-damage susceptibility in mice with retinal degeneration.³⁵ An environmental influence on the phenotype may also be possible, but is probably of limited importance, as most patients in our study originated from the same geographic area and did not show notable differences in lifestyle.

The large majority of macular lesions encountered in carriers of the p.Arg142Trp mutation had the typical aspect of a certain stage of CACD, although the age at onset and the rate of progression were variable. In CACD, the mean age at onset of visual loss as well as the degree of photoreceptor dysfunction on full-field ERG is strongly influenced by the type of *peripherin/RDS* mutation. The onset of visual disturbances is usually before the age of 40 in CACD caused by the p.Arg172Trp or p.Arg195Leu mutation in *peripherin/RDS*. In p.Arg142Trp-related CACD, visual symptoms usually become manifest in the middle of the fifth decade.^{5,6,14,16} The p.Arg142Trp mutation is associated with a CACD phenotype without progression to panretinal cone-(rod) dystrophy. The p.Arg172Trp mutation was initially reported to cause a fairly constant phenotype resembling p.Arg142Trp-related CACD, but p.Arg172Trp was subsequently shown to be associated with a more widespread cone or cone-rod dystrophy.¹⁸ The p.Arg195Leu mutation also appears to cause panretinal cone or cone-rod dysfunction, in contrast to the p.Arg142Trp mutation in our study.^{6,16}

Pathophysiology

Several histopathological studies on CACD have been published.^{1,3,36} All studies showed an absence of photoreceptors, RPE and choriocapillaris in the area of atrophy, which is

in keeping with the clinical findings in CACD. In none of the aforementioned studies, information of the underlying genetic defect was available. The clinical observations and experimental studies suggest that choriocapillaris atrophy ensues from primary RPE atrophy.^{37,38} A histopathological study on a donor eye of a patient with advanced butterfly-shaped pattern dystrophy caused by a p.Cys213Tyr mutation in *peripherin/RDS* found a central area of complete photoreceptor and RPE cell loss, with lipofuscin-containing cells in the subretinal space, and an intact choriocapillaris.³⁹ In the area surrounding this zone of profound atrophy, RPE cells were distorted and greatly distended by lipofuscin, with the overlying photoreceptors being partly atrophic.

Experimental studies on transgenic mice carrying the p.Arg172Trp mutation indicate that this mutation exerts a dominant negative effect on photoreceptors, especially cones, and that the severity of the phenotype depends on the degree of expression of the mutant allele.^{40,41} In this mouse model, genetic supplementation of wild-type *peripherin/rds* provided a partial, long-term rescue of rod function, but no permanent rescue of cone function.⁴² It is tempting to speculate that the p.Arg142Trp mutation exerts its detrimental effect through the same dominant-negative mechanism as the p.Arg172Trp mutation. A slightly less pathogenic effect might explain why this mutation primarily affects the dense, central cone population, with relative sparing of the more peripherally located photoreceptor elements.

We propose the following pathophysiological sequence of CACD associated with the p.Arg142Trp mutation in the *peripherin/RDS* gene. The amino acid substitution of a relatively large, positively charged, arginine residue to a nonpolar and hydrophobic tryptophan residue probably has a disturbing effect on *peripherin/rds* protein structure, resulting in dysmorphic cone and rod outer segments. The disturbance of normal photoreceptor function and extracellular matrix interaction leads to alterations in the normal photoreceptor outer segment - RPE interface and results in an increase in the phagocytosis of these abnormal outer segments by RPE cells.^{41,43,44} This in turn raises the level of lipofuscin and toxic by-products in the RPE, which is reflected in the findings on FAF, resulting in RPE and photoreceptor cell death through apoptosis.^{45,46} Finally, when atrophy of the choriocapillaris occurs,^{37,38} the typical ‘punched-out’ CACD lesion arises.

Central areolar choroidal dystrophy versus atrophic age-related macular degeneration

CACD and other *peripherin/RDS*-related macular dystrophies share clinical characteristics with atrophic AMD, such as geographic atrophy and drusen-like deposits.^{17,31,47,48} Our study also indicates that there may be an overlap in the age at onset of CACD and atrophic AMD. CACD in the elderly patient may be confused with AMD, especially in cases with low penetrance. This is illustrated by mutation analysis of *peripherin/RDS* in a group of 21 patients in our hospital that were categorized as having “early-onset AMD”. Three of these patients carried the p.Arg142Trp mutation in *peripherin/RDS*, and were subsequently re-diagnosed as having CACD. On the other hand, familial cases of AMD are not uncommon, and may mimic an autosomal dominant pattern of inheritance.

Several features may help to distinguish CACD from atrophic AMD. In the early stages of CACD, an oval area of hypopigmentation is observed in the macula. In contrast, early age-related maculopathy usually displays either dispersed hard and/or soft drusen clustered in the macula, or a parafoveal band of reticular hyperpigmentation. Geographic atrophy in AMD usually emanates from large, confluent atrophic drusen or from the parafoveal band of hyperpigmentation.⁴⁷ The area of geographic atrophy is often surrounded by residual drusen or smaller satellite geographic lesions in these cases. Unlike CACD, the geographic atrophy in end-stage atrophic AMD is often bordered by a band of hyperpigmentation. On FAF, geographic atrophy in atrophic AMD shows a median enlargement of 1.52 mm² per year.⁴⁹ This is significantly higher than the 0.61 mm² enlargement per 18 months in the CACD patients in our study. Drusen-like changes that may be observed especially at the edge of CACD lesions show intense FAF, whereas basal laminar drusen caused by *CFH* gene mutations and drusen in AMD generally do not display intense FAF.^{50,51} This probably reflects a difference in the pathogenesis and composition of these drusen-like lesions, despite the superficial similarity on ophthalmoscopy. Choroidal neovascularisation is rare in CACD, as it was observed in only one highly myopic patient in the present study.

Peripherin/RDS mutations probably do not play an important role in AMD, but studies on the prevalence of *peripherin/RDS* mutations in patients with atrophic AMD are sparse.⁵² The finding of a p.Arg142Trp *peripherin/RDS* mutation in 1 out of 57 asymptomatic control persons over the age of 75 years, originating from the south-eastern region of the Netherlands, also indicates that the carrier frequency of the mutation in this region may be quite high. Future studies may be directed towards investigating the incidence of *peripherin/RDS* mutations in patients with (CACD-like) atrophic AMD.

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4.3. Mutations in the *peripherin/RDS* gene are an important cause of multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus

Abstract

Objectives: To describe the phenotype and to analyse the *peripherin/RDS* gene in 10 unrelated families with multifocal pattern dystrophy simulating Stargardt disease (STGD1).

Methods: The probands of 10 families and 20 affected family members underwent an ophthalmic examination, including dilated fundus examination, fundus autofluorescence imaging and optical coherence tomography (OCT). In all probands and in selected family members, fluorescein angiography, electrophysiological testing and visual field analysis were performed. Blood samples were obtained from affected and unaffected family members for analysis of the *peripherin/RDS* gene.

Results: All 10 probands carried mutations in the *peripherin/RDS* gene. Nine different mutations were identified, including six mutations that were not described previously. All probands showed a pattern dystrophy with yellow-white flecks in the posterior pole that strongly resembled the flecks seen in STGD1, on ophthalmoscopy as well as on autofluorescence and OCT. Clinical findings in the family members carrying the same mutation as the proband were highly variable, ranging from no visible abnormalities to early-onset retinitis pigmentosa.

Conclusions: Mutations in the *peripherin/RDS* gene are the major cause of multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. This autosomal dominant disorder should be distinguished from autosomal recessive STGD1, in view of the different inheritance pattern and the overall better visual prognosis.

Introduction

The autosomal dominant pattern dystrophies constitute a group of disorders characterized by deposits of yellow, orange or grey pigment, predominantly in the macular area. In general, these disorders are relatively benign, manifesting usually in midlife with mild to moderate disturbance of central vision.¹⁻⁴ Nevertheless, severe vision loss may occur in up to 50% of the affected individuals after the age of 70, due to atrophy of the retinal pigment epithelium (RPE)-photoreceptor complex and/or the development of choroidal neovascularization.⁵⁻⁹ In addition, progression to a more widespread retinal dystrophy with characteristics of retinitis pigmentosa may develop, depending on the underlying genotype.^{10,11} In the classification according to Gass, five main categories of pattern dystrophy are discriminated, based on the pattern of pigment distribution: adult-onset foveomacular vitelliform dystrophy, butterfly-shaped pigment

dystrophy, reticular dystrophy of the retinal pigment epithelium, multifocal pattern dystrophy simulating fundus flavimaculatus, and fundus pulverulentus.¹¹ A striking inter- and intrafamilial phenotypic variability has been described by several authors, and different subtypes of pattern dystrophy have even been reported in the individual patient.^{4-7,10,12-22,22-24} Pattern dystrophy is genetically heterogeneous, but mutations in the *peripherin/RDS* gene are frequently encountered.^{5,11,23,25-28} Weleber and co-workers reported pattern dystrophy, retinitis pigmentosa and fundus flavimaculatus in a single family with family members carrying the same *peripherin/RDS* mutation.²⁹ Besides pattern dystrophy and autosomal dominant retinitis pigmentosa, *peripherin/RDS* mutations have been associated with central areolar choroidal dystrophy, cone-rod dystrophy, as well as digenic retinitis pigmentosa.^{11,25-27} The gene product of *peripherin/RDS* is the integral membrane protein peripherin/rds, which plays an important role in photoreceptor outer segment morphogenesis.³⁰⁻³² In this study, we describe the clinical findings and the underlying genetic defects of patients in 10 unrelated families with multifocal pattern dystrophy resembling Stargardt disease (STGD1)/fundus flavimaculatus.

Methods

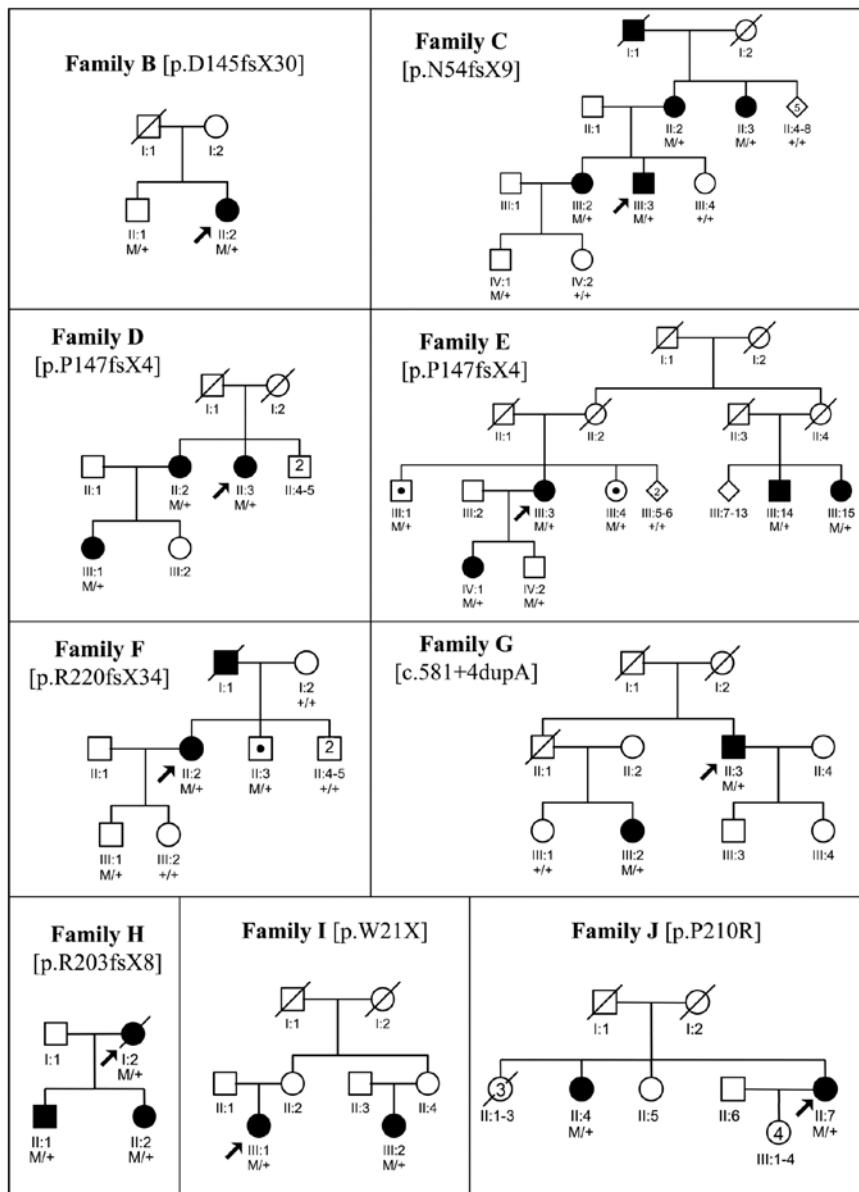
The research described in this study conformed to the tenets of the Declaration of Helsinki and was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Centre (Nijmegen). Informed consent was obtained from all participants before enrolling them in the clinical and molecular genetic studies.

Molecular genetic studies

Peripheral venous blood samples were obtained from the 10 probands and from 39 family members. Their genomic DNA was isolated as described elsewhere.³³ The three coding exons and splice junctions of the *peripherin/RDS* gene were amplified by the polymerase chain reaction (PCR), and these fragments were then subjected to sequence analysis to detect mutations.²³

Clinical studies

We examined 10 probands from 10 different families with multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus (Fig. 4.10). Seven of these patients had received the diagnosis multifocal pattern dystrophy in the past. Three of the 10 probands (E, G and H) were identified retrospectively when a group of 15 presumed STGD1 patients without *ABCA4* mutations were analysed for *peripherin/RDS* mutations. The medical histories of the 10 probands were obtained. Subsequent clinical examination included best-corrected Snellen visual acuity, indirect ophthalmoscopy and fundus photography. In addition, fundus autofluorescence (FAF) imaging (Heidelberg Retina Angiograph (HRA) 2, Heidelberg Engineering, Dossenheim, Germany) was performed in all probands using



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Figure 4.10.

Pedigrees of the examined families. The probands with multifocal pattern dystrophy are indicated with an arrow. The specific mutations have been put between brackets and are indicated by an "M" within the pedigree. Mutation carriers who showed only mild foveal abnormalities, without signs of multifocal pattern dystrophy, are indicated with a black dot. The pedigree of proband A (*p.Asp157Asn*) is not shown, as he was the only person of his family who was clinically affected and who was examined.

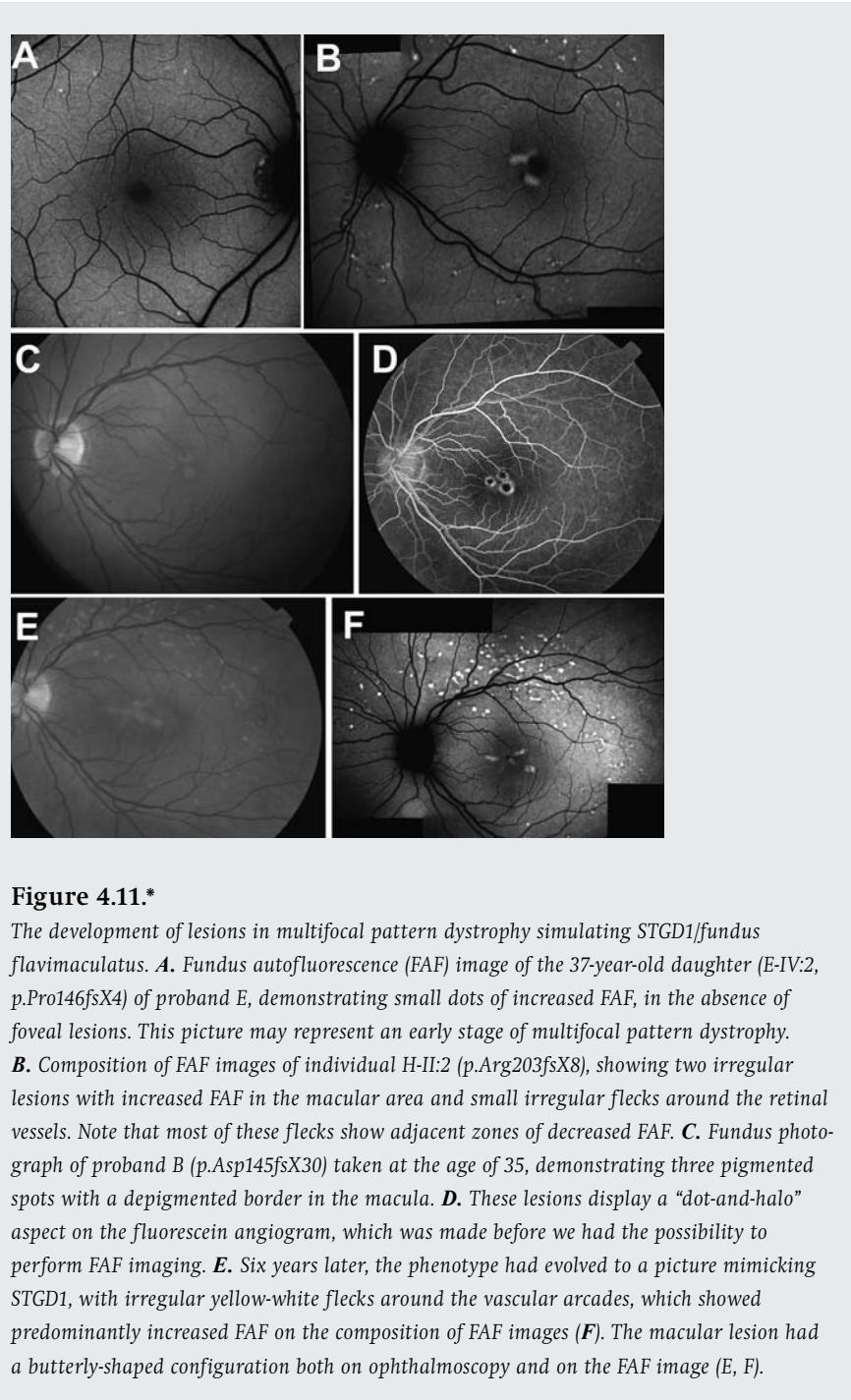


Figure 4.11.*

The development of lesions in multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus. **A.** Fundus autofluorescence (FAF) image of the 37-year-old daughter (E-IV:2, p.Pro146fsX4) of proband E, demonstrating small dots of increased FAF, in the absence of foveal lesions. This picture may represent an early stage of multifocal pattern dystrophy. **B.** Composition of FAF images of individual H-II:2 (p.Arg203fsX8), showing two irregular lesions with increased FAF in the macular area and small irregular flecks around the retinal vessels. Note that most of these flecks show adjacent zones of decreased FAF. **C.** Fundus photograph of proband B (p.Asp145fsX30) taken at the age of 35, demonstrating three pigmented spots with a depigmented border in the macula. **D.** These lesions display a "dot-and-halo" aspect on the fluorescein angiogram, which was made before we had the possibility to perform FAF imaging. **E.** Six years later, the phenotype had evolved to a picture mimicking STGD1, with irregular yellow-white flecks around the vascular arcades, which showed predominantly increased FAF on the composition of FAF images (**F**). The macular lesion had a butterfly-shaped configuration both on ophthalmoscopy and on the FAF image (E, F).

a previously described protocol,³⁴ as well as optical coherence tomography (Stratus OCT, Carl Zeiss Meditech, Inc., Dublin, CA, USA), using the “Macular Thickness Map” protocol. The probands also underwent fluorescein angiography and visual field analysis, by means of Goldmann perimetry (stimuli V4e-III4e-I4e-I3e-I2e-I1e) and Humphrey 10-2 Swedish Interactive Thresholding Algorithm (SITA)-Fast visual field analysis of the central 10° of the visual field, except for patient 8 and 10 who were unavailable for visual field analysis. Full-field electroretinography (ERG) and an electro-oculography (EOG) were performed in all probands according to the guidelines of the International Society for Clinical Electrophysiology of Vision (ISCEV), except for proband H, in whom the ERG and EOG were recorded according to the protocol by Thijssen et al.³⁵ Additionally, we examined the 20 family members who carried the same mutation as the proband of that family. The medical history was obtained in all of these 20 family members, and a clinical examination was performed including fundus photography, FAF imaging, and optical coherence tomography. Six affected family members underwent full-field ERG and EOG according to the ISCEV standards, whereas one patient underwent these examinations according to the previously mentioned protocol.³⁵

Results

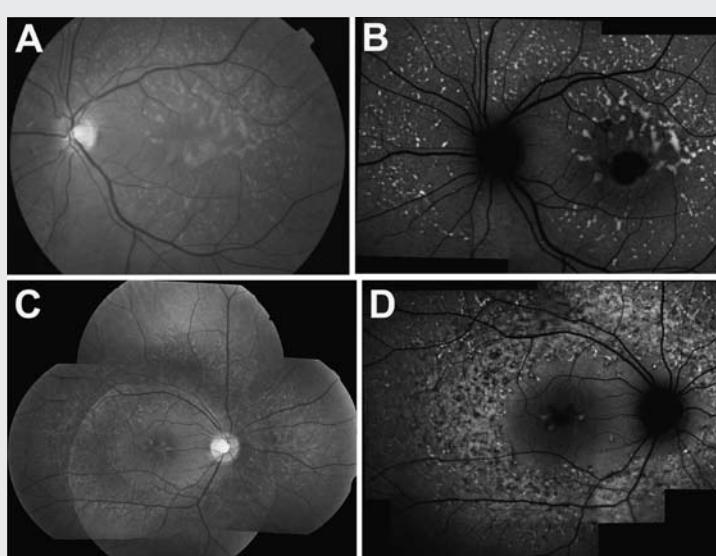
Molecular genetic findings

All 10 probands carried mutations in the *peripherin/RDS* gene. Nine different mutations were identified, including five different frameshift mutations, two missense mutations and one nonsense mutation (Table 4.7). Six of these mutations (p.Asp145fsX30, p.Asn54fsX9, p.Arg220fsX34, c.581+4dupA, p.Arg203fsX8, and p.Trp21X) have not been described previously. The splice site mutation c.581+4dupA was detected in proband G. This mutation is predicted to completely abolish splicing.³⁶ The six novel mutations were not found in 92 control individuals. The frameshift mutation p.Pro147fsX4 was identified in two unrelated probands (probands D and E). Of the 39 additional family members that were screened for the *peripherin/RDS* mutation that was found in the proband, 20 family members were shown to also carry the same mutation as the proband (Table 4.8).

Clinical findings

The clinical characteristics of the probands are summarized in Table 4.7. The mean age at onset was 45 (range: 34-55 years). The initial symptoms were metamorphopsia (6 patients), loss of visual acuity (4 patients), central scotomata (3 patients), and night blindness (5 patients). Seven probands reported additional family members with similar visual complaints. All probands retained reading vision in at least one eye, except for proband G, a 73-year-old male (c.581+4dupA). This patient experienced visual loss with metamorphopsia at age 45, in combination with night blindness. He lost the ability to read approximately 21 years later, at the age of 66 years.

All patients showed irregular yellowish flecks in the posterior pole at a certain point in time, resembling the flecks seen in STGD1. These flecks were also found in variable

**Figure 4.12.***

Advanced multifocal pattern dystrophy. **A.** Fundus photograph of proband F (p.Arg220fsX54), showing numerous yellowish flecks that are scattered throughout the posterior pole, with the larger flecks situated around a central area of well-circumscribed chorioretinal atrophy. **B.** A fundus autofluorescence (FAF) image shows increased FAF, corresponding to the yellow lesions on ophthalmoscopy, together with a complete absence of FAF in the central atrophic area. **C.** A composition of fundus photographs of proband A (p.Asp157Asn) demonstrated a central lesion typical for pattern dystrophy. A large ring-shaped, atrophic-appearing area can be seen, surrounding the macular area and optic disc. **D.** These lesions could be delineated with more detail on a composition of FAF images. The ring-shaped zone showed confluence of the flecks. Granular zones of decreased FAF were seen within this area of increased FAF, which reflects the beginning atrophy of the retinal pigment epithelium. Towards the periphery, the flecks became less confluent and could be identified individually.

size, shape and number in several asymptomatic family members who carried the same mutation as the proband of their family (Fig. 4.11). The flecks were mostly situated around the retinal vascular arcades, nasal and superior to the optic disc and in the macular area, where flecks were usually largest (Fig. 4.11 and 4.12). Probands A, C, D, G, I and J showed a variable degree of confluence and atrophy of the STGD1-like flecks over a period of probably more than 5 years, based on the comparison of consecutive fluorescein angiograms (Fig. 4.12 and 4.13). Besides these flecks, all patients displayed macular changes, ranging from a variety of patterns of yellow or greyish deposits in the foveomacular area, to atrophic lesions in proband F (p.Arg220fsX34) (Fig. 4.12 and 4.13).

Typical “dot-and-halo” lesions were observed in patient B (p.Asp145fsX30) (Fig. 4.11). The central “dot-and-halo” lesions in the left eye gradually developed into a butterfly-shaped pattern dystrophy of the macula (Fig. 4.11). None of the patients in this study developed subretinal neovascularization. Four patients (probands C, D, E and G) demonstrated pigmentary changes in the peripheral retina. All four patients suffered from a variable degree of night blindness. Typical retinitis pigmentosa characteristics were observed only in proband D (Table 4.7).

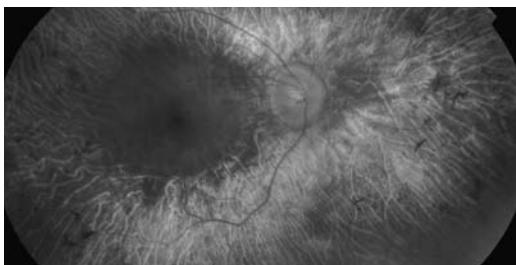


Figure 4.13.*

Fundus photograph of patient E-III:14 (p.Pro147fsX4 mutation), showing a characteristic retinitis pigmentosa phenotype.

On fluorescein angiography, the flecks were hyperfluorescent in the early and late phase of the examination, sometimes with a central hypofluorescent spot. None of the patients displayed a so-called “dark choroid” on fluorescein angiography. On FAF imaging, the flecks showed a highly increased FAF, often with small adjacent zones of decreased FAF (Fig. 4.11 and 4.12). Lesions that were yellowish and seemed to contain a lipofuscin-like substance on ophthalmoscopy, mostly corresponded with an increased FAF signal. On OCT, some of the STGD1-like flecks appeared as a highly reflective focal thickening of the hyperreflective “outer red line” (ORL), which corresponds to the photoreceptor/retinal pigment epithelium complex.³⁷ Macular lesions showed thickening of the ORL, with a variable increase in underlying reflectivity.

Full-field ERG results varied from normal to nonrecordable (Table 4.7). The photopic ERG was abnormal to nonrecordable in all patients with confluence of the yellowish flecks. The EOG also varied largely, from a normal to absent light rise, and was abnormal in 11 of 20 eyes. Humphrey 10-2 SITA-Fast analysis of the central 10° of the visual field showed normal to near-normal results in 4 cases (probands A, B, C and E), whereas proband D, F and G displayed a severely decreased sensitivity in both eyes. Proband I showed a decrease in sensitivity at the inferior and temporal edges of the central visual field of both eyes. Goldmann perimetry was normal only in proband F, whereas the other patients showed a mild to marked constriction of the peripheral visual field.

Twenty family members in whom the same mutation as in the proband was found, were examined (Table 4.8). Nine of these individuals experienced visual disturbances. The genetically affected family members of probands C, D, H, G and I all showed the phenotype of multifocal pattern dystrophy, except for patient C-IV:1, who was 34 years of age. Other phenotypes were observed in families B, E, F and J (Table 4.8), although

these individuals carried the same mutation as the proband with multifocal pattern dystrophy. Three family members showed no retinal abnormalities despite carrying a pathologic *peripherin/RDS* mutation.

Discussion

The phenotype of the multifocal pattern dystrophy described in this report is caused by autosomal dominant inheritance of mutations in the *peripherin/RDS* gene, with a variable expressivity and decreased penetrance. The type and distribution of the flecks in this pattern dystrophy strongly resembled the flecks observed in the fundus flavimaculatus phenotype of STGD1, an autosomal recessive retinal dystrophy caused by mutations in the *ABCA4* gene.⁴¹ This resemblance was evident on ophthalmoscopy as well as on FAF and OCT.⁴²⁻⁴⁶

All probands demonstrated a variable number of irregularly shaped yellow-white flecks scattered throughout the posterior pole and around the retinal vascular arcades. These yellowish flecks were preceded either by typical macular pattern dystrophy or by non-specific pigmentary changes in the fovea. The lesions were best defined by FAF imaging. Discrete retinal abnormalities were also easily identified with this technique in several asymptomatic family members carrying the same *peripherin/RDS* mutation as the proband. Extensive atrophy of the central retina tends to occur with increasing age, as demonstrated by the four probands who were over 60 years of age. The fact that multifocal pattern dystrophy, like many other ‘macular’ dystrophies, may involve the peripheral retina is demonstrated by the abnormal photopic and scotopic ERGs in almost half of the probands and an abnormal EOG in the majority of the probands.

Of the nine different mutations that were identified, there were five different frameshift mutations and one nonsense mutation, which led to a premature termination by a stop codon. This may lead to either a shorter protein product or it may induce degradation of the mRNA by nonsense-mediated decay. The mechanism of pathogenicity of the mutation may be haploinsufficiency in case of the frameshift mutations, the nonsense mutation and the splice site mutation. In case of the two missense mutations that were found, a dominant negative effect may also be possible, although there is plausible evidence that these mutations may also lead to haploinsufficiency.^{47,48}

The frameshift mutation p.Pro147fsX4 has been previously described in patients with central areolar choroidal dystrophy.³⁹ This p.Pro147fsX4 mutation was found in two unrelated probands, patients D and E. Both patients had multifocal pattern dystrophy simulating STGD1, but in patient D the phenotype progressed to a panretinal dystrophy resembling retinitis pigmentosa, with widespread involvement of both the rod and cone system. The missense mutation p.Asp157Asn has been reported previously in patients with pattern dystrophy,³⁸ whereas the p.Pro210Arg missense mutation was found in patients with adult-onset foveomacular vitelliform dystrophy.^{27,40}

In our study, a genotype-phenotype correlation could not be established. The present study again demonstrates the highly divergent phenotypes in family members who

Table 4.7.
Summary of clinical findings in the probands with multifocal pattern dystrophy.

Proband number	Sex	Age at onset (years)	Age (years)	Vistual acuity OD	Vistual acuity OS	ERG photopic* OD	ERG photopic* OS	ERG scotopic* OD	ERG scotopic* OS	EOG [†]	Macula	Peripheral retina	Mutation	Effect	Previously described
A	m	34	43	1.2	1.2	SA	A	A	N	2.6	Pattern of multiple yellow lesions (Fig. 4.12)	No abnormalities	c.469G>A	p.Asp157Asn	Yes ³⁸
B	f	35	41	0.9	0.8	A	SA	N	N	1.5	OD: multiple pigmented round lesions OS: butterfly-shaped pattern dystrophy (Fig. 4.11)	No abnormalities	c.433_434delGA	p.Asp145fsX30	No
C	m	47	56	1.2	1.0	SA	SA	SA	SA	1.7	Atrophic confluence of yellow-white lesions, surrounded by STGD1-like lesions	Discrete midperipheral granular pigmentary changes	c.163delT	p.Asn54fsX9	No
D	f	50	61	0.8	0.025	SA	SA	SA	SA	1.0	Extensive chorioretinal atrophy	(Mid-)peripheral atrophy, bone spicule and paravenous pigmentation, narrow vasculature, temporal paleness of optic disc	c.441delT	p.Pro147fsX4	Yes ³⁹

Table 4.7. continued

Proband number	Sex	Age at onset (years)	Age (years)	Visual acuity OD OS	ERG photopic* OD OS	ERG scotopic* OD OS	EOG [†] OD OS	Macula	Peripheral retina	Mutation	Effect	Previously described
E	f	48	57	1.0 0.9	N N	N A	2.0 2.0	Confluent atrophic yellow-white lesions, surrounded by STGD1-like flecks	Peripheral atrophy, reticular pigmentary pattern	c.441delT p.Pro147fsX4	Yes ³⁹	
F	f	45	51	0.5 1.0	N N	N A	2.0 1.7	Round area of chorioretinal atrophy, surrounded by STGD1-like flecks (Fig. 4.12)	No abnormalities	c.658delC p.Arg220fsX34	No	
G	m	45	73	0.05 0.05	NR NR	NR NR	2.8 2.6	Extensive chorioretinal atrophy (Fig. 4.13)	Peripheral atrophy, OD perivascular pigmentation	c.581+4dupA	Splice defect	No
H	f	48	65	0.017 0.4	N [#] N [#]	N [#] N [#]	1.7 [#] 1.9 [#]	Central atrophy, surrounded by STGD1-like yellowish lesions	No abnormalities	c.607_620delC GGTACCT GGTGGA	p.Arg203fsX8	No
I	f	44	55	0.6 0.8	SA SA	N N	1.5 1.4	Atrophic confluence of yellow-white lesions, surrounded by STGD1-like lesions	No abnormalities	c.63G>A p.Trp21X	No	

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ERG results are expressed in abbreviations that reflect the amplitude: N, normal (equal to or above the lower 5% of the range for a normal population; photopic $\geq 78 \mu V$, scotopic $\geq 263 \mu V$); A, abnormal (1-5% of normal range; photopic: $\geq 69 \mu V$ and $< 78 \mu V$; scotopic: $\geq 195 \mu V$ and $< 263 \mu V$); SA, severely abnormal (below 1% of normal range).

photopic < 69 μ V, scotopic < 195 μ V); NR, nonrecordable.

Values and Andean nations normal if > 20 non ISCEV normal if > 18

Wydawane przez Wydawnictwo Naukowe PWN w Warszawie, ul. Banacha 22, 00-006 Warszawa, w ramach programu "Naukowe Wydawnictwa PWN".

[†] Recorded prior to ISCEV regulations according to the protocol described by

AAbbreviations: ERG phot photometric ERG; ERG scot scotopic ERG; NI normal

Table 4.8.
Summary of the clinical findings in family members with the same *peripherin/RDS* mutation as the probands.

Family	Pedigree number	Mutation	Age at onset (years)	Age at (years)	Visual acuity OD	Visual acuity OS	Retinal phenotype	ERG photopic* OD OS	ERG scotopic* OD OS	ERG OD OS	EOG†
B	II:1	p.Asp145fsX30	-	39	1.2	1.0	ODS: normal	-	-	-	-
C	II:2	p.Asn54fsX9	70	85	0.017	0.0033	ODS: extensive atrophy of posterior pole, midperipheral yellowish flecks	-	-	-	-
	II:3	p.Asn54fsX9	-	80	0.9	1.0	ODS: macular pattern dystrophy changes, non-confluent STGD1-like flecks around vascular arcades	-	-	-	-
	III:2	p.Asn54fsX9	56	57	1.2	1.2	ODS: macular pattern dystrophy changes, STGD1-like flecks around vascular arcades with incipient confluence	N	N	N	2.1 2.9
	IV:1	p.Asn54fsX9	-	34	1.2	1.0	OS: normal, OD: discrete spot of parafoveal pigmentary changes	-	-	-	-
D	II:2	p.Pro147fsX4	60	67	0.8	0.9	ODS: confluence of STGD1-like lesions and patches of atrophy in posterior pole, OD slight peripheral pigmentary changes	N	N	N	-
	III:1	p.Pro147fsX4	-	41	0.9	0.9	ODS: macular pattern dystrophy changes, STGD1-like flecks around vascular arcades	N	N	N	1.8 1.6
E	III:1	p.Pro147fsX4	-	50	1.2	1.2	ODS: mild foveal pigmentary changes	-	-	-	-
	III:4	p.Pro147fsX4	-	59	0.017	0.8	ODS: mild foveal pigmentary changes, reticular pattern of pigmentary changes in peripheral retina	-	-	-	-
	III:14	p.Pro147fsX4	12	60	0.2	0.2	ODS: extensive chorioretinal atrophy outside macular area, bone spicule pigmentation, narrow retinal vessels, waxy pale optic disc	SA	SA	SA	1.0 1.0

	III:15	p.Pro147fsX4	35	65	0.6	0.6	ODS: multifocal pattern dystrophy, yellow-white flecks throughout posterior pole	-	-	-	-	-
IV:1	p.Pro147fsX4	-	37	1.2	1.0	1.0	ODS: discrete foveal pigmentary changes, small yellow-white dots around retinal vascular arcades	-	-	-	-	-
IV:2	p.Pro147fsX4	-	32	1.6	0.0033 (trauma)	ODS: normal	-	-	-	-	-	-
F	II:3	p.Arg220fsX34	43	45	1.6	1.6	ODS: small yellowish lesions in fovea, similar to lesions seen initially in proband B (Fig. 4.11)	-	-	-	-	-
	III:1	p.Arg220fsX34	-	29	1.0	1.0	ODS: normal	-	-	-	-	-
H	II:1	p.Arg203fsX8	-	49	1.2	1.0	ODS: small yellowish lesions in fovea, small STGDI-like flecks around vascular arcades	N	A	N	2.0	1.6
II:2	p.Arg203fsX8	-	45	0.9	0.8	ODS: small yellowish lesions in fovea, small STGDI-like flecks around vascular arcades (Fig. 4.11)	N	N	N	1.8	1.6	
G	III:2	c.581+4dupA	49	50	0.8	0.8	ODS: multifocal pattern dystrophy, atrophic confluence of STGDI-like flecks	-	-	-	-	-
I	III:2	p.Trp21X	30	30	0.8	0.5	ODS: small yellow lesions with pigmented centre in foveal area, similar to lesions seen in proband B, many STGDI-like flecks around vascular arcades	-	-	-	-	-
J	II:4	p.Pro210Arg	52	65	1.0	0.3	OD: butterfly-shaped pattern dystrophy, OS: foveal atrophy surrounded by pigmentary changes	N [‡]	N [‡]	A [‡]	1.5 [‡]	1.5 [‡]

*ERG results are expressed in abbreviations that reflect the amplitude: N, normal (equal to or above the lower 5% of the range for a normal population; photopic $\geq 78 \mu\text{V}$, scotopic $\geq 263 \mu\text{V}$); A, abnormal (1.5% of normal range; photopic: $\geq 69 \mu\text{V}$ and $< 78 \mu\text{V}$; scotopic: $\geq 195 \mu\text{V}$ and $< 263 \mu\text{V}$); SA, severely abnormal (below 1% of normal range; photopic $< 69 \mu\text{V}$, scotopic $< 195 \mu\text{V}$); NR, nonrecordable.

[†]Values are Arden ratios: ISCEV: normal if ≥ 2.0 , non-ISCEV: normal if ≥ 1.8 .

[‡]Recorded prior to ISCEV regulations according to the protocol described by Thijssen et al.³⁵

Abbreviations: ERG phot, photopic ERG; ERG scot, scotopic ERG; NI, normal.

carry identical *peripherin/RDS* mutations. The retinal abnormalities range from minor foveal abnormalities to multifocal pattern dystrophy simulating STGD1 and even retinitis pigmentosa. This remarkable clinical heterogeneity precludes a straightforward genotype-phenotype correlation even with larger patient series.^{5,8,10,12,16,18,20,29,49} Obviously, modifying factors besides the specific mutation in the *peripherin/RDS* gene exert an important influence on the resulting phenotype.

Zhang and co-workers reported the histopathological findings in a patient with butterfly-shaped pigment dystrophy with Stargardt-like flecks caused by a p.Cys213Tyr substitution in *peripherin/RDS*.^{8,21} Adjacent to a central area of atrophy, the RPE cells were greatly distended by lipofuscin. This is in accordance with the predominantly increased FAF signal of the lesions described in the present study. The same accumulation of lipofuscin in RPE cells has been demonstrated for STGD1.⁵⁰⁻⁵² It is likely that the lipofuscin accumulation in multifocal pattern dystrophy is less pronounced, in view of the better visual prognosis as well as the absence of choroidal blockage on the fluorescein angiogram. The small adjacent zones with a decreased FAF signal, that were seen in association with the majority of the STGD1-like flecks, are probably caused by RPE atrophy. This is reflected in the predominant hyperfluorescence of the flecks on the fluorescein angiogram. As the disease progresses, the flecks tend to evolve towards a confluent atrophic area which shows larger zones of decreased FAF intensity. The multifocal nature of this pattern dystrophy may be explained by local differences in cell interactions, distribution, morphology and metabolism.⁵³

Autosomal dominant multifocal pattern dystrophy should not be confused with autosomal recessive STGD1, as well as infrequent autosomal dominant retinal disorders such as STGD3 (*ELOVL4* gene) and STGD4.⁵⁴⁻⁵⁶ Clinical findings that may help to distinguish this pattern dystrophy from STGD1 are the autosomal dominant pattern of inheritance, the relatively late age at onset (fifth decade), the comparatively good and stable visual acuity and the absence of a “dark choroid”. However, the incomplete penetrance (10% in this study) and the variable expression may mask the dominant inheritance pattern. Conversely, due to the high carrier frequency of *ABCA4* mutations, STGD1 patients may have relatives with some form of retinal dystrophy.⁵⁷ Three of the probands in this study received the proper diagnosis only after analysis of the *peripherin/RDS* gene in a group of 15 patients with presumed STGD1, but without detectable mutations in the *ABCA4* gene. Therefore, an analysis of the *peripherin/RDS* gene should be considered in absence of *ABCA4* mutations, especially when confronted with the aforementioned phenotype.

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Chapter 5

Clinical and molecular genetic analysis of phenotypes associated with variants in the *CFH* gene

Section 5.1 is adapted from:

The spectrum of phenotypes caused by variants in the *CFH* gene. **C.J.F. Boon**, N.C. van de Kar, B.J. Klevering, J.E.E. Keunen, F.P.M. Cremers, C.C.W. Klaver, C.B. Hoyng, M.R. Daha, A.I. den Hollander.

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Basal laminar drusen caused by compound heterozygous variants in the *CFH* gene. **C.J.F. Boon**, B.J. Klevering, C.B. Hoyng, M.N. Zonneveld-Vrieling, S.B. Nabuurs, E. Blokland, F.P.M. Cremers, A.I. den Hollander.

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5.1. The spectrum of phenotypes associated with variants in the *CFH* gene

Abstract

Complement factor H (CFH) is a complement inhibitor, which is present as a soluble protein and attached to cell surfaces throughout the human body. As such, CFH is a key player in complement homeostasis, inhibiting excessive activation of the complement cascade, with an emphasis on the alternative pathway. The significance of CFH is demonstrated by the broad range of phenotypes associated with specific *CFH* gene variants. This phenotypic spectrum includes renal phenotypes, such as membranoproliferative glomerulonephritis and atypical hemolytic uremic syndrome, as well as ocular phenotypes, such as basal laminar drusen and age-related macular degeneration. In addition, several overlapping phenotypes have been described in association with *CFH* gene variants. The phenotypic outcome of these *CFH* variants depends on their differential impact on plasma- and surface-bound CFH function. Consequently, distinct genotype-phenotype correlations may be observed.

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Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; AP, alternative pathway; BLD, basal laminar drusen; CCP, complement control protein; CFH, Complement Factor H; CFI, Complement Factor I; CRP, C-reactive protein; CR1, complement receptor 1; DAF, decay accelerating factor; FHL-1, Factor H-like protein 1; GAG, glycosaminoglycan; GBM, glomerular basal membrane; HUS, hemolytic uremic syndrome; MCP, membrane co-factor protein; MPGN, membranoproliferative glomerulonephritis; RPE, retinal pigment epithelium; SCR, short consensus repeat; STEC, Shiga toxin-producing *Escherichia coli*.

1. Introduction

The complement system is a key component of innate immunity and constitutes one of the main effector mechanisms of antibody-mediated immunity.¹ The human complement system consists of nearly 40 molecules, distributed as soluble plasma proteins, as integral membrane proteins, or both. The complement system has several physiologic activities. Amongst others, it is able to eliminate microbes and damaged self cells, it forms activation products which initiate inflammation, it enhances adaptive immune reactions, and it is able to clear immune complexes (Table 5.1).

Complement is a cascade system of successive steps in three different pathways: the classical pathway, the lectin pathway, and the alternative pathway (Fig. 5.1). The classical pathway can be activated by antibodies bound to antigens on a bacterial surface. The lectin pathway is initiated either by binding of mannose-binding lectin to mannose groups on bacterial surfaces or altered self-surfaces, or by ficolins recognizing specific carbohydrate structures.² The alternative pathway (AP) is antibody-independent and involves binding of C3b (complement component 3b) to hydroxyl groups on (bacterial or host) cell-surface carbohydrates and proteins. Properdin may be directly involved in the initiation of the alternative pathway.^{3,4}

The three pathways ultimately converge, after the cleavage of C3 to form C3b. Once

Table 5.1.

The three main physiologic activities of the complement system (after Walport¹).

Activity	Complement protein responsible for activity
Host defense against infection	
-Opsonisation	Covalently bound fragments of C3 and C4
-Chemotaxis and activation of leukocytes	Anaphylatoxins (C5a, C3a, C4a); anaphylatoxin receptors on leukocytes
-Lysis of bacteria and cells	Membrane-attack complex (C5b-9)
Interface between innate and adaptive immunity	
-Augmentation of antibody responses	C3b and C4b bound to immune complexes and to antigen; C3 receptors on B cells and antigen-presenting cells
-Enhancement of immunologic memory	C3b and C4b bound to immune complexes and to antigen; C3 receptors on follicular dendritic cells
Disposal of waste	
-Clearance of immune complexes from tissues	C1q; covalently bound fragments of C3 and C4
-Clearance of apoptotic cells	C1q; covalently bound fragments of C3 and C4

sufficient C3b is generated, the common terminal complement pathway is activated, leading to the assembly of the membrane attack complex (C5b-9), which effectuates, amongst others, lysis of bacteria and modified cells. Upon triggering, the complement system is rapidly and abundantly activated and targeted onto foreign surfaces, but at the same time must be inhibited on normal host cells. There are several regulatory mechanisms and factors of the complement cascade. A delicate balance between stimulating and inhibiting factors ensures that activation of complement is focused on the elimination of invading microorganisms and unwanted cells, while limiting unwanted complement activation in normal tissues.

Complement Factor H (CFH) is the central regulator of the complement AP in the fluid phase and on cell surfaces, where it achieves complement homeostasis through the restriction of excessive AP activation. In addition, CFH also limits complement pathway amplification by C3b, through the inactivation of deposited C3b on self-cells after complement activation through any of the three initiation pathways.⁵ Through both these mechanisms, CFH exerts a potent inhibitory influence on the activity of the common complement pathway.

Mutations and variants in the *CFH* gene have been found to be associated with a broad range of human pathological conditions. This clinical spectrum of diseases includes for instance

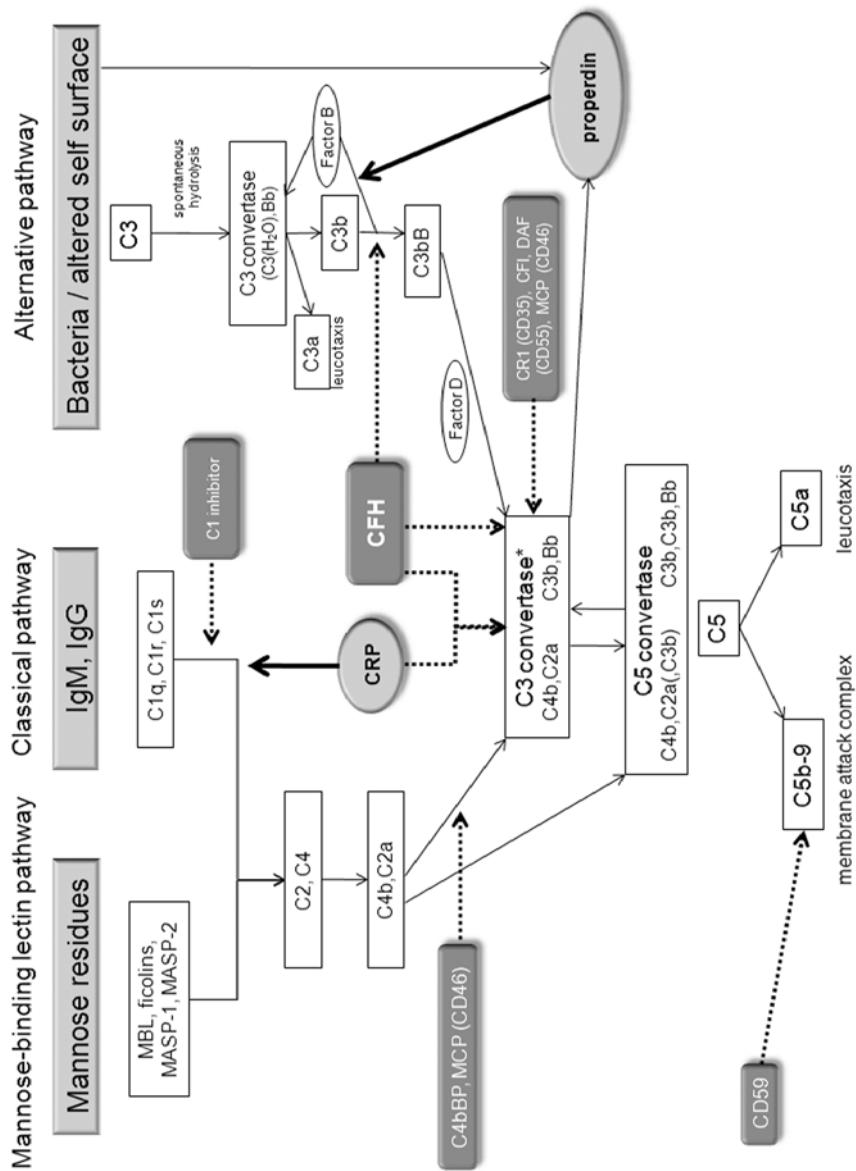
the predominantly renal phenotypes membranoproliferative glomerulonephritis (MPGN) and atypical hemolytic uremic syndrome (aHUS). However, the spectrum also comprises basal laminar drusen (BLD) and age-related macular degeneration (AMD), pathological conditions that appear to be limited to the retina. In addition, several intermediate phenotypes may be observed in association with *CFH* variants, such as glomerulonephritis with isolated C3 deposits and late-onset MPGN with AMD. The hallmark retinal lesions in AMD and BLD are drusen, which may also be observed in MPGN patients. Conversely, AMD may also be associated with plasma complement AP activation, suggesting common pathogenetic mechanisms. When studying these phenotypes and the corresponding variants in the *CFH* gene, a considerable degree of genotype-phenotype correlation may be discerned. In this review, we discuss the characteristics of the *CFH* gene and its protein product CFH. Also, the remarkably broad spectrum of *CFH*-associated phenotypes and the available information on their pathophysiology are reviewed. Finally, possible genotype-phenotype correlations are analysed, and a genotype-phenotype correlation model is proposed.

2. Molecular biology of CFH

2.1. The alternative complement pathway

The alternative complement pathway is the most ancient complement pathway, identifying, recognizing, attacking, and eliminating microorganisms and modified tissue cells. The AP is activated spontaneously in the organism by the C3 convertase that cleaves C3, resulting in C3a and C3b. C3, the central component of the complement system, is present in blood and body fluids, and can consequently act at almost every location in the body.^{1,6} Spontaneous hydrolysis of the thioester bond in C3 facilitates binding of Factor B and cleavage of Factor B by Factor D, to form the initial C3 convertase C3(H₂O),Bb.⁷ This relatively slow initial phase leads to the cleavage of C3 to C3a and C3b (Fig. 5.1). The C3b molecules subsequently bind Bb (after cleavage of Factor B by Factor D), forming the more potent C3 convertase C3b,Bb.

The formation of C3b,Bb leads to an amplification of the reaction, resulting in an exponential increase in C3a and C3b molecules. C3a is an anaphylatoxin, which triggers degranulation of mast cells and is chemotactic for leucocytes (such as macrophages). In addition, C3a shows antimicrobial activity. C3b is able to adhere to and coat the bacterial cell surface (opsonisation), which is then better recognized by phagocytic cells that possess C3b receptors. These phagocytic neutrophils and monocytes/macrophages then internalize and degrade the pathogen. Binding of C3b to C3 convertase (C3b,Bb) forms the C5 convertase, that splits C5 into C5a (a strong anaphylatoxin) and C5b. C5b initiates the assembly of the terminal complement membrane attack complex C5b-9. This membrane attack complex is able to form a pore in the bacterial cell membrane, causing cell death. The initial enzymatic components of the AP do not differentiate between activator and non-activator surfaces.

**Figure 5.1.**

The three pathways of complement activation (see also paragraph 1 and 2.1. in the text). Dotted arrow: inhibition. Bold, continuous arrow: stimulation. Abbreviations: CFH, Complement Factor H; CFI, Complement Factor I; CRP, C-reactive protein; DAF, Decay-Accelerating Factor; MASP(-1/-2), Mannose-Associated Serine Protease; MBL, Mannose-Binding Lectin; MCP, Membrane Cofactor Protein.

To prevent adverse effects on the host organism itself, a tight control of the formation and activity of the AP amplification C3 convertase C3b,Bb is essential. This is effectuated by inhibiting regulators that are present both in the plasma and on the surface of host cells. CFH and Complement Factor I (CFI) are the major down-regulators of the AP in the fluid phase. In addition, CFH also plays an important role in complement deactivation on self-surfaces such as retinal pigment epithelium (RPE) cells, erythrocytes and platelets.⁸⁻¹¹ Deactivation of complement on self-surfaces is also accomplished by the host cell surface complement regulators complement receptor 1 (CR1 or CD35), membrane co-factor protein (MCP or CD46), decay accelerating factor (DAF or CD55), and CD59. Properdin, a protein that works optimally after binding on bacterial surfaces, is able to bind C3b, C3b,B, as well as C3b,Bb, and protects these factors from catalytic cleavage by CFH and CFI.⁴

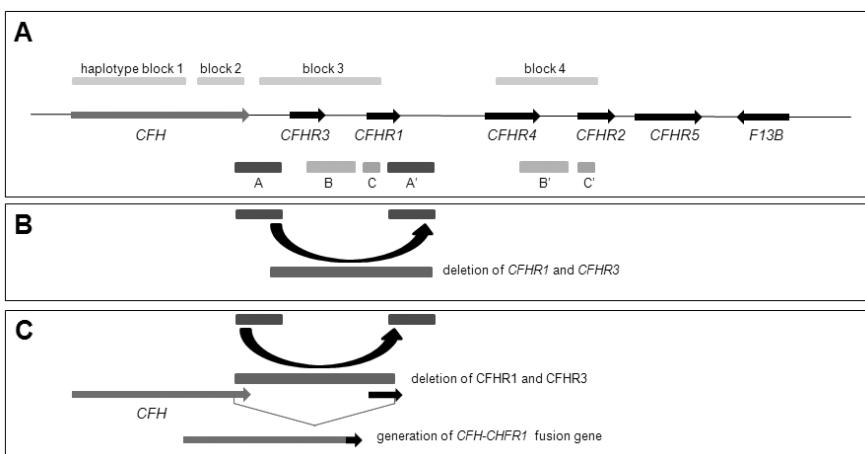


Figure 5.2.

The CFH region of the regulator of complement activation (RCA) gene cluster on chromosome 1q32. A. Genomic organization of the CFH and CFHR1-5 genes. The region contains four haplotype blocks (orange). Three large duplicated regions are present in the cluster (A and A', B and B', C and C'). B. Non-homologous recombination between the 3' ends of the duplicated regions A and A' leads to a deletion of the CFHR1 and CFHR3 genes. This rearrangement is relatively common in African and European populations, and decreases the risk of AMD. C. Non-homologous recombination between the 5'ends of the duplicated regions A and A' leads to a deletion of the CFHR1 and CFHR3 genes, and to the generation of a CFH-CFHR1 fusion gene. The fusion gene consists of the first 21 exons of CFH and the last two exons of CFHR1. This rearrangement has been found to be associated with aHUS.

2.2. The *CFH* gene

The *CFH* gene comprises 23 exons and spans more than 94 kb of genomic DNA.^{12,13} An alternative splice product of *CFH* RNA, containing exon 10, results in the synthesis of Factor H-like protein 1 (FHL-1).¹⁴ More than 550 SNPs in the human *CFH* gene region are known, that may sometimes lead to an amino acid substitution in CFH (www.ncbi.nlm.nih.gov/SNP).¹⁵ Some of these polymorphisms, for instance the Tyrosine-402-Histidine (p.Tyr402His) variant, which is associated with an increased risk for AMD, may have discrete functional consequences that will be discussed further on in this chapter. Five other genes, *CFHR1*, *CFHR2*, *CFHR3*, *CFHR4*, and *CFHR5*, encode proteins that are related to CFH. The *CFH* genomic region is organized in four SNP-haplotype blocks spanning the *CFH* and *CFHR1-5* genes (Fig. 5.2).^{15,16} In some cases, genetic rearrangements in the *CFH-CFHR1-5* region may result in the deletion of the *CFHR1* and *CFHR3* genes.^{16,17} In addition, *CFH-CFHR1* hybrid genes may be formed.¹⁸ Interestingly, the isolated deletion of *CFHR1* and *CFHR3* is a common polymorphism that is associated with a decreased risk of AMD,^{16,19} as well as an increased risk to develop aHUS.¹⁷ The presence of both the deletion of *CFHR1* and *CFHR3* and a *CFH-CFHR1* hybrid gene may predispose to aHUS.¹⁸

CFH belongs to a superfamily of structurally and functionally related proteins, named the Regulators of Complement Activation (RCA), that are encoded by a gene cluster on chromosome 1q32 (Fig. 5.2).^{13,20,21}

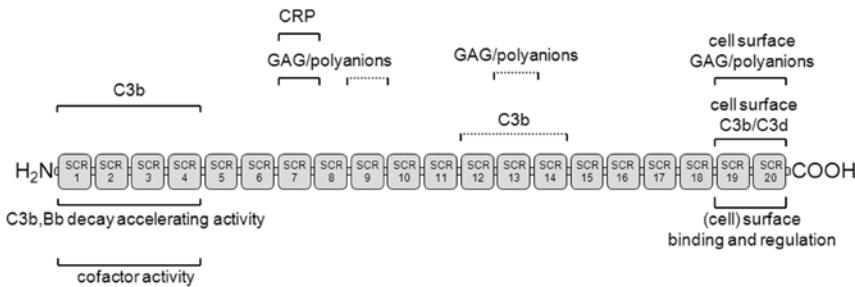
The RCA family also includes the previously mentioned CR1, DAF, and MCP, which are expressed on the surface of host cells. In addition, the RCA family contains several CFH-related proteins such as FHL-1, which is expressed in the fluid phase together with CFH itself.^{14,22-24} These proteins have overlapping and redundant activities, which may possibly explain why the absence or dysfunction of a single regulator has rather narrow tissue specificity and may not always cause clinical disease.^{11,25}

2.3. The *CFH* protein

2.3.1. *CFH* structure

The *CFH* protein was first described in 1965,²⁶ and plays a central role in the down-regulation of activation of the complement AP.^{1,27} CFH is a 155 kDa glycoprotein, which is composed of 1213 amino acid residues.²⁸ These amino acids are clustered in 20 repetitive units of approximately 60 amino acids, named short consensus repeats (SCRs).^{27,29} Each SCR is encoded by a single *CFH* exon, except for SCR 2, which is encoded by exons 3 and 4.¹⁵ These SCRs all show four invariant cysteine residues and an almost invariant tryptophan residue at identical positions.²⁷ Due to these compositional similarities, these 20 SCRs fold into 20 structurally similar three-dimensional structures called complement control protein (CCP) modules (Fig. 5.3).^{15,27} CFH contains several N-linked carbohydrates (glycans), that do not appear to be essential for its complement regulatory function.³⁰

The *CFH* protein is mainly synthesized by the liver.^{31,32} CFH is found in human plasma at highly variable concentrations, ranging from 116 to 711 µg/ml.^{33,34} These variable concentrations appear to be the result of both genetic and environmental influences.³³

**Figure 5.3.**

Functional domains of the CFH protein. The CFH protein consists of 20 short consensus repeats (SCRs) or Complement Control Protein (CCP) modules that show a certain degree of compositional similarity. However, different (clusters of) SCRs have different functional properties that are depicted in the figure. For additional discussion of these protein properties see also section 2.3. in the text. Abbreviations: CRP, C-reactive protein; GAG, Glycosaminoglycan.

For instance, plasma CFH levels increase with age and are decreased in smokers.³³ It has been estimated that roughly 60% of CFH plasma variation is due to genetic variations, for example as a result of low expression of *CFH* alleles.^{15,33} Extrahepatic synthesis of CFH is also seen in a broad range of cell types, such as RPE cells, glomerular mesangial cells, podocytes, erythrocytes, platelets, neurones, glia cells, myoblasts, and peripheral blood lymphocytes.^{8,15,35-38}

This extrahepatic synthesis, which increases local CFH concentrations, is presumed to locally protect specific host tissues against exaggerated complement activation and inflammation, for instance in the case of a local infection.¹⁵ CFH production by the RPE may be especially important to control local inflammation in this microenvironment. Interestingly, oxidative stress, an important pathogenetic factor in AMD, decreases CFH expression by the RPE.³⁸

2.3.2. CFH function

The principal function of CFH, the down-regulation of complement AP activation, is effectuated in three ways.^{11,27,39} First, CFH inhibits the assembly of an active C3 convertase, by binding to C3b and therefore inhibiting interaction with Factor B. Second, CFH is able to destroy an existing C3b,Bb convertase, by accelerating the decay of this complement activator. In the third mechanism, CFH is an essential cofactor in the cleavage and inactivation of C3b by the serine protease CFI. In addition to the down-regulation of the complement AP, CFH accelerates the breakdown of cell surface-bound C3b to iC3b after activation of any of the three initiation pathways.⁵ In mice, classical complement pathway activation by immune complexes can recruit the AP, if the AP is not effectively regulated by CFH.⁴⁰

CFH and CFH related proteins bind to bacterial and host cell surfaces through the interaction with sialic acid-rich polyanions, which is essential for its ability to effectively regulate complement AP activation on these surfaces.^{9,41-43} In addition, such cellular binding of CFH and/or its related proteins to B lymphocytes may be able to influence migration of these cells, and could influence their role in adaptive immunity.⁴⁴⁻⁴⁷ However, some bacteria are able to take advantage of this mechanism, by binding CFH on their sialic acid-covered surface and consequently evading activation of the complement AP.^{25,48,49} In addition, CFH has the capacity to bind glycosaminoglycans (GAGs) such as heparin.^{27,50} CFH may also bind fibromodulin,⁵¹ as well as adrenomedullin. An important binding partner of CFH is C-reactive protein (CRP).⁵² Ligand-bound CRP recruited from the fluid phase activates the classical complement pathway, especially the initial stage involving C1-C4, through a concentration-dependent interaction with C1q, thus stimulating the opsonisation and elimination of microbes and damaged self cells.^{53,54} Surface-bound CRP, however, interacts with CFH via SCR 7.^{52,55-58} This interaction leads to an inhibition of the complement C3 and C5 convertases, reducing the generation and deposition of the membrane attack complex, C5b-9, in the common terminal pathway. It has been proposed that the function of CRP, in the latter mechanism, is to target CFH to injured self-tissues.⁵⁷

Alterations in the function of complement regulators or induction of complement activation, for instance by altered self-surfaces or immune complexes, may lead to reduction in the levels of complement components and their function. As described above, there are three pathways for complement activation, and the level of activity of each of these pathways can be analyzed by hemolytic complement filtrations. More recently, an ELISA-based assay for the assessment of each of the three pathways was established and standardized.⁵⁹ Such assays are performed on serum samples that have to be directly processed and stored at -80°C. Consumption of complement or complement deficiencies are detected relatively easy with these complement pathway ELISA assays. CH50 is such an assay of the complete complement pathway. A low CH50 indicates depletion of complement factors, without being able to distinguish between individual complement factors. Similarly, AP50 reflects the degree of consumption of the AP. Once a defect is observed, a more detailed analysis may be performed, such as the evaluation of specific components of the early classical pathway, the AP, or the lectin pathway. Each of the components of the terminal sequence can also be assessed. In general, it is important to determine whether levels of C3 or its breakdown products are within normal limits. For analysis of subtle turnover of complement, one should especially pay attention to the assessment of C5a or C5b-9, next to antigenic determination of specific complement components. In addition, antigenic analysis of specific complement components may assess whether an abnormality can be explained by loss of functional activity or by a gain of function.

2.3.3. Structure-function correlations in CFH

A schematic overview of the functional domains of CFH is depicted in Fig. 5.3. CFH has at least two distinct C3b-binding sites: the N-terminal SCRs 1-4 and the C-terminal SCRs

19 and 20, and possibly other sites, such as SCRs 12-14.^{23,60-63} The C-terminal C3b-binding site is unique in having specific high affinity for cell surface-bound C3b, whereas SCRs 1-4 have only modest affinity for C3b, once C3b is attached to a surface. C3b,Bb decay accelerating activity as well as cofactor activity also reside in SCRs 1-4.^{23,61,64,65} Binding to GAGs and polyanions such as heparin is effectuated by SCR 7 and SCR 19-20, and possibly regions 9 and 13.⁶⁶⁻⁷⁰ SCR 7, which includes the site of the p.Tyr402His variant,⁷¹ does not only bind heparin and other GAGs, but also CRP,^{56,72} fibromodulin, DNA, and several bacteria-associated proteins.⁷³ SCRs 19-20 have multiple binding properties, as they are able to bind to C3b/C3d,^{62,63} to heparin/heparan sulphate-containing surfaces,⁶⁷ as well as to endothelial surfaces.¹⁰ A functional test of CFH on sheep erythrocytes uses these binding properties of SCRs 19-20 to look for mutations in this region of CFH. Binding of CFH to surface-associated C3b may be enhanced by CFH oligomerization.⁷⁴

3. The spectrum of diseases associated with *CFH* gene variants

3.1. Membranoproliferative glomerulonephritis

3.1.1. Clinical characteristics of membranoproliferative glomerulonephritis

The term MPGN, also known as mesangiocapillary glomerulonephritis, refers to the morphologic appearance of the typical glomerular lesions on light microscopy. Lesions are characterized by a thickening of the glomerular capillary wall, in association with mesangial cell proliferation. MPGN usually manifests in children and young adults between 5 and 30 years of age, and may present with hematuria and/or proteinuria, acute nephritic syndrome, or nephrotic syndrome. MPGN is generally associated with a chronic deterioration of renal function, leading to end-stage renal failure within 10 years of the diagnosis in approximately half of the patients.⁷⁵⁻⁷⁷ Some patients experience episodes of acute renal deterioration together with strong fluctuations in proteinuria, without obvious triggering factors. In others, the disease may remain stable for many years despite persistent proteinuria.⁷⁵

Three forms are discerned histologically, based on electron microscopic ultrastructural analysis of the glomerulus, together with immunofluorescent analysis of the presence of immunoglobulin and complement components. MPGN type I (MPGN-I) is characterized by subendothelial electron-dense deposits along the glomerular basal membrane (GBM), together with the presence of both complement and immunoglobulin within the glomerulus. Dense deposit disease, also known as MPGN type II (MPGN-II), is characterized by the dense deposition of C3 in the GBM, without the presence of immunoglobulin (as opposed to MPGN type I and III).^{39,75} Although MPGN-II is widely used as a synonym for dense deposit disease, the latter is considered a more accurate descriptive term.^{39,75} After all, the dense deposits are pathognomonic, whereas the condition is associated with capillary wall thickening and hypercellularity in only a minority of cases.⁷⁸ Differentiation

between MPGN-I and -II may be difficult as both conditions may have largely overlapping microscopic and immunofluorescence characteristics,¹¹ and *CFH* mutations have been described in both conditions.^{79,80} In MPGN type III, subepithelial and subendothelial deposits are present, as well as a variable amount of immunoglobulin and complement component C1q.

MPGN is a relatively rare cause of glomerulonephritis and accounts for approximately 4 to 7% of primary renal causes of nephrotic syndrome in children and adults, respectively.^{81,82}

A higher incidence of bacterial infection (such as *Neisseria meningitidis* meningitis) may be observed in MPGN patients, as a consequence of severe CFH deficiency and secondary depletion of C3, the central component in the complement defense mechanism.³⁹

Patients with MPGN related to homozygous or compound heterozygous *CFH* mutations show an absence of CFH in the plasma, corresponding with continuous activation of the complement AP, high C3 turnover, and hypocomplementemia. In general, low plasma C3, high C3d, low Factor B, as well as low CH50 and AP50 are indicative of this condition.^{11,79} Several studies indicate that persistent hypocomplementemia is associated with a poorer prognosis,⁸³⁻⁸⁵ whereas other studies did not find a correlation between C3 levels and the clinical course.^{86,87} MPGN-II may also be associated with acquired partial lipodystrophy, a condition with loss of subcutaneous fat in the upper half of the body, which usually precedes clinically apparent renal disease.^{88,89} However, this association has only been found in patients with MPGN-II related to the presence of C3 Nephritic Factor auto-antibodies, not in MPGN patients with *CFH* mutations.⁷⁵

Patients with MPGN-II/dense deposit disease may develop drusen, yellow-white deposits in Bruch's membrane that underlie the RPE (Fig. 5.4A).⁹⁰ Drusen contain, amongst others, inflammation-associated proteins (including complement factors).⁹⁰ As will be discussed further on in this review, drusen are the hallmark lesions in AMD.⁹¹ In MPGN-II, however, these drusen are often already detectable in the first decades of life, and they are usually smaller and more numerous spread throughout the retina, in that respect resembling the drusen observed in BLD.^{92,93} Like in BLD and AMD, drusen in MPGN-II are identified most readily on fluorescein angiography (Fig. 5.4A).^{92,94} In most cases, the retinal condition remains fairly stationary for a prolonged period of time.^{95,96} Nevertheless, with time, these drusen may enlarge and merge, sometimes evolving to macular and peripheral chorioretinal atrophy and/or subretinal neovascular membranes in the macula, resulting in severe visual loss in up to 25% of patients.⁹⁷⁻⁹⁹ In long-standing cases, electro-oculography (reflecting the function of the entire RPE) may become abnormal due to widespread RPE atrophy.^{100,101} In these advanced stages, color vision, dark adaptation, as well as the dark-adapted electroretinogram (reflecting photoreceptor function) may also become mildly abnormal.¹⁰⁰ Of note, drusen-like deposits have also been described in a case of partial lipodystrophy without MPGN.¹⁰²

An atypical case of late-onset MPGN and AMD was described in a patient with a single p.Cys431Tyr missense mutation in *CFH* (located in SCR 7, like p.Tyr402His).¹⁰³ This patient developed both neovascular AMD with BLD-like deposits and MPGN in his late 50s. Plasma analysis showed a low C3 level, a CFH level in the lower part of the normal range, and

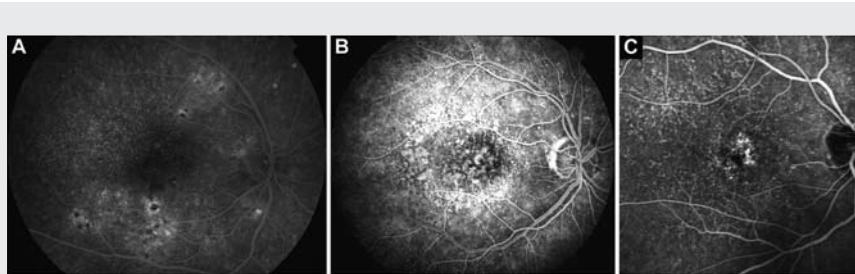


Figure 5.4.

Fluorescein angiography photographs displaying similar small and larger drusen in the posterior pole of the eye in patients with different CFH-related diseases. **A.** Excessive basal laminar drusen in a 32-year-old patient with membranoproliferative glomerulonephritis type II. **B.** Extensive drusen that enlarge and show confluence towards the macula in a 47-year-old patient with early-onset basal laminar drusen. **C.** Numerous small drusen that are indistinguishable from basal laminar drusen in an 86-year-old patient with age-related macular degeneration.

traces of C3 Nephritic Factor. Functional analyses indicated that the mutant CFH protein was not present in the plasma. In addition to this heterozygous missense mutation, the patient carried numerous risk alleles for both AMD and MPGN. In addition, this patient was a heavy smoker, which is well-known as a major environmental risk factor for the development of AMD.¹⁰⁴

There is no universally effective treatment for MPGN. Plasmapheresis and plasma exchange may be efficient in replacing deficient CFH with normal CFH, thus temporarily correcting the complement defect.^{75,77,80} Renal transplantation in MPGN ends in disease recurrence in most cases, and 5-year allograft failure is approximately 50%.^{77,105} This number is significantly higher than in the general population of renal transplant patients. Graft loss in MPGN typically occurs within 2.5 years after transplantation.⁷⁷ Non-specific therapeutic measures, such as optimal control of blood pressure and reduction of proteinuria, are beneficial to both MPGN and aHUS patients.⁷⁵

Glomerulonephritis with isolated C3 deposits, shortly named glomerulonephritis C3, is another renal disease that has recently been added to the expanding spectrum of CFH-related diseases. Glomerulonephritis C3 is a phenotype that appears to be intermediate between MPGN and aHUS.^{106,107} Two subtypes are discerned: glomerulonephritis C3 with histological features of MPGN and glomerulonephritis C3 without MPGN.¹⁰⁷ Like aHUS, glomerulonephritis C3 may be caused by heterozygous mutations in the CFH gene.¹⁰⁷ Interestingly, glomerulonephritis C3 with MPGN has been described secondary to aHUS.¹⁰⁸ Conversely, MPGN followed by aHUS may also be observed.¹⁰⁹ Apparently, CFH-related glomerulonephritis C3 is situated in a transition area between MPGN and aHUS. The pathophysiological background for such an intermediate phenotype is unclear.

3.1.2. *CFH* gene variants in membranoproliferative glomerulonephritis

Mutations in *CFH* account for a small minority of MPGN cases.^{11,75} To date, 9 patients and a total of 7 *CFH* mutations have been described in association with MPGN (Fig. 5.5). In four of these cases, homozygous mutations have been found: p.Arg127Leu (located in SCR 2),⁷⁹ p.Cys431Ser (in SCR 4),⁷⁹ p.Cys673Ser (in SCR 11),⁷⁹ and p.Lys224del (a deletion of lysine in SCR 4).⁸⁰ Another patient carried the compound heterozygous mutations p.Cys518Arg and p.Cys941Tyr.¹¹⁰ More recently, a patient with late-onset MPGN was described, who carried a heterozygous p.Cys431Tyr missense mutation in *CFH*, and he carried the p.Tyr402His AMD and MPGN risk variant homozygously.¹⁰³ In addition, he carried most of the other known AMD risk variants in several other complement genes, including *C3*, *CFB*, he was heterozygous for an AMD risk allele in ARMS2/LOC387715,¹¹¹ and he lacked the protective deletion of the *CFHR1/CFHR3* genes. Although most MPGN patients do not have disease-causing mutations in *CFH*, some polymorphisms in *CFH* (and also *CFHR5*) are significantly more frequent in MPGN patients compared to controls.¹¹² Interestingly, the p.Tyr402His variant, which is also associated with AMD and BLD, is one of these MPGN risk variants.¹¹² Besides *CFH* mutations, found in only a small number of patients, MPGN may also be caused by auto-antibodies, which are monoclonal immunoglobulin lambda light chain dimers, that inactivate the regulatory function of *CFH* in the plasma.^{113,114} Another frequent finding in MPGN is the presence of the auto-antibody C3 Nephritic Factor in plasma, which stabilizes the C3b,Bb AP convertase.^{76,115} This C3 Nephritic Factor may also be found concurrently with *CFH* mutations in MPGN.^{80,107,116,117}

3.1.3. Pathophysiology of membranoproliferative glomerulonephritis

Relatively little is known about the exact pathogenesis of *CFH*-related MPGN. In MPGN patients with homozygous or compound heterozygous *CFH* mutations, a lack of plasma *CFH* causes uncontrolled plasma complement AP activation, resulting in ubiquitous C3 deposition. The characteristic finding in MPGN-II/dense deposit disease is an intense deposition of C3 cleavage products along the glomerular capillary walls, together with electron-dense deposits in the central part of the GBM.^{39,75} In MPGN-I, a glomerular deposition of immunoglobulin is seen in addition to complement deposition, as well as subendothelial dense deposits. As the disease progresses, mesangial hypercellularity and matrix interposition occur.^{39,75,118,119} As GBM damage progresses, podocytes detach, undergo hypertrophy and ultimately die.^{75,120,121} In this stage, the final barrier to protein loss is disrupted, resulting in marked proteinuria.¹²¹⁻¹²³

Interestingly, deposits similar to the glomerular dense deposits in MPGN-II are seen in Bruch's membrane of the eye (drusen), as well as in the sinusoidal basement membrane of the spleen.^{75,124,125} Histopathological studies on drusen in MPGN-II showed both linear and focal depositions throughout the extent of the inner collagenous layer of Bruch's membrane.^{124,126} In this regard, several studies have drawn attention to the fact that the choriocapillaris-Bruch's membrane-RPE complex shows a remarkable resemblance to the capillary tuft-GBM-glomerular epithelial interface.^{75,126,127}

Disease severity in MPGN may be modified for instance by the additional presence

of C3 Nephritic Factor, which increases the damaging potential of unrestricted complement AP activation.^{80,103,115} Of note, complete CFH deficiency, with a corresponding C3 depletion, has also been found in an individual with systemic lupus erythematosus who also had a partial deficiency of C2.¹²⁸ In addition, systemic lupus erythematosus and recurrent infections (including recurrent meningococcal disease) was also described in two families with complete CFH deficiency, which, in one of those families, was caused by a homozygous p.Glu171X nonsense mutation and concurrent absence of FHL-1 and CFH.^{129,130} Systemic lupus erythematosus is usually associated with deficiencies in the classical complement pathway (especially C1q deficiency).¹³¹ However, the classical and alternative complement pathways interact and converge downstream at the level of C3,^{40,132-134} making an overlap in pathogenesis biologically plausible.

The aforementioned observations in human MPGN are mirrored by findings in a mouse and pig model. In these models, homozygous Cfh-deficient animals (*Cfh* *-/-*) had an absence of plasma CFH and a depletion of plasma C3. They developed MPGN as a result of uncontrolled complement activation and C3 deposition, whereas the heterozygous animals (*Cfh* *+/-*) did not develop a MPGN phenotype.^{39,40,135-138} In this mouse model of MPGN, an uncontrolled activation of C3 in plasma leads to MPGN.¹³⁸ The renal depositions in this mouse MPGN consist of proteolytic cleavage products derived from C3b, that are generated by CFI.¹³⁹ Such a massive C3 accumulation in the retina could also lead to retinal disorganization. When a transgenic CFH molecule that lacked SCR 16-20, mimicking a human aHUS mutation, was introduced in *Cfh* *-/-* mice, plasma CFH and C3 levels and complement AP activity were largely restored.¹⁴⁰ Instead, these animals developed a clinical picture resembling human aHUS.

3.2. Atypical hemolytic uremic syndrome

3.2.1. Clinical characteristics of atypical hemolytic uremic syndrome

Hemolytic uremic syndrome (HUS) is characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure caused by platelet thrombi in the renal microcirculation. Several etiologies have been described. Most patients with HUS are seen in childhood, in which the disease is often caused a Shiga toxin-producing *Escherichia coli* (STEC) infection.^{141,142} This STEC-associated or typical HUS manifests with watery or bloody diarrhea and has a fairly good prognosis. Non-STEC-associated or atypical HUS (aHUS) is rare and comprises a heterogeneous group of patients in whom a STEC infection can be excluded. Compared to STEC-associated HUS, aHUS has a more unfavorable outcome: a high mortality (up to 25%) and morbidity rate is seen in aHUS patients during the acute phase and the survivors frequently have recurrences, whereas 80-90% of typical HUS patients completely recover without recurrences.^{141,143} Approximately 80% of aHUS cases have an undefined or sporadic inheritance pattern, the remainder roughly corresponding to familial cases (www.fh-hus.org). aHUS may be elicited by factors such as infection, drugs, and pregnancy, although no provocative factors are identified in many cases of aHUS.¹⁴⁴ In the last decade, it has become clear

that a dysregulation of complement activation plays a major role in the pathogenesis of aHUS. So far, mutations in complement AP regulatory proteins are found in 50-60% of aHUS patients. The majority (20-30%) of these patients carry mutations in *CFH*, followed by mutations in *MCP* (10-13%), *CFHR1-CFHR3* (10%), and *CFI* (5-10%).

CFH-related aHUS usually presents earlier in childhood as compared to aHUS caused by mutations in *MCP* and *CFI*, although an adult onset may be seen in 30% of cases.^{145,146} Plasma levels of *CFH* and *C3* are often normal, as well as *CH50* and *AP50* levels, except in more severe cases caused by homozygous or compound heterozygous *CFH* mutations.¹⁴⁷ In these cases, the age at onset is earlier, often within the first two years of life.^{145,147-151} On histopathology, the predominant abnormality in aHUS is found in the renal arterioles and interlobular arteries.¹⁴⁴ These structures show endothelial swelling with retraction, resulting in exposure of the GBM. The vessel lumina are clogged up with erythrocytes and platelet fibrinoid thrombi.

Compared to aHUS caused by mutations in other complement AP genes, patients with *CFH*-related aHUS have the worst prognosis, with regard to the incidence of end-stage renal disease and death.^{145,146} Up to 60% of aHUS patients with *CFH* mutations reach end-stage renal disease or die within a year.^{145,146} Patients with *CFH* mutations show a less favorable response to plasmapheresis and plasma infusion, although these treatments may nevertheless be able to achieve remission in 67% of the cases.^{145,146,150} Recurrence of aHUS in *CFH*-related aHUS patients who underwent kidney transplantation is seen in 78-100%, often ending in graft rejection and graft failure.^{145,152,153} Contrary to *CFH*-related MPGN, no drusen or other AMD-like changes have been described in *CFH*-related aHUS to date, although vitreous and choroidal hemorrhage, as well as retinal ischemia, may be noted.¹⁵⁴

3.2.2. *CFH* gene variants in atypical hemolytic uremic syndrome

Most cases of aHUS are sporadic, but autosomal dominant and autosomal recessive familial cases have also been described.^{79,145,148,155,156} Mutations in *CFH* are found in 14-30% of aHUS patients^{145,156,157}. To date, more than 70 *CFH* mutations have been described in aHUS (for a comprehensive overview, see www.flh-hus.org).¹⁵⁸ Contrary to MPGN, aHUS is usually caused by heterozygous *CFH* mutations. Most of these mutations are located in the C-terminal region of *CFH*: approximately 65% is found in SCR 15-20, and 27% is situated in SCR 20 (Fig. 5.5).^{147,148,159} Missense mutations account for the majority (approximately 55%) of *CFH* mutations in aHUS, followed by deletions (11%), and nonsense mutations (7%). aHUS-associated mutations in the C-terminal region of *CFH* may also be the result of gene conversion. Gene conversion between *CFH* and *CFHR1*, for instance, results in the replacement of *CFH* SCR 20 by *CFHR1* SCR 5.¹⁶⁰ Another example is a hybrid gene, in which exons 1-21 are derived from *CFH*, whereas exons 22-23 originate from *CFHR1*, resulting in a heterozygous disease-causing hybrid *CFH-CFHR1* mutant.¹⁸ As mentioned previously, a deletion of *CFH*-related genes *CFHR1* and *CFHR3*, sometimes in combination with *CFH* auto-antibodies, is also associated with aHUS.^{17,161}

Despite the predominance of heterozygous missense mutations in aHUS, homozygous

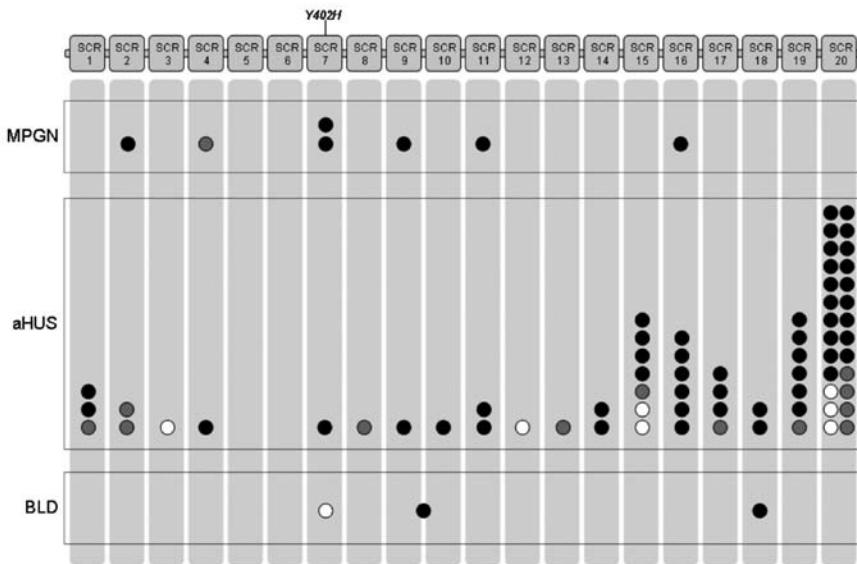


Figure 5.5.

Overview of the disease-associated mutations in the CFH gene. Only the mutations in the protein-coding sequence are depicted, excluding splice site mutations. In BLD, the depicted mutations are found in combination with the p.Tyr402His variant on the other allele. Black dot: missense mutation. Grey dot: deletion or duplication. White dot: nonsense mutation.

Abbreviations: *aHUS*, atypical hemolytic uremic syndrome; *BLD*, basal laminar drusen; *MPGN*, membranoproliferative glomerulonephritis; *SCR*, short consensus repeat.

CFH mutations,^{79,145,148} as well as compound heterozygous mutations have also been reported in early-onset aHUS.^{147,149-151,157,159} As expected, homozygous mutations are often found in offspring from consanguineous parents. The p.Tyr899X nonsense mutation was found homozygotously in several young patients with aHUS.^{79,145,150} Again, these mutations generally affect the most C-terminal SCRs, with a predilection for SCR 20. Another study has found a heterozygous *CFH* mutation together with a *CFI* mutation in glomerulonephritis C3.¹⁰⁶ This renal phenotype may show overlapping features with both aHUS and MPGN.^{106,107} Heterozygous *CFH* mutations in aHUS show a high degree of non-penetrance, indicating that additional genetic and/or environmental factors are required to develop the phenotype.¹⁶² In this regard, several aHUS risk polymorphisms have been identified in *CFH* and other complement-associated genes.^{17,155,157,162-164} In addition to *CFH* mutations, aHUS is also associated with variants in several other genes involved in the complement AP, including *CFI*,¹⁶⁵⁻¹⁶⁷ *MCP*,^{168,169} *C3*,^{15,170} as well as *CFB*.¹⁷¹ Like MPGN, aHUS may also be caused by auto-antibodies, which are directed against CFH in these cases.¹⁷² These antibodies block the same C-terminal part of CFH that is often affected in aHUS caused by mutations in the *CFH* gene, and thus have a similar effect.¹⁷³

HELLP syndrome (Hemolysis, Elevated Liver enzymes, Low Platelets), a complication of pregnancy-related hypertension, has recently been shown to be associated with mutations in complement AP genes, including one p.Arg303Gln mutation in SCR 5 of the *CFH* gene.¹⁷⁴ Like aHUS, HELLP syndrome is associated with hemolytic anemia, thrombocytopenia, and acute renal dysfunction (in up to 50% of patients). However, liver involvement is uncommon in aHUS, whereas it is a hallmark feature in HELLP syndrome. HELLP syndrome and aHUS are therefore two distinct but related disease entities, and HELLP syndrome may be considered another member of the family of disorders related to dysregulation of the complement AP.

3.2.3. Pathophysiology of atypical hemolytic uremic syndrome

In aHUS, heterozygous (often missense) mutations that generally cluster in SCR 15-20 generally do not preclude normal secretion of CFH into plasma and normal plasma regulatory activity of CFH.^{148,159} This corresponds to the often normal plasma C3 levels and AP activity in these patients, although some patients appear to require an additional decrease in plasma CFH to develop aHUS.^{147,175} Instead, *CFH* mutations in aHUS compromise cell surface-associated protective functions of CFH.⁴² Normally, prevention of the propagation of the complement AP amplification loop on cellular surfaces of the host is effectuated by inactivation of C3b on endothelial cells through the interaction of CFH with sialic acids and heparin-like glycosaminoglycan polyanions on self-surface, and through membrane-bound complement regulators such as MCP, and CFI.^{68,74} This mechanism is compromised in aHUS through the dysfunction of the C3b-binding SCRs 19-20 of CFH and/or a lack of recognition of polyanionic markers that identify host surfaces.^{68,74}

In early-onset aHUS caused by homozygous or compound heterozygous *CFH* mutations, plasma CFH and C3 may be severely diminished, contrary to later-onset aHUS caused by single heterozygous *CFH* mutations.^{79,148,176} The interaction with specific self-surface polyanions appears necessary for CFH to adopt a configuration that allows the inhibition of C3b activation.^{68,177} Endothelial cell damage may result in an additional procoagulant situation.¹⁷⁸ A disruption of these mechanisms may therefore lead to the attack of erythrocytes, platelets, and (especially renal) endothelial cells by the complement AP. As a result, the clinical triad of hemolytic anemia, thrombocytopenia, and renal failure is observed. In the cases of plasma CFH-deficient aHUS, there was no evidence of dense deposits in the GBM such as in MPGN.³⁹

Two especially interesting cases of aHUS with low plasma CFH and C3 have been described, in which one 11-month-old patient carried a homozygous p.Tyr899X nonsense mutation in *CFH*, whereas the other 16-month-old patient carried a compound heterozygous p.Asn767LysfsX7 *CFH* mutation together with a p.His183Arg missense mutation in the *CFI* gene.¹⁰⁶ Several months to years after they underwent a renal transplantation, these patients developed glomerulonephritis C3 in the allograft. These results indicate that glomerulonephritis C3 and aHUS are different expressions of the same condition.

3.3. Basal laminar drusen

3.3.1. Clinical characteristics of basal laminar drusen

The phenotype of BLD is also referred to as “cuticular drusen” or “early adult onset, grouped drusen”. The mean age at onset in BLD is 50 years, but ranges from 35 to 70 years, and patients may remain asymptomatic for a long period.¹⁷⁹ In BLD caused by *CFH* variants, the age at onset appears to be even later, with a mean of 57 years, making the differential diagnosis with AMD even more challenging.¹⁷⁹ BLD is characterized by a pattern of small (25 to 75 µm), slightly raised, yellow subretinal drusen with slightly pigmented borders, randomly scattered throughout the macula and peripheral retina.^{179,180} Drusen in *CFH*-related BLD are usually smaller and more numerous than in “typical” AMD. Although these small drusen may be identified on ophthalmoscopy, additional investigations, such as fluorescein angiography, fundus autofluorescence, and infrared reflectance photography may assist in showing the true extent of the lesions.¹⁷⁹⁻¹⁸¹ On fluorescein angiography, BLD displays a typical “stars-in-the-sky” picture (Fig. 5.4B).¹⁸⁰

The drusen in BLD may remain relatively stationary and may be compatible with a fairly good visual acuity. In later stages, the drusen may become more numerously scattered throughout the retina, often clustered in groups. Both in the macula and peripheral retina, these drusen may show confluence and evolve to patches of chorioretinal atrophy.¹⁷⁹ In the macula, these confluent drusen may lead to a serous, “pseudovitelliform” RPE detachment, which may compromise visual acuity. Such an RPE detachment may show spontaneous resolution with retention of a relatively good visual acuity, but may also become atrophic and/or end up in the formation of subretinal neovascularization.^{179,180} In this stage, the resulting visual loss is often severe. As mentioned previously, extensive small hard drusen like in BLD may not only be observed as an isolated BLD phenotype, but highly similar structures are also regularly observed in MPGN and AMD patients (Fig. 5.4).

Different clinical subtypes of drusen are discerned, based mainly on ophthalmoscopic characteristics.¹⁸²⁻¹⁸⁴ Histopathological data show that BLD are ultrastructurally and compositionally similar to other types of drusen, which are also located between the RPE and Bruch’s membrane, suggesting a similar pathway for drusen biogenesis.¹⁸⁵ In this respect, the term “basal laminar” drusen is not entirely correct, as the deposits do not seem to correspond to nodular or diffuse thickenings of Bruch’s membrane.¹⁸⁵ On histopathology, drusen in BLD show a high degree of confluence, whereas they often appear as numerous small but separate lesions on ophthalmoscopy. This apparent discrepancy is less pronounced on fluorescein angiography, on which the number, extent, and degree of confluence of the hyperfluorescent lesions outnumber the lesions visible on ophthalmoscopy.

Routine laboratory testing of blood and urine in *CFH*-related BLD patients does not show obvious renal dysfunction.¹⁸⁵ To date, no reports on complement concentrations in BLD caused by *CFH* variants have been published. Of note, preliminary data in *CFH*-related BLD caused by a p.Gln408X nonsense mutation in conjunction with the p.Tyr402His

variant in *CFH* indicate that this phenotype is also associated with mild systemic complement AP activation (personal observation, C.J.F. Boon and M.R. Daha). There are currently no data on the treatment outcome of choroidal neovascularization in BLD.

3.3.2. *CFH* gene variants in basal laminar drusen

Most cases of BLD are sporadic, but familial cases have also been described.¹⁷⁹ Roughly 17% of BLD patients appear to carry *CFH* mutations, and these patients often have a positive family history for macular degeneration.¹⁷⁹ Like in AMD, the p.Tyr402His variant also appears to play a prominent role in BLD. The allele frequency of p.Tyr402His in BLD patients is up to 70%,^{179,186,187} compared to approximately 55% in “typical” AMD patients and 34% in healthy white control subjects.^{36,188,189} In a subset of BLD patients, the combination of at least one p.Tyr402His variant together with a specific *CFH* variant leads to an early-onset BLD phenotype.¹⁷⁹ To date, four *CFH* variants have been described that may cause BLD when expressed together with the p.Tyr402His variant (Fig. 5.5): a p.Gln408X nonsense mutation, a p.Arg1078Ser missense variant (located in SCR18), a p.Arg567Gly missense variant (located in the interdomain hinge region between SCR9 and SCR10), and a c.350+6T→G splice site variant.¹⁷⁹ Of note, one study indicates that missense variants in the *fibulin 5* gene may also be involved in BLD.¹⁹⁰

3.3.3. Pathophysiology of basal laminar drusen

The specific *CFH* variants that are found in some BLD patients, together with the *CFH* p.Tyr402His variant, apparently have specific pathological consequences for the retina. To date, there are no histopathological studies on drusen in BLD caused by such *CFH* gene variants. As mentioned previously, histopathological studies indicate that drusen in AMD, BLD (without genotyping), and MPGN are quite similar on the level of ultrastructure and molecular composition.^{124,185} Drusen in MPGN, as well as in BLD and AMD, contain complement factors and other inflammatory components.^{36,124,185,191-193} The comparable clinical and histopathological findings indicate that drusen in MPGN, BLD and AMD are a highly similar expression of a common underlying local inflammatory reaction. This leads to a complement deposition between the RPE and Bruch’s membrane, with or without a variable degree of systemic inflammation and complement activation (Fig. 5.4). It remains to be unravelled if the exact composition of drusen in AMD, BLD, and MPGN is different, or if their constitution is identical and there is merely a difference in the time of appearance of these drusen.

3.4. Age-related macular degeneration

3.4.1. Clinical characteristics of age-related macular degeneration

Drusen are also the hallmark lesions in AMD, the most common cause of legal blindness above the age of 60 in the Western world.⁹¹ The mean age at onset of visual loss in AMD is in the 8th decade. In persons over 80 years of age, the prevalence of legal blindness due to AMD may rise to more than 10%.¹⁹⁴ Several clinical classifications have been developed

for AMD, based mainly on the findings on ophthalmoscopy.^{182,195-198} In the later stages of AMD, when visual loss becomes manifest, a distinction is made between atrophic or “dry” AMD, and neovascular or “wet” AMD. Atrophic AMD is characterized by patches of profound, chorioretinal (“geographic”) atrophy. Neovascular AMD, on the other hand, is caused by aberrant choroidal neovascular vessels that grow under or into the retina. These vessels form a neovascular membrane that leaks fluid, which compromises normal photoreceptor architecture and thus retinal function. Distortion of the normal photoreceptor arrangement leads to metamorphopsia, a symptom that is often the first manifestation of AMD. When photoreceptor function is further compromised and involves the fovea, visual acuity may decrease dramatically. Hemorrhages may occur within these lesions, and the neovascular complex may evolve to a scar. Contrary to atrophic AMD, which may take years to cause severe visual loss, neovascular AMD may end up in legal blindness within months.¹⁹⁹ Both the atrophic and neovascular forms may be found in the same patients in random sequence.⁹¹

The diagnosis of AMD is generally established with ophthalmoscopy, but fluorescein angiography and/or optical coherence tomography (OCT) are often used to determine the exact location, size, and type of neovascularisation.²⁰⁰ Like in MPGN- and BLD-related drusen, fluorescein angiography is particularly suitable to determine the size and extent of drusen in AMD (Fig. 5.4C). Relatively recent imaging techniques, such as fundus autofluorescence and high-resolution spectral domain-OCT, are able to provide further insight into AMD lesions and may help to determine and evaluate the clinical outcome and therapeutic options.²⁰¹⁻²⁰⁴

AMD patients show increased plasma levels of complement AP activation products compared to control subjects.³⁴ Most AP activation determinants are elevated, but this is especially true for C3d and Ba, which reflect chronic complement AP activation. Contrary to for instance MPGN and some subtypes of aHUS, the levels of CFH and C3 are comparable in AMD patients and controls.³⁴ Factor D, an essential protein in the activation of the complement AP, also appears to be significantly elevated. Preliminary studies indicate that systemic complement AP activation in AMD is associated with the presence of specific AMD risk alleles in complement genes, including the p.Tyr402His risk variant in *CFH*.³⁴ In addition, the presence of p.Tyr402His and another *CFH* risk polymorphism may be associated with a reduction in the glomerular filtration rate.^{205,206}

Current treatment modalities in AMD are aimed at the prevention of geographic atrophy and neovascular AMD, and the arresting or reversal of choroidal neovascularization once it has occurred.²⁰⁷ In addition, advice and support to stop smoking may be effective in significantly reducing the risk of AMD.¹⁰⁴ Results from the Age-Related Eye Disease Study (AREDS) suggest that increased dietary intake or supplementation with vitamin C, vitamin E, zinc, and beta carotene, in patients with moderate to advanced AMD in one (the most affected) eye, may reduce the risk of advanced AMD in the fellow eye.²⁰⁷⁻²⁰⁹ Recent data also suggest a protective role for omega-3-polyunsaturated fatty acids in the prevention of neovascular AMD.²¹⁰⁻²¹³ However, the current body of evidence does not support the use of antioxidant vitamin or mineral supplements in the general

population.²¹⁴ Anti-angiogenic therapy, for instance through the intravitreal injection of an anti-vascular endothelial growth factor (anti-VEGF) such as ranibizumab, reduces the risk of visual loss and may even reverse visual loss in patients with neovascular AMD.²¹⁵

3.4.2. *CFH* gene variants in age-related macular degeneration

The p.Tyr402His amino acid substitution, located in SCR 7, was the first *CFH* variant shown to be strongly associated with AMD in the Caucasian population.^{36,188,216,217} Estimates of the relative risk of AMD amongst carriers of this polymorphism, as compared to non-carriers, range from 2.45 in heterozygotes to 7.4 (in homozygous carriers).^{36,188,216,217} This relative risk of late AMD increases to 34.0 when homozygous carriers are current smokers.²¹⁸ The p.Tyr402His variant appears to be much more frequent in people from Caucasian and African descent (allele frequency of approximately 35%) than in Hispanics and Asians (allele frequencies of approximately 17% and 7%, respectively).^{189,218,219} The p.Tyr402His variant shows no or only marginal association with AMD in the Chinese,²²⁰⁻²²³ Japanese,^{224,225} and Korean populations.²²⁶ Apart from p.Tyr402His, several other *CFH* polymorphisms and haplotypes have been reported to confer an increased risk of AMD, both in Caucasians and Asians.^{36,188,223,226-228} Several other haplotypes of *CFH* and adjacent regions, one of them containing the previously mentioned deletion of *CFHR1* and *CFHR3*,¹⁶ lower the risk of AMD.^{19,36,228} One of these haplotypes (*CFH* haplotype H2), which carries a p.Val62Ile variant in the N-terminal region, lowers the risk not only of AMD, but also of aHUS and MPGN.^{15,17,140}

Variants in other genes involved in the complement system also alter the risk of AMD and its progression, including *C3* (encoding complement component 3),²²⁹⁻²³¹ *CFB* (encoding Factor B), and *C2* (encoding complement component 2).²³²⁻²³⁵ Recent evidence also suggests a role for the classical pathway of complement activation in AMD, as genetic variants in the *SERPING1* gene, which encodes the C1 inhibitor, alter the risk of AMD.²³⁶⁻²³⁸ However, these findings could not be replicated by another large study.²³⁹

3.4.3. Pathophysiology of age-related macular degeneration

In AMD, the *CFH* p.Tyr402His variant shows decreased affinity for GAGs and CRP. GAGs, present in the retina and Bruch's membrane,²⁴⁰ may be used as markers of self-surface by *CFH*. With age, relative levels of GAGs change in Bruch's membrane and the RPE.^{241,242} The ability of the p.His402 and p.Tyr402 *CFH* variants to recognize and protect Bruch's membrane may therefore fluctuate and differ with age.²⁴³ In addition, an age-related increase of oxidative influences, such as lipofuscin and its pro-oxidative constituents, may lead to a decrease of *CFH* expression by the RPE, and may trigger changes that eventually lead to AMD.^{38,244-246} Therefore, the p.Tyr402His variant may decrease the ability of *CFH* to locally protect the RPE. CRP, an acute phase reactant that has both pro- and anti-inflammatory properties, plays an important role in the down-regulation of the complement AP through the interaction with *CFH*.^{52,58,71,247} Such an interaction of CRP with *CFH* appears to be impaired by the p.Tyr402His change.^{71,243,248} Patients with AMD that carry the p.Tyr402His variant homozygously have elevated levels of CRP both

in serum and choroid.^{218,249-252} In addition, individuals carrying both *CFH* p.Tyr402His and specific *CRP* haplotypes, resulting in elevated serum CRP levels, have an increased risk of AMD,²¹⁸ although results are somewhat conflicting on this matter.²⁵³ This suggests that elevated levels of CRP in AMD are associated with normal pro-inflammatory and reduced anti-inflammatory characteristics, possibly due to the p.Tyr402His variant in *CFH*.⁷¹ This could lead to the observed local and systemic chronic inflammation and complement AP activation,^{34,218,249,252} which predisposes to the deposition of inflammatory debris and drusen formation in the macula, as will be discussed further on in this review.⁹⁰ The aforementioned findings illustrate the pathophysiological consequences of the *CFH* p.Tyr402His variant that may lie at the basis of the development of AMD. However, these findings should be seen against the background of other genetic and environmental modifying factors, as the p.Tyr402His variant is neither sufficient nor necessary to develop AMD.

Various animal models of AMD have been developed, though none of these animal models exactly copy AMD.²⁵⁴ One of the major problems is that a macula is only found in humans and primates. However, since rod photoreceptor degeneration occurs prior to cone photoreceptor death in human AMD,²⁵⁵ the rod-dominated mouse retina may, to a certain extent, be a suitable model for the study of photoreceptor loss in AMD. One must bear in mind, however, that the enormous metabolic rate that is characteristic of the human macula, is not reflected in the mouse retina. To date, seven mouse models are available that display features of both atrophic and neovascular AMD: the *Ccl2/Ccr2* knockout mouse,²⁵⁶ the *ApoE* mouse,²⁵⁷ the *Sod1* knockout mouse,²⁵⁸ the *Ccl2/Cx3cr1* double knockout mouse,^{259,260} the *Ceruloplasmin/Hephaestin* double knockout mouse,²⁶¹ and the *Efemp1* p.Arg345Trp knock-in mouse.^{262,263} In mice immunized with carboxyethylpyrrole-modified mouse serum albumin, in order to mimic oxidative damage in AMD,^{264,265} auto-antibodies were formed against this hapten.²⁴⁵ As a result, the complement system was activated, and early AMD-like sub-RPE lesions appeared.²⁴⁵ These mouse models may show drusen, complement deposition, lipofuscin accumulation, Bruch's membrane disruption, RPE atrophy, as well as choroidal neovascularization. These mouse models also confirm findings in humans that indicate that pro-inflammatory lifestyle characteristics, such as obesity, a high fat diet, lower antioxidant intake, as well as elevation of inflammatory biomarkers such as CRP, increase the risk of the development of AMD and the evolution towards advanced AMD.^{218,252,254,266}

AMD and BLD appear to be associated with systemic complement AP activation, like MPGN and some cases of aHUS, albeit in a more low-grade, chronic fashion.³⁴ This could indicate that AMD (and possibly BLD), too, is a systemic disease that becomes clinically apparent only in the macula, which appears to be especially sensitive to this kind of damage. An interesting finding in this regard is that the estimated glomerular filtration rate is reduced in individuals with the p.Tyr402His AMD and MPGN risk variant, and several other *CFH* polymorphisms.²⁰⁵ Although speculative, these findings could suggest discrete subclinical *CFH*-related renal alterations in p.Tyr402His carriers. In cases of complement activation in the plasma, tissue surfaces that have a specific predisposing

anatomical structure (such as the eye and kidney) are probably particularly sensitive to complement deposition and damage. An additional explanation for this anatomical predisposition may be a relative lack of membrane-bound complement regulators on the GBM and, possibly, ocular structures such as the RPE basement membrane and Bruch's membrane.⁷⁴

A significant association of the p.Tyr402His *CFH* risk variant, as well as a lack of protective *CFH* alleles, was also found in multifocal choroiditis.²⁶⁷ Multifocal choroiditis is a retinal phenotype that typically affects persons younger than 50 years, and is presumed to have an autoimmune inflammatory pathogenesis.^{268,269} Like in AMD, choroidal neovascularization is often seen in multifocal choroiditis. The common genetic risk factors therefore suggest some shared disease mechanism between these diseases.

3.5. Cardiovascular disease and Alzheimer's disease

Some studies indicate that the *CFH* p.Tyr402His allele increases the risk of coronary heart disease, myocardial infarction, ischemic stroke, cardiovascular mortality, as well as mortality at old age in general.^{251,270-274} The cardiovascular risk may possibly be modified by the degree of hypertension.²⁷⁴ However, this association of p.Tyr402His and cardiovascular disease is refuted by several other studies, that either find no association,^{275,276} or even an inverse association.²⁷⁷

The same applies to Alzheimer's disease. In one large study, the *CFH* p.Tyr402His variant conferred an increased risk of Alzheimer's disease, an association that was only evident when individuals also carried the *ApoE* ε4 allele.^{278,279} Plasma *CFH* levels also appear to be increased in patients with Alzheimer's disease, when compared to control subjects.²⁸⁰ Interestingly, the *ApoE* ε4 allele decreases the risk of AMD.²⁸¹⁻²⁸⁴ Other studies, however, did not find any association of p.Tyr402His and Alzheimer's disease.^{285,286}

Because of these contradictory study results, the possible association of cardiovascular disease and Alzheimer's disease with the p.Tyr402His *CFH* variant will not be further discussed in this review.

4. Pathophysiology of drusen and drusen-associated complications

Histopathologically, drusen may arise from so-called basal laminar and basal linear deposits, which are located between the basal membrane and the basement membrane of the RPE.^{287,288} One of the major components of drusen is lipofuscin,^{179,201,289} an autofluorescent mixture of fluorophores that contains toxic waste products of the visual cycle.^{246,289} This lipofuscin may find its origin in the overlying RPE, through the exocytosis of residual phagocytic material at the RPE basolateral membrane.²⁹⁰ In addition, drusen contain a broad range of components that are often related to inflammation.^{90,291} Of special interest to this review is the finding of numerous complement components in drusen, including *CFH*, C3, C5 and the membrane attack complex C5b-9.^{90,291} The accumulation of complement factors in drusen, especially C5b-9, could be precipitated

by the decreased interaction between the CFH p.Tyr402His variant and CRP, as discussed previously.^{52,71,243,248} The choroid is a source of many of the complement factors, whereas the RPE appears to have a more defensive function, as it produces CFH and carries several membrane-bound downregulators of the complement AP.^{35,90,292} Blue-light induced A2E, the major toxic fluorophore of lipofuscin in RPE and drusen, as well as other sources of oxidative stress, are able to initiate activation of the complement AP, possibly through the suppression of CFH expression by RPE cells.^{38,245,266}

When present at increased concentrations, CFH may self-associate into oligomers that might facilitate the formation of protein precipitates within drusen.²⁹³ In this respect, it is worth noting that the CFH p.Tyr402His variant shows a higher propensity to oligomerise.²⁹⁴ Lipid components such as apolipoprotein E and amyloid P are also found in drusen, as well as proteins such as TIMP3 and amyloid-beta. In addition, HLA-DR has been identified in drusen, together with a core of dendritic cells, which are antigen-presenting cells that may participate in the induction of immune responses.⁹⁰ As such, drusen are the first clinical manifestation of a web of molecular and cellular activities that result from a complex interplay of multiple genetic and environmental factors, including chronic inflammation and complement AP activity. As a result of inflammation, oxidative damage and accumulation of a mixture of degradative and inflammatory debris, RPE cells and photoreceptors are damaged and die through apoptosis.^{207,245,295-297} Photoreceptors overlying and adjacent to drusen show a decreased density and display morphologic and molecular signs of degeneration.²⁹⁸

Drusen are the precursors of geographic atrophy in MPGN, BLD, and AMD.²⁹⁹ The evolution of drusen takes place over many years, in which large amounts of small hard drusen may further increase in number and show confluence, to form larger, soft drusen.^{299,300} These soft drusen, which are actually small detachments of the RPE, may then further enlarge and evolve to larger RPE detachments with hyperpigmentation. Ultimately, these drusenoid RPE detachments result in profound atrophy of the overlying RPE and photoreceptors,^{298,301} resulting in the appearance of geographic atrophy. Therefore, geographic atrophy is viewed upon as the end stage of the process of drusen formation and progression. It should be noted that this end stage is reached in only a minority of patients with drusen.^{299,300} Geographic atrophy often appears first in the parafoveal area and may spare the fovea for a prolonged period of time.³⁰² On histopathology, an absence of the RPE is seen, with secondary loss of photoreceptors and a variable degree of atrophy of the choriocapillaris.³⁰³

In some patients, the aforementioned process of drusen evolution is complicated by the formation of choroidal neovascularization (neovascular AMD). It is unknown why some individuals develop such choroidal neovascularization along the way, while others do not. Such a difference may find its origin in interindividual differences in the balance of pro- and anti-angiogenic factors, as well as discrete differences in the anatomical microenvironment of the retina. Local production of vascular endothelial growth factors (VEGFs) stimulates the formation and growth of aberrant neovascular vessels,^{304,305} a mechanism that is attacked in the case of anti-VEGF therapy in neovascular

AMD. Choroidal neovascularization may also be promoted by complement components C3a and C5a, which are present in drusen and are able to induce VEGF expression.^{306,307} Early choroidal neovascularization in AMD is characterized by diffuse membranous deposits under the RPE, together with an infiltration of macrophages.³⁰⁸ In addition, ingrowth of vessels, surrounded by enlarged pericytes, into and through the damaged Bruch's membrane and in the sub-RPE space is observed.^{295,308} The tips of these vessels are not covered by pericytes, allowing to leak fluid, erythrocytes and fibrin into this sub-RPE space.³⁰⁸ This results in the characteristic sub- and/or intraretinal fluid, hemorrhage, and scar formation.

5. Disease-associated *CFH* variants, their consequences for protein structure and function, and genotype-phenotype correlations

Most homozygous or compound heterozygous *CFH* mutations in MPGN, as well as the single heterozygous p.Cys431Tyr mutation in late-onset MPGN, are missense mutations that lead to the loss of a cysteine residue (Fig. 5.5).^{79,103,110} All four cysteines of each SCR in CFH form structurally important disulfide bonds.³⁰⁹ Mutations involving these residues may therefore lead to a considerable change in the secondary structure of CFH. Such a structurally altered protein is presumed to be retained and degraded intracellularly, without being secreted into the plasma.^{110,309} Contrary to the aforementioned mutations, the homozygous p.Lys224del mutation in SCR 4 does not preclude secretion of this mutated CFH into the plasma.⁸⁰ Lysine residues are important determinants of the binding capacity of CFH for C3b.³⁰ In the case of this specific p.Lys224del mutation in MPGN, the circulating mutant CFH proteins are unable to bind C3b and regulate plasma complement AP activation.⁸⁰ Thus, the pathogenetic mechanisms leading to MPGN are different between the non-secreted CFH proteins and the secreted but functionally deficient p.Lys224del mutation. The consequence - uncontrolled plasma AP activation - is the same in both cases. However, because circulating mutant CFH has some residual function in the case of the homozygous p.Lys224del mutation, the age at onset is somewhat later (6 to 11 years, versus below 2 years of age in most cases of absent plasma CFH).^{79,80} Contrary to aHUS, the p.Lys224del mutation does not affect the binding of CFH to cell surfaces and its recognition of other ligands.

More than 90% of *CFH* mutations in aHUS are located in the C-terminal region, the majority clustering in SCR 20 (Fig. 5.5).^{147,148} Firm evidence suggests that the two last C-terminal domains, SCRs 19-20, contain a C3b binding site and a heparin/polyanion binding site, which appear essential for the association of CFH with host endothelial and other surface cells, as well as surface-bound cofactor activity of CFH.^{10,67,69,74,147,148,175,310,311} A loss of SCRs 19-20 deprives CFH of the capability to degrade endothelial cell-bound C3b.³¹⁰ In this area of CFH, amino acids 1183, 1191, 1197, and 1210 correspond to mutational "hot spots", and appear to be involved in binding of CFH to surface-bound

C3b, to heparin, as well as to endothelial cells.^{10,175,312}

In glomerulonephritis C3 without MPGN, two *CFH* mutations have been described. The first mutation, a heterozygous p.Arg1210Cys missense mutation situated in SCR 20, presumably reduces binding of CFH to C3b as well as to endothelial cells.^{10,107,312} This mutation has also been identified in aHUS patients.^{10,312} The other mutation, a heterozygous p.Pro76X *CFH* nonsense mutation, leads to a truncated protein that is not secreted in the plasma, causing a decreased plasma CFH concentration due to haploinsufficiency.¹⁰⁷ This mutation has also been reported repeatedly in aHUS (www.fh-hus.org). In a case of glomerulonephritis C3 with MPGN, a heterozygous p.Gly650Val mutation was found, situated in SCR 11 close to a C3b-binding domain. Again, several mutations nearby this domain have also been found in aHUS.^{79,107,156}

In BLD, the heterozygous p.Gln408X nonsense mutation causes an early-onset drusen phenotype.¹⁷⁹ The p.Gln408X mutation may lead to nonsense-mediated decay, preventing the expression of the protein. Alternatively, should the protein be translated, it is probably not secreted in the plasma or, if it is secreted, it will probably be degraded rapidly. The p.Arg1078Ser and p.Arg567Gly *CFH* missense variants in BLD have, on the basis of homology models, been hypothesized to alter the affinity for several binding partners of CFH, such as C3b and CRP.¹⁷⁹ The c.350+6T→G *CFH* splice site mutation in BLD is predicted to severely affect splicing.¹⁷⁹ The aforementioned mutations most likely cause early-onset BLD only when they are inherited in association with the p.Tyr402His variant.¹⁷⁹

The p.Tyr402His risk variant is situated in SCR 7 (Fig. 5.5), which binds heparin and polyanions, as well as it interacts with CRP and group A streptococcal M protein.^{56,57,66,72} The p.Tyr402His amino acid change results in little structural alterations.^{71,243} However, the p.Tyr402His variant does decrease the interaction of CFH with CRP,^{71,177,243,248} GAGs, and possibly heparin.^{71,177,243,313} A decreased ability of CFH to bind GAGs on self-surfaces and microbial surfaces could compromise its ability to regulate complement AP activation on such surfaces. An impaired interaction between CFH and CRP, the latter aiding C3 and C5 convertase inhibition, could lead to an increased production and deposition of C5b-9, the membrane attack complex. In addition, the p.Tyr402His variant shows increased self-association to oligomers.²⁹⁴

The aforementioned findings illustrate that the severity of disease roughly corresponds to the underlying defect(s) in the *CFH* gene. As a result, we propose a genotype-phenotype correlation model for *CFH*-related disease, which is summarized in Figure 5.6. In this model, early-onset MPGN and early-onset aHUS result from specific homozygous or compound heterozygous *CFH* mutations, that result in excessive plasma complement AP activation and - in the case of aHUS - a decreased cell surface activity of CFH leading to an attack of self-surfaces. Later-onset aHUS and MPGN are caused by single heterozygous *CFH* mutations, in addition to modifying genetic and environmental factors. Intermediate phenotypes, such as glomerulonephritis C3 with or without MPGN, may also be caused by specific single heterozygous *CFH* mutations. Early-onset BLD is caused by a specific *CFH* mutation together with a heterozygous or homozygous p.Tyr402His variant. An

increased risk of AMD with or without small drusen that resemble (late-onset) BLD is associated with the p.Tyr402His variant or other specific *CFH* variants or haplotypes. In this genotype-phenotype model, the degree of systemic complement AP dysregulation, as well as the consequences for renal function, decreases towards AMD, whereas additional genetic and environmental modifying factors may become more important.

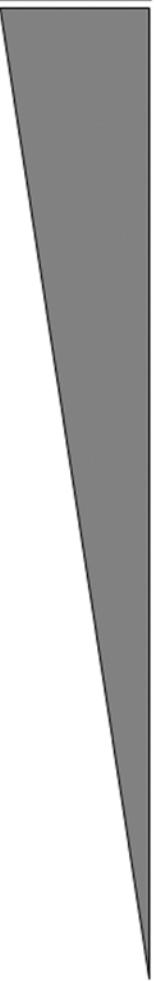
As in most diseases with a genetic background, it should be noted that a straightforward genotype-phenotype correlation is not consistently seen in *CFH*-related disease. For instance, abnormally low plasma levels of CFH and C3 are not invariably caused by homozygous *CFH* nonsense mutations, but may also be seen in patients with (compound or isolated) heterozygous missense mutations in the *CFH* gene.^{103,149} Patients with BLD do not have renal disease on routine laboratory screening, despite carrying one allele with a presumably severe *CFH* mutation. Apparently, a single wild-type *CFH* allele and its protein product are sufficient to prevent excessive complement AP activation on blood and kidney cells. It is also sufficient to prevent the excessive plasma complement AP activation and C3 deposition in the glomeruli that cause MPGN, despite harboring the p.Tyr402His variant, that not only increases the risk of AMD and BLD, but also of MPGN. The *CFH* variants in BLD appear to have more specifically damaging consequences for the retina, without clinically affecting the kidneys.

The aforementioned considerations thus provide only part of the explanation of the phenotypic differentiation of *CFH*-related disease. Obviously, genetic and environmental modifying factors, as well as for instance interindividual differences in cell surface characteristics, may be an important additional player in these cases. The proposed genotype-phenotype model and its graphic representation in Figure 5.6 therefore merely aims to serve as an overview, highlighting and categorizing the broad range of *CFH*-related disorders.

6. Phenotypic variability, variable expression, and non-penetrance

The issue why some individuals that carry a heterozygous *CFH* variant remain disease-free is largely unresolved. It is also unclear why some patients with homozygous *CFH* variants that result in complete CFH deficiency develop MPGN, whereas others experience an early-onset aHUS. Interestingly, the morphological appearance of MPGN - a non-specific, histologically defined glomerular lesion - may develop into a picture of chronic aHUS.³⁹

As indicated previously, several factors in addition to the type of *CFH* mutation could influence the resultant phenotype in *CFH*-related diseases like aHUS, such as the degree of *CFH* expression, the residual function of the affected and unaffected *CFH* domains, the presence of *CFH* splice variants, as well as the presence of auto-antibodies such as C3 Nephritic Factor.^{107,115,116} Different *CFH* risk alleles may differentially increase the susceptibility to MPGN, aHUS, as well as AMD.³¹⁴ The p.Tyr402His variant, for instance, is associated with an increased risk of AMD and MPGN, but does not alter the risk of



Disease	Typical age at onset (years)	CFH variant(s)
early-onset MPGN	5 – 30	homozygous or compound heterozygous (cysteine residues, Lys224del)
early-onset aHUS	< 5	homozygous or compound heterozygous (C-terminal nonsense and/or missense)
aHUS	< 30	heterozygous (mostly C-terminal, missense) + additional genetic / environmental susceptibility factors
glomerulonephritis C3 with MPGN	around 30? (broad range)	heterozygous (Gly560Val in SCR 11)
glomerulonephritis C3 without MPGN	around 40? (broad range)	heterozygous (Arg1210Cys in SCR 20, Pro76X in SCR 2)
late-onset MPGN with BLD-like AMD	late 50s	heterozygous missense (Cys431Tyr) + additional genetic/environmental risk factors
BLD	around 55 (broad range)	heterozygous nonsense, missense, or splice site mutation + at least one Tyr402His risk allele
AMD	> 60	Tyr402His risk allele or other CFH risk variants + additional genetic and environmental risk factors

Figure 5.6.

Genotype-phenotype correlation model for diseases associated with variants in the *CFH* gene. In this model, the degree of systemic complement alternative pathway (AP) dysregulation, as well as the consequences for renal function, decreases towards age-related macular degeneration (AMD), whereas additional genetic and environmental modifying factors may become more important. Although atypical hemolytic uremic syndrome (aHUS) is placed

Consequences	Plasma parameters	References
plasma CFH absent or defective function → glomerular C3 deposition + drusen	CFH ↓/↓↓, C3 ↓↓, CFB ↓, CH50 ↓↓, AP50 ↓↓, C3d ↑↑ (+/- C3 Ne F)	Ault et al., 1997; Dragon-Durey et al., 2004; Licht et al., 2006
plasma CFH low/absent + decreased CFH cell surface activity	CFH ↓-↓↓, C3 ↓-↓↓, (CFB ↓↓), CH50 nl-↓↓, (AP50 ↓), (C3d ↑↑)	Caprioli et al., 2001; Richards et al., 2001; Cheong et al., 2004; Dragon-Durey et al., 2004; Licht et al., 2005; Caprioli et al., 2006; Saland et al., 2006; Cho et al., 2007
decreased CFH cell surface activity → attack of self-cells	CFH nl-↓(half), C3 nl-↓	Caprioli et al., 2001; Perez-Caballero et al., 2001; Richards et al., 2001; Dragon-Durey et al., 2004; Caprioli et al., 2006; Sellier-Leclerc et al., 2007
glomerular C3 deposition	CFH nl, C3 nl-↓	Servais et al., 2007
glomerular C3 deposition	CFH nl-↓(half), C3 nl-↓, CFB ↓ (+/- C3 Ne F)	Servais et al., 2007; Boyer et al., 2008
decreased plasma CFH → glomerular C3 deposition + drusen	CFH low-nl, C3 moderate ↓, CFB nl (C3 Ne F)	Montes et al., 2008
drusen	(CFH mildly ↓, C3 mildly ↓, C3d mildly ↑)	Boon et al., 2008
chronic complement AP activation in plasma + drusen	CFH nl, C3 nl, C3d ↑, Ba ↑, Factor D ↑	Boon et al., 2008; Patel et al., 2008; Scholl et al., 2008

below membranoproliferative glomerulonephritis (MPGN), this does not implicate that this is a milder phenotype. It merely indicates that the disease is usually associated with heterozygous CFH mutations, and that it usually does not severely impact plasma CFH and C3 levels, contrary to MPGN. Additional abbreviations and symbols: nl, normal; C3 Ne F, C3 Nephritic Factor; ↓, decreased; ↓↓, markedly decreased.

aHUS.^{112,315} A deletion of *CFHR1* and *CFHR3* decreases the risk of AMD,^{16,19} whereas it increases the risk of aHUS.¹⁷ It has been estimated that approximately 60% of the phenotypic variance of CFH levels in aHUS is due to additional genetic modifiers.^{157,163}

As for environmental factors, CFH plasma levels show an age-dependent increase, and are decreased in smokers.³³ Oxidative insults such as blue-light induced damage by toxic fluorophores have been shown to decrease *CFH* expression by the RPE.^{35,38} On the other hand, expression of Factor B by the RPE increases with advancing age, promoting complement AP activation.³¹⁶ These findings indicate that, in the aging retina, the RPE and Bruch's membrane are subjected to increasing levels of complement activation and its pro-inflammatory consequences. aHUS may be triggered for instance by infection, which may precede aHUS in up to 70% of cases,¹⁴⁵ immunosuppressive agents, oral contraceptives, as well as pregnancy.^{145,148} In BLD, the p.Tyr402His variant appears to be required to develop an early-onset BLD phenotype.¹⁷⁹ AMD is the example par excellence of complex multifactorial disease related to *CFH*. After all, carrying the p.Tyr402His variant and/or other *CFH* risk haplotypes significantly increases the risk of AMD, but the presence of these factors is not strictly required to develop AMD. The risk of AMD is modified by numerous additional genetic factors, both in complement and non-complement genes,^{91,315} as well as by environmental modifying factors, such as smoking and possibly *Chlamydia pneumoniae* infection.^{104,317,318} A predictive model based on 5 common polymorphisms in the *CFH*, *ARMS2/LOC387715*, and *C2/CFB* genes estimates that persons who carry all risk genotypes have a 14-fold higher AMD risk compared to the general population, and a 285-fold greater risk than the lowest risk group.^{228,319}

Another striking illustration of the influence of genetic modifiers and environmental factors on the phenotypic outcome is the previously mentioned case of a patient with AMD with BLD-like deposits and late-onset MPGN.¹⁰³ This patient carried only one missense mutation in *CFH*, which would normally not be expected to cause MPGN on itself. However, in addition to this mutation, this patient carried several AMD and MPGN risk variants, the plasma showed traces of C3 Nephritic Factor, and he was a heavy smoker. All these additional genetic and environmental "hits" may eventually lead to clinical disease, although with a later-than-usual onset.

7. Future perspectives

Especially in aHUS and AMD, which may be considered prototypes of multifactorial disease, the classical concepts of causality are challenged. In AMD, for instance, gene therapy does not seem an attractive option, as too many variables influence the pathogenesis and outcome of the disease.³²⁰ In such a scenario, the short- and long-term risks of gene therapy may outweigh the potential benefit. In this regard, it should be carefully considered that the complement AP has important physiological functions in the human body, such as protection against infection.¹ The modification of exogenous factors such as smoking appears a more feasible and effective goal in this stage. In *CFH*-

related diseases that follow a more straightforward causal relationship, such as early-onset MPGN due to homozygous *CFH* nonsense mutations, gene therapy may prove to be a promising future treatment. In the case of AMD, on the other hand, it seems rational to apply more direct therapeutic approaches aimed at preserving and/or restoring central retinal function.

Faster and more effective treatments in MPGN, aHUS, as well as in AMD and BLD, are urgently needed. The outcome of current treatments in aHUS and MPGN is generally poor, whereas morbidity and mortality are high, especially aHUS and MPGN caused by *CFH* mutations.^{145,146,152} As patients with *CFH*-related aHUS often display a rapid evolution towards end-stage renal disease, and have a high risk of mortality within a few years, effective treatment should be initiated as soon as possible particularly in these patients.^{145,146} Therefore, the rapid identification of the underlying genetic or functional defect, for instance using hemolytic assays, is of the utmost importance. Not only do these results have prognostic implications, they may also expedite the start of the appropriate therapy, such as plasma supplementation with normal CFH.^{175,321,322}

There are currently no gene therapeutic options to restore CFH deficiency due to *CFH* mutations in aHUS and in MPGN. In *Cfh*^{-/-} mice, the MPGN phenotype may be corrected when mice are concurrently knocked out for Factor B (*Cfb*^{-/-}), as the absence of Factor B prevents the formation of C3b,Bb.¹³⁸ Knocking out C5 in *Cfh*^{-/-} mice also resulted in a reduction in glomerular cellularity, serum creatinine levels and mortality,³²³ as C5 activation appears to be a particularly important component of renal injury during acute inflammatory episodes.^{39,119} Consequently, treatment with an anti-C5a antibody such as eculizumab may prove to be effective.^{77,324}

In AMD, the impact of the genetic background also becomes more and more apparent. Determining an individual's genetic risk profile,^{228,319} together with behavioural risk factors,^{104,318} and possibly serological complement activation markers,³⁴ may provide valuable insight in AMD risk assessment. The *CFH* p.Tyr402His variant may predispose to certain subtypes of AMD,^{325,326} and it influences the risk of progression in AMD.³²⁷ An individual's response to treatment with nutritional supplements such as antioxidants and zinc,^{328,329} as well as the response to anti-angiogenic therapy,³³⁰⁻³³² may also depend on the underlying *CFH* genotype. Especially in multifactorial diseases such as AMD and aHUS, the underlying palette of genetic (and environmental) factors may turn out to be essential in ones likelihood to respond to a specific therapeutic intervention. The development of agents that combat local complement overactivation could mean a huge step forward towards a marked risk reduction in for instance AMD. An important issue in this respect is to maintain a basal complement activation, to prevent for instance infectious complications that could result from excessive complement inhibition.

The aforementioned studies are the first steps in *CFH*-related pharmacogenetics. Current and future studies face the challenging task to further unravel the complex network of contributing genetic and environmental factors. In this perspective, knowledge about the underlying genotype may exert a considerable influence on the prognosis, as well as the choice and efficacy of specific preventive measures and treatments.

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Chapter 5

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5.2. Basal laminar drusen caused by compound heterozygous variants in the *CFH* gene

Abstract

Objective: To evaluate the role of the *CFH* gene in 30 probands with early-onset basal laminar drusen.

Methods: Thirty probands were diagnosed with basal laminar drusen, based on ophthalmoscopic and fluorescein angiographic findings. In addition to an extensive ophthalmologic evaluation, blood samples were obtained for analysis of the coding region of the *CFH* gene. The effect of amino acid variants were evaluated by molecular modeling.

Results: We identified heterozygous nonsense, missense and splice variants in *CFH* in five families. The affected individuals all carried the p.Tyr402His AMD risk variant on the other *CFH* allele. This supports an autosomal recessive disease model, where individuals who carry a *CFH* mutation on one allele and the p.Tyr402His variant on the other allele, develop drusen. Patients with these *CFH* variants show confluent macular drusen and many small drusen in the (mid-)peripheral retina, that may show an evolution to extensive chorioretinal atrophy.

Conclusion: Our findings strongly suggest that monogenic inheritance of *CFH* variants can result in basal laminar drusen in young adults, which can progress to maculopathy and severe vision loss later in life.

Introduction

Age-related macular degeneration (AMD) is the most common cause of blindness in the Western world, with a prevalence of 12% after 80 years of age.¹ The presence of macular and/or extramacular drusen is an important risk factor for the development of advanced AMD.² Different clinical subtypes of drusen have been described, but all drusen appear to be similar on the level of ultrastructural and molecular composition.³ AMD is a multifactorial disorder and variants in several genes have shown to be associated with the disease.^{1,4} Strong association is seen with the p.Tyr402His variant and several non-coding variants in the *CFH* gene.⁵⁻⁹ The *CFH* protein has been detected in drusen and acts as an inhibitor of the alternative complement cascade.¹⁰

“Basal laminar drusen” (also termed “cuticular drusen” or “early adult onset, grouped drusen”) refers to an early-onset drusen phenotype, which shows a pattern of uniform small (25 to 75 µm), slightly raised, yellow subretinal nodules that are randomly scattered in the macula. The term “basal laminar drusen” is widely used, but may be considered a misnomer, as these deposits do not appear to correspond with nodular or diffuse thickenings of the Bruch’s membrane.³ In later stages, these drusen often

become more numerous, with clustered groups of drusen scattered throughout the retina. On fluorescein angiography, a typical “stars-in-the-sky” picture may be observed (Fig. 5.7).¹¹ In time, these small basal laminar drusen may expand and ultimately lead to a serous pigment epithelial detachment of the macula, which may result in visual loss.¹¹ The basal laminar drusen phenotype has an even stronger association with the p.Tyr402His variant than AMD, with an allele frequency of 70%, compared to 55% in a cohort of typical AMD patients and 34% in controls.¹² Basal laminar drusen are also seen in patients with membranoproliferative glomerulonephritis type II (MPGN-II), a severe early-onset renal disease caused by an uncontrolled systemic activation of the alternative pathway of the complement cascade, which can be caused by highly penetrant recessive mutations in the *CFH* gene.^{13,14} MPGN-II associated drusen appear to be morphologically and compositionally similar to drusen in AMD.¹⁵ Mutations in *CFH* have been also associated with atypical hemolytic uremic syndrome (aHUS), a severe disease frequently leading to end-stage renal failure.^{16,17}

Methods

Clinical studies

In this study, we evaluated 30 probands diagnosed with basal laminar drusen maculopathy. At early stages the diagnosis was based on the biomicroscopic observation of small yellow-white deposits (“basal laminar drusen” or “cuticular drusen”) in the macula, sometimes extending towards the (mid-)peripheral retina, in combination with a “stars-in-the-sky” appearance on fluorescein angiography (Fig. 5.7). The occurrence of confluent (“soft”) drusen of variable size in the central macular region and subsequent development of a serous detachment of the retina and/or retinal pigment epithelium (RPE) were considered typical for the later stages of this disorder. A final feature in the later stages is geographic atrophy of the RPE that is frequently observed following resolution of the RPE detachment. An informed consent was obtained from all probands and participating family members. The study adhered to the tenets of the Declaration of



Figure 5.7.

Typical fluorescein angiogram of a patient with advanced drusen maculopathy resembling basal laminar drusen (patient 9). Note the typical “stars-in-the-sky” appearance, with central confluence of drusen and the small pseudo-vitelliform pigment epithelial detachment.

Helsinki and was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Centre (Nijmegen, the Netherlands).

Molecular genetic studies

Peripheral venous blood samples were obtained from the 30 probands, as well as from 18 family members from families A, B, and C. After isolation of their genomic DNA, we evaluated the role of *CFH* in the 30 probands by sequence analysis of the 22 coding exons and splice junctions.

Results

Clinical findings

An overview of the clinical characteristics of these patients is given in Tables 5.2 and 5.3. Four patients did not experience visual complaints prior to the diagnosis (the finding of retinal abnormalities was coincidental), whereas 26 patients had noticed a decrease in visual acuity, with an age at onset ranging from 35 to 70 years (mean, 50 years). Eight patients reported family members with macular disease or visual disturbances compatible with macular disease. In 12 patients (40%), many small drusen were also seen in the (mid-)peripheral retina. In 28 patients (93%), the development of large, soft drusen could be observed in the central macular area. A pseudovitelliform yellow serous retinal or RPE detachment of the central macula with associated loss of visual acuity, was seen in 8 patients (27%). Ten patients (33%) showed chorioretinal atrophy of the macula. On fluorescein angiography, individual drusen as well as the larger exudative drusen were hyperfluorescent. Choroidal neovascularization was seen in 8 eyes of 5 patients (13% of 60 eyes).

Molecular genetic findings

The p.Tyr402His variant was present in 50% of *CFH* alleles (Tables 5.2 and 5.3), which is lower than the 70% reported elsewhere in patients with basal laminar drusen,¹² but higher than in healthy control subjects (34%). More importantly, we found that 7 patients in two families (A and B) were compound heterozygous for the nonsense mutation p.Gln408X and the AMD risk allele p.Tyr402His (Fig. 5.8). The *CFH* variants in the deceased individual A-II.3 were deduced upon CA-marker analysis in the right branch of family A. The clinical characteristics of the affected family members are shown in Figure 5.9 and Table 5.3. Two family members (A-IV.1 and A-IV.2) carrying only the p.Gln408X allele did not develop drusen. In a third family (C), we identified three individuals with drusen who carried a heterozygous variant (p.Arg1078Ser) on one allele, and the p.Tyr402His variant on the other *CFH* allele (Fig. 5.8). The p.Arg1078Ser variant occurs in the 18th short consensus repeat (SCR 18) of CFH. To predict the effect of this variant, we constructed a homology model for SCR 18 (Fig. 5.10). As SCR 18 shares 40% homology with SCR 19, model building was straightforward using the SCR 19-20 structure as a template.¹⁸ The model shows that the amino acid p.Arg1078 is solvent exposed, and the change to serine at this

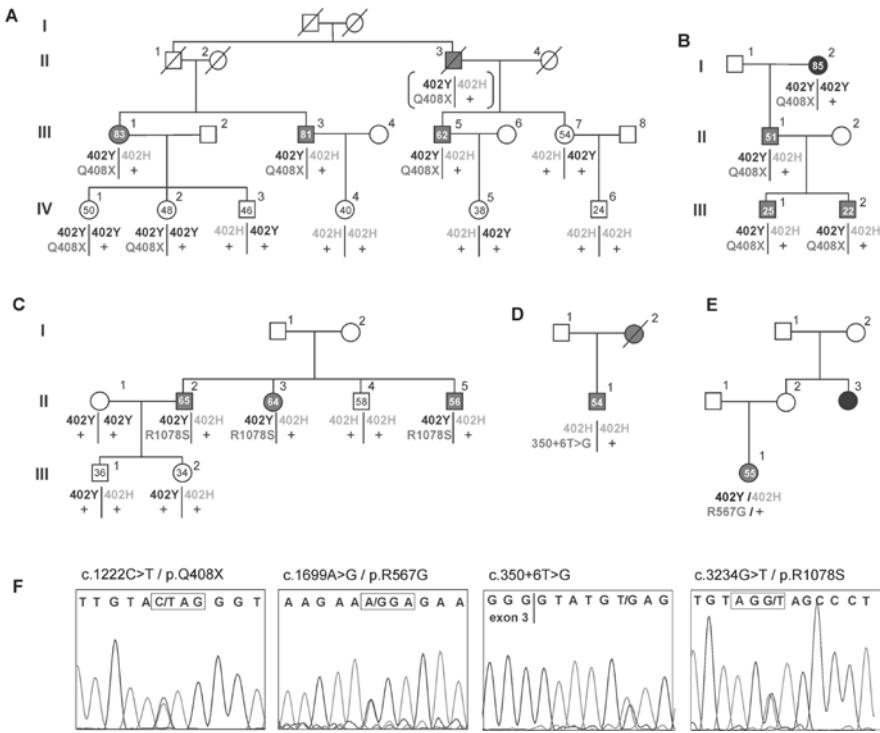
position is not expected to have a large structural effect on SCR 18 itself. However, SCR 18 neighbors the C-terminal SCR domains 19 and 20, which were shown to have many different interaction partners, including the major opsonin C3b, glycosaminoglycans and endothelial cells. The p.Arg1078Ser variant introduces a smaller side chain in SCR 18, and abolishes the negative charge at this position. This change might interfere with the different interactions in which CFH participates. Notably, mutation of a nearby residue (p.Gln1076Glu) was identified in a patient with aHUS (FH aHUS mutation database).

In patient D, we identified the p.Tyr402His variant homozygously, and a heterozygous variant in the splice donor site of exon 3 (c.350+6T>G) of *CFH* (Fig. 5.8). This variant is predicted to severely affect splicing, since the splice prediction score is reduced from 0.97 to 0.59 (NNSPLICE version 0.9).¹⁹ Moreover, this residue is completely conserved between human and all species for which the genomic sequence of *CFH* is available (rhesus macaque, mouse, rat, dog, horse, opossum, platypus and elephant). The deceased mother of patient D was diagnosed with macular and peripheral drusen by a local ophthalmologist at the age of 43.

A patient in a fifth family (E) carried a heterozygous amino acid variant (p.Arg567Gly), and was heterozygous for the p.Tyr402His variant (Fig. 5.8). Other family members were unavailable for examination or segregation analysis. The p.Arg567Gly variant occurs in the interdomain hinge region between the short consensus repeats SCR 9 and SCR 10. As no experimental structure of SCR 9-10 is known, we built a homology model of this hinge region and the two surrounding domains, using the crystal structure of the CFH SCR domains 19-20 as a template.¹⁸

In our model, p.Arg567 is located on the protein surface and hydrogen bonds from the hinge region to the backbone of SCR 10 (Fig. 5.10). As such, p.Arg567 could play a structural role in defining the orientation of SCR 9 with respect to SCR 10. This hypothesis is supported by p.Glu566 and p.Glu568, which both hydrogen bond to a tyrosine residue in SCR 9 and SCR 10, respectively. As the charged p.Arg567 in our model also appears to be partially exposed to the surface, it could play a role in one of the many interactions in which CFH can engage. For example, CFH has been shown to bind complement factor C3b via interaction sites in SCR domains 1-4, 9, 12-14 and 19-20.^{20,21} Furthermore, CFH can bind C-reactive protein (CRP) via SCR 7, but also in the region spanning SCR 8-11.²² This would place p.Arg567 close to a C3b binding site and in a CRP-binding region. p.Arg567Gly removes a positive charge in the interdomain hinge region between SCR 9 and SCR 10, and is likely to result in an increased mobility of the linker between these repeats. Based on these findings, we speculate that p.Arg567Gly might result in an altered affinity of CFH for C3b and possibly also CRP.

The new *CFH* variants identified in these families were not identified in 92 ethnically matched control subjects, nor in 90 controls between 65 and 85 years of age who did not have any signs of maculopathy. In two other patients, we identified p.Asn1050Tyr and p.Glu936Asp heterozygously in *CFH*. The p.Asn1050Tyr variant was previously found in 4% of control alleles¹⁶, and the p.Glu936Asp variant was found in 18% of controls.²³ Although proband 24 in our study was compound heterozygous for the p.Asn1050Tyr

**Figure 5.8.**

Molecular genetic analysis of the *CFH* gene in families affected with drusen maculopathy.

A and B. Seven affected individuals in two families are compound heterozygous for the nonsense mutation p.Gln408X and the AMD risk allele p.Tyr402His. Brackets flanking the *CFH* variants of A-I.3 indicate that they were deduced through CA-marker analysis. **C.** Three affected individuals in a third family are compound heterozygous for the missense variant p.Arg1078Ser and p.Tyr402His. **D.** Patient D is homozygous for the p.Tyr402His variant, and in addition carries a heterozygous variant in the splice donor site of exon 3 (c.350+6T>G). **E.** A patient in a fifth family carries a heterozygous amino acid variant (p.Arg567Gly), and is heterozygous for the p.Tyr402His variant. Segregation analysis could not be performed. The grey pedigree symbols in panels A-E denote patients with basal laminar drusen. The black symbols denote two females affected with AMD. Numbers in the pedigree symbols reflect current age. **F.** Sequences of heterozygous variants detected in the *CFH* gene.

and p.Tyr402His alleles, his affected father carried the p.Asn1050Tyr variant without p.Tyr402His, indicating that compound heterozygosity of *CFH* variants does not cause the disease in this family.

Table 5.2.
Clinical characteristics of drusen patients without compound heterozygous *CFH* mutations.

Patient number	Sex	Family history*	Age at onset† (years)	Age (years)	Visual acuity‡ OD OS	Retinal phenotype§,¶	<i>CFH</i> variants	<i>CFH</i> pTyr402His
1	f	no	-	53	20/20 20/20	ODS: extensive macular drusen	-	Tyr/Tyr
2	f	no	-	40	20/20 20/20	ODS: extensive confluent macular drusen	-	Tyr/His
3	f	no	53	54	20/70 20/50	ODS: numerous small drusen in posterior pole	-	Tyr/His
4	m	no	51	57	20/70 20/70	ODS: macula: confluent drusen surrounded by small drusen. PED, chorioretinal atrophy, many small drusen in peripheral retina	-	Tyr/His
5	f	no	49	50	20/32 20/70	ODS: extensive confluent and atrophic drusen of the posterior pole, PED, OD: occult CNV, OS: minimally classic CNV	-	His/His
6	f	no	35	35	20/20 20/20	ODS: many drusen throughout (mid-)peripheral retina, confluent drusen in posterior pole	-	Tyr/Tyr
7	f	yes	44	47	20/32 20/32	OD: confluent drusen with large exudative PED and large occult exudative CNV, OS: large confluent drusen with surrounding small drusen	-	Tyr/His
8	m	no	45	58	20/25 20/100	ODS confluent macular drusen and chorioretinal atrophy	-	Tyr/His
9	f	no	43	46	20/25 20/25	ODS confluent macular drusen, PED (Fig. 5.7)	-	Tyr/His
10	f	no	43	57	20/125 20/200	ODS: very extensive atrophic, confluent macular drusen and (mid-) peripheral drusen, OD: chorioretinal atrophy, OS: fibrotic scar	-	Tyr/His
11	f	no	53	54	20/20 20/25	ODS: confluent macular drusen, small midperipheral drusen	-	Tyr/His
12	m	no	50	54	20/32 20/20	ODS: confluent macular drusen, small midperipheral drusen	-	Tyr/His
13	m	no	48	60	20/32 20/250	ODS: confluent macular drusen, PED	-	Tyr/His

14	f	no	57	57	20/32	20/25	ODS; extensive confluent macular drusen, pseudovitelliform PED	-	Tyr/His
15	m	no	70	74	20/25	20/25	ODS; confluent macular drusen	-	Tyr/Tyr
16	f	no	43	56	20/20	20/20	ODS; extensive drusen in posterior pole, confluent macular drusen	-	Tyr/His
17	f	no	44	44	20/25	20/100	ODS; extensive drusen in posterior pole, confluent macular drusen	-	His/His
18	m	no	56	58	20/25	20/160	OD; confluent macular drusen, OS: macular drusen, large pseudovitelliform PED	-	Tyr/His
19	f	no	43	43	20/25	20/100	ODS; confluent macular drusen	-	Tyr/His
20	f	no	-	38	20/20	20/20	ODS; large confluent drusen in posterior pole and midperipheral retina, macular pigmentary changes	-	Tyr/His
21	f	yes	51	51	20/40	20/2000	ODS; extensive macular and (mid-)peripheral drusen, confluent macular drusen and chorioretinal atrophy	-	Tyr/His
22	f	no	-	69	20/20	20/20	ODS; confluent macular drusen with parafoveal chorioretinal atrophy	-	Tyr/His
23	f	no	47	48	20/80	20/80	ODS; extensive confluent macular drusen, numerous small (mid-)peripheral drusen, OD: large occult CNV	-	Tyr/His
24	f	yes	44	45	20/32	20/20	ODS; innumerable drusen throughout retina, confluent macular drusen	p.Asn1050Tyr	Tyr/His
25	f	?	62	63	20/50	20/30	ODS; confluent macular drusen and geographic atrophy, innumerable (mid-)peripheral drusen, patches of chorioretinal atrophy, reticular pattern of hyperpigmentation	p.Glu936Asp	Tyr/His

* A positive family history was defined as reported family members with macular disease.

†, no visual loss reported.

‡(A), amblyopia.

§ PED, pigment epithelial detachment.

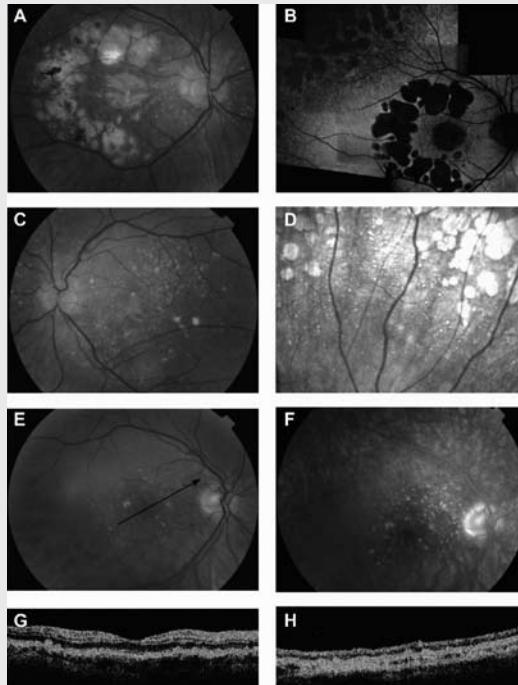
|| CNV, choroidal neovascularization.

Table 5.3.
Clinical characteristics of drusen patients and family members carrying specific compound heterozygous *CFH* variants.

Patient code*	Sex	Family history†	Age at onset‡ (years)	Age (years)	Visual acuity§	Retinal phenotype¶,#	<i>CFH</i> variant	<i>CFH</i> p.Tyr402His
			OD	OS				
A-III.5 (P)	m	yes	45	62	20/400	20/25	OD: confluent macular drusen, chorioretinal atrophy, (mid-)peripheral retina: cystoid drusen, chorioretinal atrophy (Fig. 5.9A,B) OS: confluent macular drusen, (mid-)peripheral retina: cystoid drusen, chorioretinal atrophy (Fig. 5.9C,D)	p.Gln408X His/Tyr
A-II.3	m	yes	?	deceased	20/25	20/200	ODS: extensive confluent drusen and chorioretinal atrophy OS: extensive confluent drusen and chorioretinal atrophy	p.Gln408X His/Tyr
A-III.1	f	yes	63	83	20/800	20/800	ODS: chorioretinal atrophy of posterior pole, large atrophic drusen, subretinal fibrosis, extensive drusen in atrophic (mid-)peripheral retina	p.Gln408X His/Tyr
A-III.3	m	yes	67	81	20/50	20/400	ODS: confluent atrophic macular drusen and chorioretinal atrophy, extensive drusen in (mid-)peripheral retina	p.Gln408X His/Tyr
A-IV.1	f	yes	-	50	20/20	20/20	ODS: normal	p.Gln408X Tyr/Tyr
A-IV.2	f	yes	-	48	20/20	20/20	ODS: normal	p.Gln408X Tyr/Tyr
B-II.1 (P)	m	yes	48	51	20/25	20/25	ODS: confluent macular drusen, (mid-)peripheral retina: small drusen and discrete pigmentary changes (Fig. 5.9E-H)	p.Gln408X His/Tyr
B-I.2	f	yes	68	85	20/400	20/32	ODS: confluent macular drusen and chorioretinal atrophy, (mid-)peripheral retina: small drusen and discrete pigmentary changes	p.Gln408X Tyr/Tyr
B-III.1	m	yes	-	25	20/20	20/20	ODS: very discrete midperipheral cystoid drusen, clearly visualized on fluorescein angiography	p.Gln408X His/Tyr

B-III.2	m	yes	-	22	20 20	20 20	ODS: barely discernable macular and peripheral drusen, clearly visualized on fluorescein angiography [Fig. 5.9I,] ^j	p.Gln408X	His/Tyr
C-II.2 (P)	m	yes	57	65	20 40	20 200	ODS: innumerable small drusen throughout retina, (mid-)peripheral chorioretinal atrophy and reticular pattern of hyperpigmentation. OD: large confluent macular drusen, PED, occult CNV, chorioretinal atrophy. OS: atrophic scar in macula, surrounded by large confluent drusen	p.Arg1078Ser	His/Tyr
C-II.3	f	yes	-	64	20 25	20 25	ODS: confluent macular drusen and PED, numerous small drusen in (mid-)peripheral retina	p.Arg1078Ser	His/Tyr
C-II.5	m	yes	-	56	20 16	20 16	ODS: barely discernable (mid-)peripheral drusen, clearly visualized on fluorescein angiography	p.Arg1078Ser	His/Tyr
D	m	yes	54	54	20 20	20 125 (A)	ODS: large confluent macular drusen, cystoid drusen in midperipheral retina	c.350+6T>G	His/His
E	f	yes	54 (aunt)	55	20 32	20 70	ODS: confluent macular drusen surrounded by small drusen, PED, chorioretinal atrophy, small drusen in peripheral retina	p.Arg567Gly	His/Tyr

^{*}(P) proband.[†]A positive family history was defined as reported family members with macular disease.[‡], no visual loss reported.[§](A), amblyopia.[¶]CNV, choroidal neovascularization.[#]PED, pigment epithelial detachment.

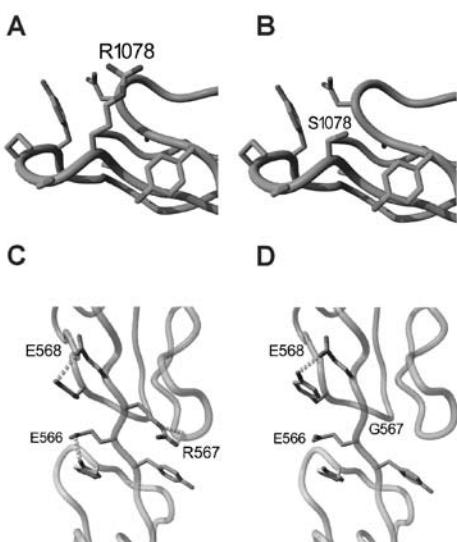
**Figure 5.9.***

Retinal phenotypes of drusen patients carrying the combination of a p.Gln408X mutation and the p.Tyr402His AMD risk variant. (A-D) Retinal phenotype of patient A-III.5. **A.** Fundus photograph of the right eye, showing extensive chorioretinal atrophy of the posterior pole. At the first examination, 17 years earlier, these areas corresponded with large confluent drusen with incipient atrophy. **B.** The short-wavelength fundus autofluorescence (FAF) image shows an absence of FAF corresponding with the patches of chorioretinal atrophy, not only in the macular area, but also in the midperipheral retina. Moreover, diffuse changes in FAF intensity can be observed.

C. Fundus photograph of the left eye, showing large confluent drusen and mild chorioretinal atrophy. **D.** An infrared reflectance photograph clearly visualizes small, round midperipheral drusen scattered between the patches of chorioretinal atrophy. The drusen on infrared reflectance corresponded with discrete, round, yellow-white drusen with a slightly pigmented border on ophthalmoscopy. (E-H) Retinal phenotype of patient B-II.1. **E.** Fundus photograph showing confluent macular drusen. **F.** The macular drusen can be more easily detected on the fluorescein angiogram. **G.** Optical coherence tomography (OCT, oblique section) showing small dome-shaped elevations of the “outer red line”, corresponding with visible drusen on ophthalmoscopy (arrow). **H.** Like in patient A-III.5, small midperipheral drusen were also seen in this patient, which had a similar aspect on OCT as the macular drusen. (I-J) Patient B-III.2, the 22-year-old asymptomatic son of patient B-II.1 showed tiny hyperfluorescent drusen in the parafoveal area (**I**) and in the (mid-)peripheral retina (**J**) on fluorescein angiography, which were difficult to discern on ophthalmoscopy. His 25-year-old brother (B-III.1) had similar midperipheral lesions, but to a lesser extent.

Discussion

Previous studies have shown that the p.Tyr402His variant in CFH is strongly associated with AMD and basal laminar drusen.^{5-7,12} Our findings strongly support a recessive disease model in a subgroup of patients with basal laminar drusen. In these families,

**Figure 5.10.**

Molecular modeling of missense variants detected in CFH. **A and B.**

Model of the SCR 18 domain, depicting the effect of the p.Arg1078Ser variant. Model of **A**, the wildtype sequence (p.Arg1078) and **B**, the p.Arg1078Ser variant.

C and D. Model of the SCR 9 and SCR 10 domains, depicting the effect of the p.Arg567Gly variant. Model of **C**, the wildtype sequence (p.Arg567) and **D**, the p.Arg567Gly variant.

The SCR 9 domain is shown in yellow, SCR 10 in orange, and the linker region between the two domain is shown in blue

(see original article for color version of the figure). Hydrogen bonding partners for the residues in the linker region are indicated with dotted lines. All figures were made using Yasara.

individuals develop drusen when they carry a *CFH* mutation on one allele and the p.Tyr402His variant on the other allele. The presence of a *CFH* mutation in the absence of the p.Tyr402His variant, however, might contribute to the development of AMD at later age (e.g. in individual B-I.2). Compound heterozygosity was confirmed in four families (A, B, C and D), whereas in one family (E) segregation analysis could not be performed. Affected individuals of families A, B, C and D presented with many small drusen with a slightly pigmented border peripheral to the vascular arcades. These peculiar drusen were most readily observed on ophthalmoscopy, fluorescein angiography and infrared reflectance photography (Fig. 5.9C,D,J). On optical coherence tomography (OCT), the appearance of the drusen peripheral to the vascular arcades was similar to the drusen located in the macula.

Mutations in the *CFH* gene can cause severe renal failure in patients with MPGN-II and aHUS. We did not observe signs of a renal disorder on routine laboratory testing of blood and urine in the drusen patients with compound heterozygous *CFH* variants. It is possible that in these patients, the residual complement factor H activity sustains sufficient complement regulatory activity for normal renal function, while the ocular environment may be more sensitive to deposition and damage. Subclinical renal abnormalities, however, remain possible.²⁴

There are a few examples where heterozygosity for a mutation implicated in a Mendelian disorder appeared to result in an increased risk for a complex disease.²⁵ In this study we show that monogenic inheritance of *CFH* variants can result in basal laminar

drusen in young adults, which can progress to maculopathy and severe vision loss later in life. Our findings indicate that basal laminar drusen and AMD belong to a spectrum of diseases, associated with either monogenic or multifactorial inheritance of variants in the *CFH* gene.

Web resources

Online Mendelian Inheritance in Man; <http://www.ncbi.nlm.nih.gov/Omim>

FH aHUS mutation database; <http://www.fh-hus.org/>

Yasara; <http://www.yasara.org>

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Chapter 6

General discussion

Overview

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1. Ocular phenotypes caused by mutations in the *BEST1* gene

This thesis illustrates that Best vitelliform macular dystrophy (BVMD), which is caused by mutations in the *BEST1* gene (formerly known as *VMD2*),^{1,2} is a highly variable phenotype. Even within a single family, the phenotype may range from absence of retinal abnormalities to classical BVMD, as well as multifocal vitelliform dystrophy. The phenotypic differences in and between BVMD and *BEST1*-associated multifocal vitelliform dystrophy are most likely due to additional unknown genetic modifiers and/or environmental modifiers. Possible genetic modifiers will be discussed in paragraph 1.4 of this discussion, whereas possible variation in anatomical and environmental influences is discussed in paragraph 4.

1.1. Fundus autofluorescence and optical coherence tomography in Best vitelliform macular dystrophy

The use of fundus autofluorescence (FAF) and optical coherence tomography (OCT) was shown to be very helpful in obtaining additional information about the shape and composition of BVMD lesions. The subretinal space in BVMD lesions contains auto-fluorescent material. This auto-fluorescent material most likely includes the fluorophores A2-PE-H₂ and its oxidized form, A2-PE. These bisretinoid molecules are fluorescent associations of two all-*trans*-retinal molecules (A2) and phosphatidylethanolamine (PE), that originate in the shed photoreceptor outer segment (OS) discs.³⁻⁵ In addition, histopathologic studies on the eyes of BVMD patients have shown that a significant accumulation of lipofuscin and A2E occurs in the retinal pigment epithelium (RPE) cells,⁶⁻¹⁰ despite the lack of apposition to the photoreceptor OS discs.^{6,8,10,11} Like in *peripherin/RDS*-associated retinal dystrophies and Stargardt disease (STGD1), this correlates with the highly increased FAF signal that is observed in many BVMD patients (Chapter 3). The studies in this thesis show that FAF is able to visualize even small amounts of vitelliform material that are not readily detected on ophthalmoscopy. With FAF, the dynamic process of accumulation and apparent resorption of auto-fluorescent vitelliform material can be monitored easily and non-invasively. Additional information can be obtained with OCT, giving insight into the characteristics of the lesion in the anteroposterior dimension. OCT is able to visualize for instance the site of accumulation of vitelliform subretinal material, the morphology of a scar, as well as its position relative to the neuroretinal detachment. FAF and OCT, especially when used in combination, also appear to give information about BVMD lesions beyond the information obtained with fluorescein angiography.

FAF and OCT appear useful in the clinical assessment and follow-up of BVMD, giving additional insight in the disease process and its course. When therapy for BVMD becomes available, these imaging modalities may prove to be useful parameters in the evaluation of the treatment effect. It should be noted, however, that FAF and time domain (Stratus) OCT are of limited value in the differential diagnosis of other diseases that may present with vitelliform lesions. In chronic detachment of the neuroretina in central serous

chorioretinopathy, a picture of increased FAF may be observed that is quite similar to BVMD, and OCT findings are also quite comparable.^{4,12,13} The same holds true for the other phenotypes that need to be differentiated from BVMD. Adult-onset foveomacular vitelliform dystrophy (AFVD),¹⁴⁻¹⁹ drusen-derived “pseudovitelliform” lesions,²⁰⁻²² and acute exudative polymorphous vitelliform maculopathy may all display FAF and OCT characteristics that are sometimes hard to distinguish from BVMD and/or multifocal vitelliform dystrophy.^{23,24} The degree of FAF in the vitelliform-appearing lesions in these diseases probably depends on the amount of accumulated subretinal material, as well as the relative proportion of autofluorescent components within this material.

In these cases, a similar clinical appearance obviously does not automatically reflect an identical pathogenesis. These findings rather indicate that the retina may have a limited set of responses to genetic defects and environmental influences. The clinical expression of a genetic background, whether or not modified by environmental factors, may converge in common final pathways.²⁵ High resolution imaging techniques such as high resolution OCT may be able to reveal more subtle differences between apparently similar conditions, that are not seen on the time domain OCT, which has a lower resolution. In drusenoid pseudovitelliform RPE detachments, for instance, the RPE is included in the pseudovitelliform detachment, contrary to for instance BVMD lesions. Such differences can be more clearly shown on high-resolution OCT (Fig. 6.1). Contrary to BVMD lesions, the yellowish material in these drusen-derived pseudovitelliform lesions is not primarily derived from shed photoreceptor OSs under the neuroretina, but likely consists of heterogeneous inflammation-associated material, which will be discussed in Section 3.6.

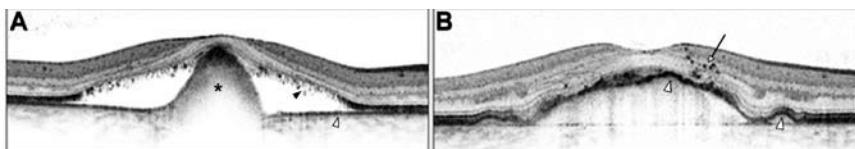


Figure 6.1.

Ultrastructural differences between lesions that both appear vitelliform on ophthalmoscopy.

A. High-resolution OCT image of a lesion in Best vitelliform macular dystrophy (BVMD, combined cicatricial/vitelliruptive stage). A prominent scar is seen, with its tip in contact with the fovea. This scar appears to originate from the retinal pigment epithelium (RPE) cell layer (white arrowhead). Hyperreflective accretions under the photoreceptor layer (black arrowhead) probably correspond to accumulations of photoreceptor outer segments that fail to be phagocytosed by the distant RPE. **B.** Drusenoid pseudovitelliform RPE detachment in a patient with age-related macular degeneration. Contrary to the BVMD lesion in panel A, the RPE is included in the elevated retinal lesion (white arrowheads). Within the retina, hyperreflective spots are visible (white arrow), that probably correspond to the intraretinal hyperpigmented spots that are often observed on ophthalmoscopy in such drusenoid RPE detachments.

1.2. Genetic and pathophysiologic characteristics of Best vitelliform macular dystrophy and other *BEST1*-associated diseases

Best vitelliform macular dystrophy

The bestrophin-1 protein is situated in the basolateral plasma membrane of the RPE and probably functions as a volume-sensitive Ca^{2+} -dependent Cl^- channel that modulates ion flow across the RPE.^{1,26,27} In addition, it is involved in the regulation of intracellular voltage-dependent Ca^{2+} channels.²⁸⁻³¹ Most mutations in *BEST1*-associated AFVD, BVMD, and multifocal BVMD are heterozygous missense mutations, although other mutations including homozygous missense mutations have also been described.^{6,19} Some of these mutations (such as the p.Ala243Val missense mutation) are deleterious, for example, through a haploinsufficiency mechanism. These mutations generally cause a milder phenotypes including AFVD.³² Most mutations, however, exert a dominant negative effect on wild-type bestrophin-1.³³ In the case of a dominant negative mechanism, a markedly reduced to absent Cl^- channel function is observed in animal and *in vitro* studies.³⁴⁻³⁶ On the other hand, haploinsufficiency mechanisms often preserve the Cl^- current to a larger extent,^{33,36} which possibly accounts for the milder phenotype. Another proposed mechanism is a “gain of function”, possibly affecting intracellular Ca^{2+} concentrations through voltage-dependent Ca^{2+} channels.^{28,31}

The propensity of lesions in BVMD (as well as in *BEST1*-associated AFVD) to develop in the macula may be explained by topographic differences in bestrophin-1 protein.⁸ The finding that mutant and wild-type bestrophin-1 are more abundant in the peripheral retina suggests that BVMD results from a relative insufficiency of wild-type bestrophin-1 in the macula.⁸ The macula has unique anatomical and functional features that differ from the peripheral retina. Not only does the macula have the highest photoreceptor and RPE cell density of the entire retina, with a corresponding high metabolic rate, but the choroidal blood flow also reaches a maximum in the macula. Bruch's membrane is thinnest in the macula, compared to more peripherally located retina. These characteristics could make the macular region more vulnerable to this lack of wild-type bestrophin-1. The defective RPE ion conductance would then result in the accumulation of subretinal fluid and a lack of apposition between the photoreceptors and the RPE. The enormous metabolic demand of the densely packed photoreceptors poses a heavy burden on the macula, and requires a highly effective interaction between the different retinal layers and the underlying choroid. These normal interactions are obviously compromised by the separation of the neuroretina from the RPE.

However, the visual acuity in BVMD patients may be unexpectedly high for many years,³⁷⁻³⁹ despite the fact that the photoreceptors are separated from the underlying RPE. There are several possible explanations for this phenomenon. Photoreceptor OS turnover appears to be slower in cones than in rods,⁴⁰ and the shed OS are able to accumulate on the outer neuroretinal surface and in the subretinal space. Part of these OS may still reach the RPE, where they are phagocytosed, albeit in an inefficient way. The lack of photoreceptor apposition would also complicate 11-cis-retinal recovery via the RPE. This

problem may be at least partially bypassed, as the cones have an alternative route for visual pigment regeneration via the Müller cells.^{41,42} In addition, intact surrounding rods are able to secrete rod-derived cone viability factor, which can also promote cone survival.^{43,44}

Although this relative sparing of visual acuity in BVMD may be observed especially in the earlier stages, visual acuity tends to show a slowly progressive decline in BVMD patients over a period of several decades.³⁸ In other cases, visual acuity can show a sharp decline in visual acuity within a few years. Apart from possible detrimental influences of mutant bestrophin-1 on RPE cell function, a progressive build-up of toxic fluorophores in the subretinal space and the RPE, as seen on FAF and in histopathological studies, may cause damage to the photoreceptors and RPE.^{6,8} Toxic properties of oxidized A2E precursors can damage the adjacent photoreceptors,^{45,46} and A2E derivatives are known to have a wide range of damaging effects on the RPE,⁴⁷⁻⁵² including an inhibition of normal photoreceptor OS phagocytosis.^{53,54} Histopathological studies have shown that a degeneration of the photoreceptors and other elements of the neuroretina may precede RPE atrophy.^{6,8,11} Damage to the RPE can ultimately lead to fibrous metaplasia, scarring, initiation of apoptosis, and RPE atrophy.^{4,5,55,56} In rare instances, BVMD is complicated by choroidal neovascularization, resulting in more rapid visual loss.⁵⁷

The fact that the bestrophin-1 protein is located in the RPE and influences RPE ion conductance is mirrored by the hallmark electrophysiological abnormality in BVMD: a severely decreased or absent light rise on the electro-oculogram (EOG).⁵⁸ However, the EOG response appears to be only indirectly dependent on bestrophin-1 function,^{27,28} and some Best1 mutations are associated with a normal EOG.^{33,59,60} Bestrophin-1 may still influence the EOG, for instance through the influence on other ion channels in the RPE.^{28,29,61}

Autosomal recessive bestrophinopathy, autosomal dominant vitreoretinochoroidopathy, and the microcornea, rod-cone dystrophy, cataract, posterior staphyloma (MRCS) syndrome

Autosomal recessive bestrophinopathy (ARB) is caused by homozygous or compound heterozygous missense or nonsense mutations in *BEST1*.⁶² ARB is not limited to the macula, unlike AFVD and BVMD, but shows panretinal abnormalities, with retinal edema and subretinal fluid. FAF imaging in ARB shows widespread abnormalities. Contrary to AFVD and BVMD, an abnormal full-field electroretinogram (ERG) is seen, reflecting photoreceptor dysfunction in the entire retina, in addition to an abnormal EOG. Moreover, this condition does not only affect the retina and RPE, but also appears to be associated with hyperopia and an increased risk of acute angle-closure glaucoma.⁶² Specific homozygous or compound heterozygous missense or nonsense mutations in ARB preclude normal whole-cell Cl⁻ conductance and normal activation of L-type voltage-dependent Ca²⁺ channels, as there is no wild-type *BEST1*.⁶² In addition, the compound heterozygous missense mutations that have been found in patients with ARB are often located more C-terminally in bestrophin-1 than most *BEST1* missense mutations in BVMD, suggesting that these C-terminal mutations might affect a specific function encoded by this region.⁶²

In autosomal dominant vitreoretinochoroidopathy (ADVIRC), vitreoretinal abnormalities are also accompanied by a certain degree of additional ocular developmental abnormalities, including nanophthalmos, microcornea, and congenital cataract.^{63,64} This anterior segment dysgenesis accounts for the high incidence of angle-closure glaucoma in ADVIRC patients. Like ARB, the full-field ERG is often abnormal, as well as the EOG.^{63,64} In one family, rod-cone dystrophy and posterior staphyloma were additionally observed, corresponding with microcornea, rod-cone dystrophy, cataract, posterior staphyloma (MRCS) syndrome.^{64,65} ADVIRC and the MRCS syndrome are also caused by heterozygous missense mutations, like BVMD. However, contrary to BVMD-associated missense mutations, the ADVIRC/MRCS syndrome-associated missense mutations, situated between c.704 and c.709 in *BEST1* (except for p.Val86Met), alter the regulation of normal exon splicing.^{64,66} This abnormal splicing regulation results in functionally abnormal protein isoforms.

The ocular developmental abnormalities in ARB, ADVIRC, and the MRCS syndrome indicate that bestrophin-1 is much more than just a regulator of the ionic microenvironment of the RPE. In addition, some region(s) within bestrophin-1 apparently have an important role in ocular development. These data also suggest that the distinction between “developmental” and “late-onset” genes may sometimes be rather artificial.⁶⁴ In this respect, it is important to recall that the RPE appears to fulfill a critical role in the regulation of growth signals to both the choroid and the sclera.⁶⁷ Therefore, bestrophin-1 could also take part in the web of events in ocular development.

1.3. Genotype-phenotype correlations in *BEST1*-associated disease

BEST1-associated ocular disease obeys to a genotype-phenotype correlation model. In this model, AFVD is caused by mild heterozygous missense mutations that affect the RPE to such an extent that the EOG remains relatively unaffected. In BVMD and multifocal vitelliform dystrophy, moderately severe heterozygous mutations, mostly missense mutations, diffusely affect the RPE to an extent that the EOG is severely disturbed. In ARB, specific compound heterozygous missense or nonsense mutations cause a phenotype that not only affects the entire RPE and neuroretina, but may also be associated with additional ocular developmental abnormalities that also affect the anterior segment of the eye. Finally, some of the heterozygous missense mutations in *BEST1* that severely affect splicing may not only adversely affect the neuroretina and RPE, but also normal development of the entire eye, including the anterior segment, the crystalline lens, vitreous, choroid and sclera. These mutations result in ADVIRC or the MRCS syndrome, two phenotypes that appear to form a continuum.

1.4. Phenotypic variability, non-penetrance, and possible genetic modifiers associated with *BEST1*-associated disease

The findings in Chapter 3 demonstrate that family members with an identical *BEST1* mutation, for instance the p.Lys194_Ala195insVal mutation, can have either extensive multifocal vitelliform dystrophy or unifocal BVMD, but may also remain completely

disease-free, even with a normal EOG. Such variation can not be solely explained by environmental or age differences. On the contrary, the asymptomatic individual with a normal EOG in the case of the p.Lys194_Ala195insVal mutation was the 44-year-old father of a daughter with early-onset multifocal vitelliform dystrophy. His son had a large BVMD lesion at the time of diagnosis, at age 5. These children both had a markedly abnormal EOG.

Variations in other genes in addition to the *BEST1* variant are probably responsible for this apparently illogical phenotypic variation. The identification of such modifier genes is challenging, as the effect of those genes may be very complex and indirect, and several genetic factors may be simultaneously involved.⁶⁸ Before starting an expensive and time-consuming search for genetic modifiers, one must ascertain that the phenotypic variability is not entirely due to environmental influences and other mechanisms such as genetic heterogeneity.⁶⁸ Several strategies for modifier gene identification are possible, such as linkage analysis, association studies, genome-wide screening, and candidate gene testing. For many genetic disorders, this quest for genetic modifiers is complicated by the rarity of the disease, and the correspondingly small size of the study population.

Experiments in transgenic mice and *in vitro* studies indicate that the level of *BEST1* expression is controlled by the *OTX* gene,⁶⁹ the *MITF* gene,⁶⁹⁻⁷¹ and possibly the *CRX* gene.⁶⁹ These genes encode essential transcription factors that orchestrate ocular development,⁷²⁻⁷⁴ and these factors bind to the *BEST1* promotor region.⁶⁹⁻⁷¹ Variations in these genes and their protein products may alter their influence on *BEST1* promotor activity and therefore the level of *BEST1* expression. Genetic variations in the interacting *BEST1* promotor region itself may also contribute to this variable expressivity. *OTX*, *MITF*, and *CRX* are also of paramount importance in the development and differentiation of the photoreceptors, RPE and numerous other ocular tissues (such as the anterior segment structures). A disturbed interaction of these factors with *BEST1* could therefore also be involved in the pathogenesis of ADVIRC, the MRCS syndrome, and ARB.

1.5. Gene therapy prospects in phenotypes caused by *BEST1* mutations

In BVMD and AFVD phenotypes caused by haploinsufficiency, the delivery of wild-type *BEST1* gene may prove to be sufficient. The null phenotype ARB also appears a suitable candidate for wild-type gene supplementation, and the *cnr* dog model -the canine counterpart of ARB- could be of great help in the development of such a strategy.^{62,75} In gain-of-function *BEST1* mutations, that have a dominant-negative effect on wild-type bestrophin-1, additional methods are required to abolish this effect. These methods could include RNA interference techniques that destroy the mutant messenger RNA.^{76,77} The optimal choice of gene therapeutic approach in ADVIRC and the MRCS syndrome is unclear, as the functional consequences of the mutant protein are not known. In this regard, the prevention of ocular developmental abnormalities in ADVIRC, the MRCS syndrome, but also in ARB, with gene therapy would probably require treatment in the first years of life. This would further complicate decision making on this matter, given the fact that variable expressivity is also seen in for instance ADVIRC.⁶⁶

When effective and as safe as possible gene therapeutic strategies have been developed for BVMD and *BEST1*-related AFVD, these should be reserved for patients that are beyond the carrier stage, as many carriers never develop symptomatic disease. Once the cicatricial and/or atrophic stage with photoreceptor and RPE cell death is reached, with a corresponding drop in visual acuity, gene therapeutic intervention will probably be of no or limited benefit. The results of this thesis illustrate that the staging and proper phenotypic evaluation of BVMD is complex and should not rely on ophthalmoscopy alone. Numerous parameters may prove to be essential in decision making about the usefulness of gene therapy in BVMD and *BEST1*-related AFVD, such as visual acuity, (high resolution) OCT, FAF, and retinal function tests such as multifocal ERG. After all, a combination of these parameters will provide the most reliable and detailed information on the viability of the macular photoreceptors and RPE.

2. Retinal dystrophies caused by mutations in the *peripherin/RDS* gene

The peripherin/RDS protein is a structural photoreceptor protein, that is essential for the formation of normally shaped photoreceptor outer segment (OS) discs and lamellae. Mutations in the *peripherin/RDS* gene are associated with extreme phenotypic heterogeneity (Chapter 4). Phenotypes may range from central, predominantly macular dystrophies, such as central areolar choroidal dystrophy (CACD), to multifocal pattern dystrophy simulating STGD1, cone-rod dystrophy, as well as typical retinitis pigmentosa (RP). Even within the subgroup of central *peripherin/RDS*-related phenotypes, a broad phenotypic range can be observed, sometimes mimicking atrophic age-related macular degeneration (AMD) and/or neovascular AMD to a large extent. This predominantly macular phenotypes include CACD, MPD, butterfly-shaped pigment dystrophy, AFVD, and AMD-like late-onset maculopathy. Another puzzling observation is the broad range of phenotypes within a single family, and even a different phenotype between the two eyes of a single patient, associated with an identical *peripherin/RDS* mutation. These fascinating phenomena have to be explained by additional modifying genetic, anatomic, and environmental factors, that will be discussed in the following sections.

2.1. Fundus autofluorescence and lipofuscin accumulation in *peripherin/RDS*-associated retinal dystrophies

All of the aforementioned *peripherin/RDS*-associated phenotypes initially show a certain degree of increased FAF, which implicates the accumulation of lipofuscin. Chapters 2 and 4 show that FAF may be a useful tool in the clinical evaluation of CACD and the follow-up of its progression, as well as in the diagnosis (and possibly the follow-up) of multifocal pattern dystrophy simulating STGD1. Lipofuscin accumulation was directly shown to be present in the only histopathological study on *peripherin/RDS*-associated disease that has been published to date, in a patient with butterfly-shaped pigment

dystrophy.⁷⁸ The light-dependent formation of lipofuscin and its toxic constituents such as A2E may compromise RPE cell function in many ways, as discussed in Chapters 1 and 2 of this thesis. These mechanisms may eventually lead to cell death through apoptosis, resulting in the chorioretinal atrophy that is seen in many advanced retinal dystrophies. Absent FAF in profound chorioretinal atrophy, for instance in stage IV CACD, is a direct consequence of RPE cell death.

If peripherin/RDS is chiefly a structural protein, why is it that its mutations are associated with lipofuscin accumulation and increased FAF? The answer probably lies in the fact that an optimal photoreceptor OS structure is mandatory for efficient phototransduction and a normal lifespan of these OSs. Mouse models indicate that, whether the detrimental effect of a *peripherin/RDS* mutation is due to a haploinsufficiency or dominant-negative mechanism, the result of this mutant allele is a disorganization of the photoreceptor OSs.^{79,80} These malformed photoreceptors in mutant *rds* mice are more vulnerable to light, and have reduced phototransduction efficiency.⁸⁰⁻⁸³ Contrary to Stargardt disease/STGD1, mutant peripherin/Rds does not appear to exert a strong direct influence on the visual cycle and all-*trans*-retinal accumulation. In the *rds* mouse model, heterozygously affected mice have irregularly shaped photoreceptor OSs, that are shed earlier and at a higher rate than in wild-type mice.⁸⁴⁻⁸⁶ Correspondingly, an increased formation rate and a higher load of phagosomes is observed in the RPE.^{82,83} These effects are more pronounced after prolonged light exposure and in sparsely pigmented eyes.^{82,83} This is not surprising, as light exposure accelerates photoreceptor OS shedding,⁸⁷ and melanin is photoprotective as well as anti-oxidative.^{88,89}

As it is well-established that lipofuscin and A2E primarily originate from photoreceptor OS degradation products,^{51,90} the increased accumulation of lipofuscin and A2E in the RPE in autosomal dominant *peripherin/RDS* phenotypes appears to be secondary to abnormal photoreceptor OS structure and shedding. Provided that these mouse experiments could be extrapolated to the human situation, which is currently unclear, the aforementioned results also indicate that *peripherin/RDS* mutation-carrying patients that are frequently exposed to high levels of light are more likely to accumulate larger amounts of lipofuscin. The same could hold true for patients with lightly pigmented eyes. As a consequence, these patients could have earlier disease progression, given the fact that RPE lipofuscin accumulation may be an important factor in *peripherin/RDS*-related disease pathogenesis.

2.2. Central areolar choroidal dystrophy versus atrophic age-related macular degeneration

Similarities between central areolar choroidal dystrophy and atrophic age-related macular degeneration

CACD and atrophic AMD show clinical similarities that can make the differential diagnosis between these disorders challenging. *Peripherin/RDS*-associated CACD may show a markedly decreased penetrance, which can mask the autosomal dominant pattern of inheritance. Conversely, familial cases of AMD may simulate an autosomal dominant

inheritance pattern, especially when the disorder manifests at a relatively early age. Some *peripherin/RDS* mutations associated with CACD, such as the p.Arg142Trp mutation described in Chapter 4, show a decreased expressivity. In these patients, the age at onset of visual loss may be well over the age of 55. The age at onset of visual loss may therefore overlap with that of atrophic AMD. Some CACD lesions even remain asymptomatic. A minority of CACD lesions may be associated with drusen-like abnormalities on ophthalmoscopy, which further complicates the distinction between CACD and AMD. In addition, geographic chorioretinal atrophy may be observed in stage III and IV CACD. Chorioretinal atrophy in both CACD and AMD shows a tendency to spare the fovea until relatively late in the course of the disease.⁹¹ Such initial foveal sparing may also be observed for instance in STGD1 and retinal dystrophy related to maternally inherited diabetes and deafness.⁹²⁻⁹⁴ There are several possible explanations for this intriguing phenomenon of foveal sparing, such as the observed increased vulnerability of parafoveal rods and S (“blue”) cones compared to the central M and L cones,⁹⁵⁻⁹⁸ as well as the presence of photoprotective macular pigment in the central fovea.^{94,99,100} Unlike rods, cones do not exclusively depend on the RPE for the regeneration of 11-cis-retinal, which could also contribute to their extended survival.^{41,101} In addition, cone outer segment turnover appears to be slower than rod outer segment turnover.⁴⁰

Differences between central areolar choroidal dystrophy and atrophic age-related macular degeneration

Several differences between CACD and atrophic AMD may help to discriminate between these two conditions. In typical CACD cases, the age at onset is before the age of 55, and an autosomal dominant pattern of inheritance is observed. Early stages of atrophic AMD often show typical and numerous drusen or hyperpigmentation in the centre of the macula. Stage I and II CACD, on the other hand, are almost invariably characterized by an area of hypopigmentation. In contrast to CACD, areas of well-demarcated chorioretinal (geographic) atrophy in atrophic AMD are usually bordered by drusen and/or hyperpigmentation, whilst lying in a round to oval zone of more discrete hypopigmentation in CACD. On FAF, CACD shows intensely increased FAF outside the areas of chorioretinal atrophy, whereas these areas often show less intense FAF in atrophic AMD.

In addition, the rate of progression of geographic atrophy (corresponding to absent FAF) appears to be more than double as high in atrophic AMD compared with CACD.¹⁰² This is somewhat surprising, as one would intuitively expect early-onset hereditary macular dystrophies to progress at a higher rate. In AMD, large, soft, indistinct drusen with focal hyperpigmentation and/or a large RPE detachment, due to confluence of such soft drusen, highly increase the risk of development of geographic atrophy.¹⁰³⁻¹⁰⁵ The development of such advanced large drusenoid lesions generally requires a time span of several decades. Once these lesions have formed and have had the time to adversely influence the overlying RPE and photoreceptors, the “breaking point” of viability to irreversible apoptosis may be rapidly reached in this large area.^{106,107} Possibly, the RPE

is more resistant to the accumulation of A2E and its detrimental consequences in *peripherin/RDS*-related CACD, as compared to a sub-RPE accumulation of drusenoid toxic and inflammatory substances, which will be discussed in section 3 of this discussion. Still, this may at best be only part of the explanation of the observed differences in geographic atrophy progression between CACD and AMD.

2.3. Multifocal pattern dystrophy simulating Stargardt disease/fundus flavimaculatus versus Stargardt disease/fundus flavimaculatus

Similarities between multifocal pattern dystrophy simulating Stargardt disease (STGD1)/fundus flavimaculatus and Stargardt disease/fundus flavimaculatus

Stargardt disease (STGD1) is among the most common hereditary retinal dystrophies.¹⁰⁸ It has an autosomal recessive inheritance pattern, and is caused by mutations in the ABCA4 gene.¹⁰⁹ Depending on the precise combination of genetic abnormalities in the ABCA4 gene, other retinal disorders may emerge, such as cone-rod dystrophy or retinitis pigmentosa.¹¹⁰ A conspicuous retinal abnormality in the fundus flavimaculatus subtype of STGD1 is the presence of irregularly shaped (“fish-tail” or “pisciform”) yellow-white flecks at the level of the RPE of the posterior pole, in the macula but also along and beyond the retinal vascular arcades.^{92,111} In time, these flecks may extend towards the equator, eventually merge, and become atrophic.

In *peripherin/RDS*-associated multifocal pattern dystrophy simulating STGD1 (MPD), similar flecks are observed that may show a similar evolution (Chapter 4). As shown in this thesis, these flecks in STGD1 and MPD have the same aspect on FAF. In both conditions, these lesions initially have a highly increased FAF signal.¹¹² As mentioned previously, the photopic and scotopic full-field ERG eventually become affected in advanced MPD. Many STGD1 patients initially also have normal full-field ERGs,¹¹³⁻¹¹⁵ but may eventually develop generalized cone dysfunction,^{92,114,116-118} together with a more discrete panretinal rod dysfunction.^{114,116,119,120} Like in MPD, the presence or absence as well as the onset of full-field ERG abnormalities in STGD1 probably depends on the specific underlying mutations. Abnormalities of the EOG, indicative of generalized RPE dysfunction, have also been observed in significant numbers of MPD and STGD1 patients.^{92,111,115,121}

Differences between multifocal pattern dystrophy simulating Stargardt disease (STGD1)/fundus flavimaculatus and Stargardt disease/fundus flavimaculatus

Even though there are striking similarities between MPD and STGD1, there are also obvious differences. First of all, MPD is caused by autosomal dominantly inherited *peripherin/RDS* mutations, whereas STGD1 follows the autosomal recessive inheritance pattern of ABCA4 mutations. STGD1 patients, especially when affected with the fundus flavimaculatus subtype, often experience a severe decrease in visual acuity between the first and third decade.^{92,93,122-124} In MPD patients, on the other hand, the age at onset is generally in the 5th decade, and these patients generally retain a fairly good visual acuity for several decades. This corresponds well with the findings on FAF. After an early period of increased central

FAF in STGD1 lesions, these central lesions soon show largely decreased FAF, due to RPE atrophy. Central macular FAF changes in MPD, on the other hand, are often more discrete at first and are dominated by an increased FAF signal for a more prolonged period. This is compatible with increased RPE lipofuscin content without marked RPE atrophy in the earlier stages.^{51,125} The fluorescein angiogram in STGD1 often shows markedly reduced choroidal background fluorescence, the so-called “dark choroid”, whereas the choroidal background fluorescence appears normal in MPD.

These differences between STGD1 and MPD may be explained by the essentially different pathogenetic processes in these two retinal dystrophies. Peripherin/RDS mainly has a structural function, whereas the ABCR protein functions as a retinoid transporter, which transports all-trans-retinal from the intradiscal to the extradiscal space, which is essential for proper visual pigment regeneration.¹²⁶ This function is compromised in STGD1, and the resultant accumulation of all-trans-retinal in the photoreceptor outer segments leads to the formation of A2-PE in the photoreceptor outer segments. The overload of toxic substances in the photoreceptors probably compromises photoreceptor function more directly and quickly. After being phagocytosed by the RPE, this A2-PE is transformed to A2E and other toxic derivatives. Consequently, RPE levels of lipofuscin and A2E in STGD1 patients are 6 to 12 times higher than in normal control subjects,¹²⁷⁻¹³⁰ corresponding with increased FAF in early STGD1.^{112,128} Changes in the size and distribution, and probably the total amount of RPE lipofuscin throughout the retina, may also account for the previously mentioned dark choroid on fluorescence angiography in STGD1.^{114,128-130} In STGD1, photoreceptors may degenerate secondary to atrophy of the lipofuscin-laden RPE, although primary toxic effects on photoreceptor function and viability, for instance inflicted by a high load of toxic A2-PE or A2-rhodopsin, are also plausible.^{48,131-133} The absence of a dark choroid on fluorescein angiography in MPD may be an indirect reflection of the difference in the level of RPE lipofuscin accumulation. As a result, the RPE may be able to cope with this stress and support the photoreceptors for a more prolonged period of time in MPD than in STGD1. The generally later onset of primary and/or secondary dysfunction of the central cones in MPD therefore likely accounts for the comparatively better visual acuity and prognosis of MPD.

Thus, although MPD and STGD1 share several characteristics, STGD1 often leads to marked visual loss earlier in life, due to different disease pathways. Quantitative FAF measurements, together with histopathological studies, may prove to be a useful non-invasive imaging method to further differentiate these conditions. The finding of specific FAF patterns may also have prognostic implications, especially when combined with other functional information such as the full-field and multifocal ERG.^{112,118,134} In addition, FAF may prove to be valuable as an outcome parameter in future treatment trials in MPD and STGD1, as it is able to provide both morphological and functional information. At present, however, the quantitative measurements and quantitative comparison of FAF encounters several difficulties. Factors that currently complicate FAF quantification are for instance the variability in the settings of the imaging camera, changes in the relative positioning of the patient and the camera, instability of the laser power over time,

interindividual variability of for instance refractive errors and optic media opacification, as well as variability in the dynamic metabolic processes influencing RPE lipofuscin.

2.4. Phenotypic variability, non-penetrance, and possible genetic modifiers associated with *peripherin/RDS*-associated disease

Differential role of peripherin/RDS in cones versus rods

Although the peripherin/RDS protein is essential for normal photoreceptor OS formation in both cones and rods, peripherin/RDS appears to have distinct functions in cones versus rods.^{80,135} Consequently, *peripherin/RDS* mutations may also affect the phenotype differentially, ranging from preferential rod involvement (in RP), roughly equal cone and rod damage (in cone-rod dystrophy and MPD), to predominant cone involvement (in CACD). For example, rods are less able to cope with a lack of wild-type peripherin/RDS than cones.^{84,86,136} In turn, peripherin/RDS intermolecular disulfide bonding appears more important in cones than in rods.¹³⁵ This is most likely due to the differential structural requirements of the closed rod OS discs, that are covered by plasma membrane, compared to the open cone OS lamellae. Interaction of peripherin/RDS with ROM1 seems to be more important for cone lamellae maintenance.¹³⁵ In addition, peripherin/RDS trafficking to the cone OSs is different from that in rods, and seems to be coupled to proper cone opsin localization.¹³⁵ This is in contrast to rhodopsin trafficking in rods, that is independent of peripherin/RDS transport. Apart from its important structural role in photoreceptor OS formation, there are indications that peripherin/RDS could also be involved in signalling pathways, possibly even in the phototransduction cascade.¹³⁷⁻¹⁴⁰ These functions may also be differentially affected in cones and rods. In the light of these substantial differences, the variable phenotypic consequences of different *peripherin/RDS* mutations makes perfect sense.

In this perspective, the preferential involvement of central cones in CACD caused by the p.Arg142Trp *peripherin/RDS* mutation in this thesis is fascinating, especially when considering the fact that the great majority of *peripherin/RDS* mutations also cause significant rod and peripheral cone dysfunction, as reflected by full-field ERG measurements. The cause for such central cone involvement in p.Arg142Trp-related CACD may be extrapolated from experiments in the mouse models.

The amino acid change of a basic, polar, large arginine residue to the neutral tryptophan with its very different aromatic side chain could significantly affect peripherin/RDS structure. The p.Arg142Trp change occurs near the cysteine residue on position 150 (p.Cys150), which is essential for proper intermolecular disulfide bonding and subsequent higher-order peripherin/Rds oligomer formation in cones but not rods. In addition, it enables the interaction of peripherin/Rds with Rom1 in cones, which seems less important in rods.¹³⁵ In cones, such biochemical interactions enable the normal formation of OS rim structures and lamellae, which is a prerequisite for normal photoreceptor function and phototransduction. A logical consequence is that the p.Cys150Ser mutation causes early-onset cone dystrophy in mice through a dominant-

negative mechanism.¹³⁵ The p.Arg142Trp mutation could therefore be envisioned to affect cone function in a dominant-negative manner by hindering the normal disulfide interactions of the nearby p.Cys150. The dominant-negative effect of p.Arg142Trp may depend on the degree of expression of the mutant allele, just like it was shown for the p.Arg172Trp mutation, that also predominantly affects the cones.¹⁴¹ The propensity for central cone involvement in p.Arg142Trp-associated CACD may be at least partially explained by the huge metabolic demand posed on these specific cones, which would make an optimal configuration of cone OS lamellae essential. The fact that cones do not rely as heavily on normal peripherin/RDS function compared to rods may account for the relatively late onset of visual loss in *peripherin/RDS*-related CACD and MPD, as compared to for instance STGD1.^{80,141}

Genetic modifiers of peripherin/RDS-related disease

Still, the aforementioned findings do not explain the extreme intrafamilial variability that is observed with many *peripherin/RDS* mutations. Such observed phenotypic variability also can not be explained simply by age differences. Therefore, largely unidentified genetic and environmental factors must exert an additional influence on the resultant phenotypic outcome. At present, this makes it difficult to establish genotype-phenotype correlations, even in supposedly monogenic retinal dystrophies. Apart from possible differences in dietary and smoking habits, as well as variations in light exposure,¹⁴² which all probably contribute only marginally, genes that modify the expression and severity of *peripherin/RDS* mutations have to be implicated.

A plausible candidate modifying gene is the ROM1 gene, which encodes the direct binding partner of peripherin/RDS in the assembly of heterotetramers that promote photoreceptor OS formation. However, our findings and other studies indicate that ROM1 does not play an important role as a modifier of *peripherin/RDS* expression,¹⁴³⁻¹⁴⁵ besides its role in digenic RP.^{146,147}

Another possible genetic modifier is the RPE65 gene, for instance through the modulation of the susceptibility to light-induced retinal degeneration and rhodopsin regeneration.¹⁴⁸⁻¹⁵⁰

Finally, another candidate modifier is the NR2E3 gene, which is a transcription factor that plays a role in photoreceptor (especially rod) differentiation and maintenance. This gene, which -when mutated- can cause enhanced S cone syndrome or autosomal dominant RP in humans,¹⁵¹⁻¹⁵³ directly regulates *peripherin/Rds* expression in mice.¹⁵⁴ Interestingly, NR2E3 itself acts downstream and depends on the presence of the CRX and NRL gene, which are also key transcription factors involved in photoreceptor gene expression.⁷² Human CRX mutations are associated with autosomal dominant cone-rod dystrophy,^{155,156} autosomal dominant RP,^{156,157} as well as Leber congenital amaurosis.¹⁵⁸ Autosomal dominant and autosomal recessive NRL mutations may also cause photoreceptor dystrophies with predominant rod degeneration.^{159,160} Therefore, *peripherin/RDS* expression may not only rely on a single genetic modifier, but may also be the result of the expression of several transcription factors.

2.5. Gene therapy perspectives in phenotypes caused by *peripherin/RDS* mutations

First of all, the delivery of the gene in gene therapy must be feasible in a safe and effective manner, with long-term results, for instance through a viral vector or via nanoparticles. After this is accomplished, the choice of a gene therapeutic approach depends on the type of *peripherin/RDS* mutation.

In haploinsufficiency phenotypes, such as autosomal dominant RP caused by the p.Cys214Ser *peripherin/RDS* mutation,¹⁶¹ merely supplying enough wild-type *peripherin/RDS* to the retina will probably be effective in rescuing the phenotype.^{162,163}

In dominant-negative *peripherin/RDS* mutations, such as the p.Arg172Trp and presumably p.Arg142Trp that mainly affect cones, the effect of the dominant mutant *peripherin/RDS* protein needs to be eliminated. This could be accomplished with for instance ribozymes or small interfering/small hairpin RNA techniques.^{76,79,141,164} These methods may be especially effective in the dystrophies that are dominated by cone dysfunction, such as CACD, as cone photoreceptors do not rely as heavily on the amount of wild-type *peripherin/RDS* as rods do.^{80,86}

In dominant-negative *peripherin/RDS* mutations that are most harmful to rods, such as p.Pro216Leu in autosomal dominant RP,¹⁶⁵⁻¹⁶⁷ the same RNA interference method can prevent the mutant protein from being formed, thus eliminating the dominant-negative component. However, as rods are more vulnerable to the remaining haploinsufficiency state, such phenotypes would probably also require gene replacement with wild-type *peripherin/RDS*.⁷⁹

Besides gene therapy, the delivery of trophic factors such as ciliary neurotrophic factor or rod-derived cone viability factor may also prove to be beneficial.^{43,44,168-171}

Currently, the marked phenotypic variability of a single *peripherin/RDS* mutation and the lack of insight in its cause(s) would complicate decision making in which patients should be treated with gene therapy and when this treatment should commence.

3. Drusen and phenotypes characterized by drusen

Drusen are focal deposits of extracellular debris located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane.¹⁷²⁻¹⁷⁴ Although they are the characteristic lesions of age-related maculopathy and AMD,¹⁷⁵ they are not specific for AMD. Drusen may be found in several other retinal phenotypes, as well as in association with systemic disease such as membranoproliferative glomerulonephritis (MPGN). Phenotypes displaying drusen(-like) lesions are associated with variants in a broad range of known genes (Table 7.1), and other thus far unidentified genes. Drusen in basal laminar drusen (BLD), AMD, and in MPGN type II have been reported to be quite similar on the level of ultrastructure and molecular composition.^{174,176,177} This is not surprising, as these three conditions are all associated with variants in the *CFH* gene.¹⁷⁸ Different subtypes of drusen, such as hard and soft drusen, in for example AMD, may also show regional

Table 6.1.

Retinal diseases other than AMD associated with drusen or drusen-like lesions.

Disease	OMIM phenotype number	Mode of inheritance	Associated gene	Reference(s)
basal laminar drusen	126700	AR	<i>CFH</i>	this thesis
Doyne honeycomb retinal dystrophy (malattia leventinese)	126600	AD	<i>EFEMP1</i> (<i>fibulin-3</i>)	¹⁸²
Sorsby fundus dystrophy	136900	AD	<i>TIMP3</i>	¹⁸³
central areolar choroidal dystrophy	215500	AD	<i>peripherin/RDS</i>	this thesis
AMD-like late-onset maculopathy	-	AD	<i>peripherin/RDS</i>	¹⁴³
North Carolina macular dystrophy	136550	AD	unknown (MCDR1 locus)	¹⁸⁴

AD, autosomal dominant; AMD, age-related macular degeneration; AR, autosomal recessive; OMIM, Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>).

differences in morphology, composition and physical properties.¹⁷⁹ Drusen in conditions like Sorsby fundus dystrophy and malattia leventinese are probably compositionally different from drusen in *CFH*-related phenotypes, being dominated by accumulation of the specifically mutated proteins (TIMP-3 and EFEMP1, respectively).^{180,181}

3.1. Age-related macular degeneration

AMD is the most frequently encountered retinal disease associated with drusen. The formation and evolution of drusen is a continuum: hard drusen may gradually become more numerous, merging and enlarging to soft indistinct drusen, which may further enlarge to form a large, “pseudovitelliform” drusenoid RPE detachment, which is often accompanied by pigmentary changes.^{103,104} Eventually, the latter lesions are often incompatible with normal retinal function, and generally lead to the death of photoreceptors and RPE cells, resulting in visual loss. Geographic atrophy is considered the end stage of drusen evolution.^{91,103} Drusen evolution and geographic atrophy formation may be complicated by choroidal neovascularization, which then further accelerates the process of visual loss.

However, only a minority of persons with drusen actually develop late AMD, which is defined as geographic atrophy and/or neovascular AMD. It is estimated that individuals with hard drusen have a 1-3% chance to develop late AMD within a follow-up period of 30 years.¹⁰⁴ In contrast, patients with soft, indistinct drusen, have a 15-year cumulative

incidence of late AMD of at least 18%.^{104,185,186} However, the interindividual period of time needed for such an evolution is widely variable, sometimes even between both eyes of the same individual.¹⁰³

Apart from the *CFH* p.Tyr402His variant, several other *CFH* polymorphisms and haplotypes are associated with an increased risk of AMD.¹⁸⁷⁻¹⁹² Several other haplotypes of *CFH* and adjacent regions, one of them containing a deletion of *CFHR1* and *CFHR3*,¹⁹³ lower the risk of AMD.^{188,191,194} Interestingly, one of these haplotypes (*CFH* haplotype H2), which may carry a p.Val62Ile variant in the N-terminal region, lowers the risk not only of AMD, but also of atypical hemolytic uremic syndrome (aHUS) and MPGN.¹⁹⁵⁻¹⁹⁷ Many other genes, including several other complement-associated genes, have been found to be associated with AMD. A summary of these genes is given in Table 1.2 of the General Introduction. This broad range of genetic associations already alludes to the interesting, complex, and incompletely understood pathogenesis of AMD, which will be discussed in section 3.6 of this general discussion.

It would be very relevant to reliably identify the small subgroup of patients with hard drusen that are at a high risk to progress to late AMD. After all, such information could give direction to the employment of therapeutic and, more importantly, preventive strategies. Despite the complex and multifactorial etiology of AMD, predictive models based on genetic risk factors, plasma inflammatory parameters, and/or environmental factors, may be very helpful in the assessment of an individual's risk to develop AMD. A predictive model based on 5 common polymorphisms in the *CFH*, ARMS2/LOC387715, and *C2/CFB* genes estimated that persons who carry all risk genotypes have a 14-fold higher AMD risk compared to the general population, and a 285-fold greater risk than the lowest risk group.^{191,198} Alternatively or, more likely, in addition to such genetic prediction models, systemic levels of for instance complement factors, CRP, and interleukin-6 may improve the risk assessment in AMD.¹⁹⁹⁻²⁰² Finally, the inclusion of environmental influences such as cigarette smoking and dietary habits may further enhance this predictive accuracy.^{199,203-205}

3.2. Basal laminar drusen

Basal laminar drusen (BLD), also known as cuticular drusen or early adult onset, grouped drusen, is characterized by the presence of numerous small, "hard" drusen in the macula and often also in the (mid-) peripheral retina (Fig. 5.7). The diagnosis is often established at an earlier age than AMD, although the condition is not infrequently encountered by coincidence during a routine ophthalmological examination. BLD shows a characteristic "stars-in-the-sky" appearance on the fluorescein angiogram. Both the macular and peripheral drusen often show an abnormal FAF pattern, and the peripheral drusen resemble those in the macula on OCT. In our study, 32% of BLD patients had a positive family history for macular disease.

As discussed in Chapter 5, BLD is also a genetically heterogeneous disorder. We demonstrate that BLD may be sporadic, without a clearly identifiable inheritance pattern and without mutations in the *CFH* gene. However, our findings also strongly suggest that

the BLD phenotype may be caused by autosomal recessive inheritance of a specific *CFH* variant together with the p.Tyr402His AMD risk variant. These specific BLD-associated *CFH* variants include p.Gln408X, p.Arg1078Ser, c.350+6T→G, and p.Arg567Gly.²¹

3.3. Basal laminar drusen versus age-related macular degeneration

Similarities between basal laminar drusen and age-related macular degeneration

Drusen in BLD are highly similar to the small hard drusen that are seen in AMD (Fig. 5.4), also on the ultrastructural level.^{175,177} Hard drusen in AMD and BLD may both display a slightly pigmented border.²⁰⁶ Like in AMD, the small drusen in BLD may also merge and enlarge to soft drusen in the macula. With time, these soft drusen may coalesce to form a larger pigment epithelial detachment. Such an RPE detachment sometimes closely mimics vitelliform lesions that are seen in BVMD and AFVD both on ophthalmoscopy and on FAF, where it displays increased FAF intensity. The surrounding drusen on funduscopy and fluorescein angiography are important in the differentiation of this pseudovitelliform lesion from the aforementioned vitelliform lesions, as well as the normal EOG in contrast to BVMD. Like in AMD, BLD patients may eventually develop geographic chorioretinal atrophy or choroidal neovascularization, leading to significant visual loss.

The overlapping range in age at onset as well as the phenotypic similarities can complicate the differential diagnosis between BLD and AMD. This is not surprising, as we and others have shown that BLD and AMD may have a similar genetic and pathophysiological background. Not only is BLD associated with specific *CFH* mutations, it is also strongly associated to the p.Tyr402His AMD risk variant. The allele frequency of p.Tyr402His in BLD is 50-70%, compared to 55% in typical AMD, and 34% in normal controls.²⁰⁷ It can be difficult to recognize the postulated autosomal recessive pattern of inheritance in *CFH*-related BLD. This may be especially difficult in small families, but also in large families, as there may a pseudo-dominant inheritance pattern. This is due to the high carrier frequency of p.Tyr402His in the general population: 30-36% in the Northern European Caucasian population.¹⁹⁹ In Asian populations, the p.Tyr402His carrier frequency is much lower, with 8% in Chinese,²⁰⁸ and 6% in Koreans.²⁰⁹ Besides these similarities between BLD and AMD, there are several clinical differences that may aid in the differentiation of these two retinal diseases.

Differences between basal laminar drusen and age-related macular degeneration

A number of differences help to distinguish between the *CFH*-associated BLD phenotype and typical AMD. One should realize that “typical AMD” is a somewhat artificial term, as AMD itself shows considerable clinical heterogeneity. Nevertheless, the age at onset appears to be earlier in BLD compared to AMD. In atrophic AMD, the age at onset of visual loss is generally beyond 60 years, and often after the age of 70 years.^{210,211} Patients with neovascular AMD often are beyond 70 years of age when first experiencing visual loss.^{210,212,213} In BLD, we have found a mean age at onset of 50 years in the general group.

In the group of symptomatic patients with *CFH* mutations, the age at onset was 57 years. However, it should be noted that the range of the age at onset in BLD was broad, ranging from 35 to 70 years of age, and some patients did not experience visual loss at all.

A striking feature in BLD is the large number of “uniform small (25 to 75 µm), slightly raised, yellow subretinal nodules randomly scattered in the macula”, as described by Gass, that may extend to the (mid-)peripheral retina.²¹⁴ The small drusen in BLD have been described to appear translucent on ophthalmoscopy with retroillumination,²⁰⁶ but this is a feature that may be easily overlooked. It is mainly the large number of macular and often peripheral drusen, as well as the clustering of densely packed drusen at a relatively young age that makes BLD a recognizable and separate clinical entity. In the patients with *CFH*-related BLD, these large amounts of BLD in the peripheral retina could be associated with numerous patches of profound chorioretinal atrophy (Fig. 5.9). This a feature that is not typically observed in AMD. When these peripheral changes in BLD are pronounced, they are easily identified on ophthalmoscopy. The affected sons (B-III.1 and B-III.2) of patient B-II.1 in Section 5.2 of Chapter 5 also illustrate, however, that early BLD changes may be overlooked on ophthalmoscopy, because they are more discrete and may mainly be located in the (mid-)peripheral retina. In these cases, fluorescein angiography with peripheral photographs is very useful, as these basal laminar drusen are clearly hyperfluorescent (Figs. 5.4 and 5.9I,J).

Interestingly, some patients with AMD may also show numerous small peripheral drusen, as illustrated in Figure 5.4 of Chapter 5. Apart from the later age at onset, such an AMD phenotype is actually hard to distinguish from BLD. These BLD-like AMD phenotypes appear to be situated in the grey zone that overlaps with BLD. Although at present speculative, this subgroup of AMD patients may also have specific underlying genetic determinants that overlap with BLD.

3.4. Fundus autofluorescence in basal laminar drusen and age-related macular degeneration

Drusen in both BLD and AMD are associated with FAF abnormalities.^{215,216} Macroscopically, areas of increased and decreased FAF can be seen, that may display specific patterns.²¹⁷ The degree of FAF intensity will largely depend on the level of lipofuscin accumulation in the RPE, as well as the degree of RPE atrophy. When compared to retinal dystrophies such as STGD1, *peripherin/RDS* dystrophies, and BVMD, areas of increased FAF are generally less intensely autofluorescent in BLD and AMD. This probably corresponds to a milder elevation of lipofuscin and A2E concentrations. Individual hard and soft drusen often show a central area of decreased FAF, in most cases surrounded by an annulus of increased FAF corresponding to the outer dimension of the drusen.²¹⁵ There are several possible explanations for this specific FAF pattern.²¹⁵

First of all, the RPE stretched over a druse may contain a thinner layer of lipofuscin granules, therefore corresponding with reduced FAF.¹⁷³ A second possibility may be that the central overlying RPE cells may release lipofuscin granules, which may then be phagocytosed by the RPE cells at the periphery of the druse.²¹⁵ A third explanation

may be that drusen are associated with incipient atrophy of the overlying RPE.²¹⁸ The observation that the overlying RPE also shows altered FAF suggests lipofuscin-related changes in RPE metabolism, that may eventually affect photoreceptor function.^{215,219,220} This is supported by the finding that retinal areas of FAF abnormalities overlying drusen show dysfunction on microperimetry and scotopic and photopic fine matrix mapping with a modified Humphrey central visual field analyser.^{221,222} Histopathologic studies have shown that rod and cone outer segments overlying drusen show abnormalities that correlate with drusen size.¹⁰⁶ A 30% reduction in photoreceptor density is seen in areas overlying drusen.¹⁰⁷ Confluent drusen may become atrophic and may eventually lead to geographic chorioretinal atrophy, which itself is associated with several different FAF patterns.^{102,223,224} It is currently unclear if these different FAF patterns are the result of variable genetic backgrounds. Despite these interesting findings, the role of FAF imaging in clinical practice in AMD is currently negligible. The genotype-phenotype correlations and prognostic implications associated with specific FAF patterns in drusen and geographic atrophy have not been firmly established at present. The same applies to the role of FAF in neovascular AMD.

3.5. Genotype-phenotype correlations in diseases associated with variants in the *CFH* gene

CFH-related disease: a phenotypic spectrum. *CFH* variants are associated with AMD, BLD, the severe renal diseases MPGN and atypical hemolytic uremic syndrome (aHUS), as well as intermediate phenotypes.^{195,225} As discussed in Chapter 5, the broad range of phenotypes associated with *CFH* variants constitutes a spectrum of diseases that have a common pathogenetic origin involving dysregulation of the alternative pathway of complement activation, whether in the fluid phase (plasma) or on the endothelium. Although at present speculative, certain variants in the *CFH* gene (and possibly other genes involved in the alternative complement cascade) could predispose to a retinal phenotype, whereas other variants principally cause renal dysfunction (Chapter 5).

Genotype-phenotype correlation model. In this thesis, we propose a genotype-phenotype correlation model for *CFH*-associated disease (Fig. 5.6). In this model, early-onset MPGN and aHUS, which have an onset in the first years of life and have a high mortality rate, are located at the severe end of the disease spectrum. These diseases are associated with pronounced systemic dysregulation of the complement alternative pathway. Early-onset MPGN and aHUS are the result of specific homozygous or compound heterozygous *CFH* mutations that lead to an absence of CFH in the serum. The clinical consequences are disastrous, as CFH is essential for the inhibition of the complement alternative pathway. Later-onset MPGN and aHUS, as well as other intermediate renal phenotypes, are caused by single heterozygous mutations, and their phenotypic outcome is modified by other genetic and environmental influences. Early-onset BLD is caused by a *CFH* mutation together with a heterozygous or homozygous p.Tyr402His *CFH* risk variant. AMD (with or without late-onset BLD) is associated with the p.Tyr402His risk variant or other specific *CFH* variants or haplotypes. In addition to these *CFH* variants, AMD requires other genetic

and environmental factors. The latter two diseases are situated at the milder end of the spectrum of *CFH*-related diseases. The classification “mild” refers to mortality rates and the degree of systemic and/or renal complement activation. Of course, the impact of the possible visual handicap caused by BLD and AMD can by no means be judged as mild. As in every gene that shows genotype-phenotype correlation, the proposed genotype-phenotype correlation model for *CFH* is a simplification, that is meant as a global classification of the complex spectrum of diseases related to specific variants in this gene.

Complement activation in AMD. Systemic complement activation does not only play a role in severe early-onset renal *CFH*-related phenotypes like MPGN: chronic systemic complement activation has also been found in AMD.²²⁶ Serum complement C3 activity may also be raised in AMD, although it may not be necessarily associated with the presence or absence of the p.Tyr402His risk variant.²²⁷ It is therefore possible that AMD associated with *CFH* and other complement gene variants is in fact a systemic disorder that becomes clinically manifest only in the eye. Alternatively, or in addition, AMD associated with complement gene variants may be the result of the triggering of the pathophysiological events only in a specific local environment with unique features - in this case the macula - that make it particularly susceptible. It should be noted, however, that the *CFH* p.Tyr402His variant also increases the risk of MPGN.²²⁸ In addition, p.Tyr402His may also be associated with an increased risk of cardiovascular disease, as well as Alzheimer’s disease,²²⁹⁻²³⁵ although these findings are at present not firmly established, as they are contradicted by other studies.²³⁶⁻²³⁹ The influence of the p.Tyr402His variant could therefore reach well beyond the macula.

Genetic susceptibility to AMD. Preliminary studies indicate that carrying specific risk variants in the *CFH* gene may predispose to specific subtypes of AMD.^{207,240,241} Still, one must realize that AMD is a multifactorial disease, being the result of a complex interplay between the genetic background and environmental risk factors.²⁴² The risk will increase with the summation of the number of inherited genetic susceptibility and protective variants, and may be modified by environmental influences. One theory, the “common disease/common variant” hypothesis, proposes that relatively high-frequency alleles within the population cause predisposition to common diseases.²⁴³ In the case of AMD, this has been clearly shown for the p.Tyr402His AMD risk variant in the *CFH* gene. Another theory is the “common disease/rare variant” hypothesis. This hypothesis states that the search of disease-causing genes for common diseases in large groups of unrelated patients is often pointless, as extensive allelic heterogeneity in such populations precludes the finding of strongly disease-associated genes.²⁴⁴ Proponents of this theory advocate large family studies to find gene variants that truly show strong disease association.

Our analysis of the *CFH* gene in BLD families shows that these theories are not mutually exclusive. Several families with an early-onset drusen phenotype (BLD) in our study (Chapter 5) developed drusen only when carrying the common *CFH* p.Tyr402His risk variant in combination with a severe *CFH* gene alteration, such as the p.Gln408X nonsense mutation. The aHUS phenotype is another example of *CFH*-associated disease

with a high degree of non-penetrance and variable expressivity, that requires multiple factors to become clinically manifest. The risk of aHUS is also modified by common genetic risk polymorphisms.²⁴⁵⁻²⁴⁸ In addition, this disease may be triggered for instance by infection, pregnancy, and immunosuppressive agents.²⁴⁸⁻²⁵⁰ Another striking example of multifactorial *CFH*-related disease was provided by a study that described a patient who developed MPGN with an exceptionally late onset.²⁵¹ This patient also developed “AMD” with BLD-like drusen and choroidal neovascularization. This patient carried numerous genetic risk factors for AMD, in addition to carrying a p.Cys431Tyr missense variant, supposedly a mutation, in exon 9 of *CFH*. In addition, this patient was a heavy smoker. Interestingly, proteomic and functional analyses detected plasma levels of CFH in the lower normal range and decreased levels of C3, suggesting a chronic activation of the alternative complement pathway.

These studies illustrate that carrying a rare genetic variant or mutation in *CFH* may not be sufficient to cause a certain disease. However, when these *CFH* variants are co-inherited with several other common genetic susceptibility factors, they may result in clinical disease, especially when predisposing environmental factors are present. The multiple genetic and environmental factors that are assumed to be important in the pathogenesis of AMD are highlighted in the next section. An insight in the complex and captivating ontogenesis of AMD is deemed important, as it may serve as a paradigm for other complement-related phenotypes associated with drusen and their vision-threatening complications, such as *CFH*-related BLD and drusen in MPGN.

3.6. Pathogenesis of age-related macular degeneration: a complex paradigm for other phenotypes associated with drusen and *CFH* gene variants

3.6.1. Possible roles of *CFH*, complement activation, and other factors involved in inflammation

CFH and alternative complement pathway activation. *CFH* is expressed in several ocular structures including the retina, RPE and choroid, at a level that is comparable to the highest levels in the liver, where plasma *CFH* is synthesized.²⁵² In vitro studies show that oxidized photoreceptor outer segments and oxidative stress in general decreases *CFH* expression by the RPE.^{253,254} However, individuals homozygous for the *CFH* p.Tyr402His AMD risk variant do not appear to have different levels of *CFH* transcription and *CFH* protein in the RPE and choroid, as compared to individuals without this risk allele.²⁵⁵ They do have an approximately 2.5-fold higher level of C-reactive protein (CRP) in the choroid.²⁵⁵ CRP is a serum biomarker for chronic inflammation,²⁵⁶ and increased CRP levels significantly increase the risk of both cardiovascular disease and AMD.^{201,231,257} CRP and *CFH* are binding partners, and the p.Tyr402His risk polymorphism is located within the CRP binding site of *CFH*.²⁵⁶ As a consequence, the p.Tyr402His allele variant may impair proper CRP binding.²⁵⁸ Interaction of CRP with *CFH* has an inhibitory effect on complement AP activation, which may to a certain extent be impaired by the p.Tyr402His variant of *CFH*.^{199,256} Eventually, these pro-inflammatory changes and complement

activation lead to damage to host cells, especially in individuals with CRP haplotypes that result in higher levels of CRP in plasma and/or choroid.¹⁹⁹ The increased levels of choroidal CRP may thus reflect a state of chronic inflammation, as a result of diminished complement-inhibitory activity in subjects carrying the p.Tyr402His AMD risk allele. Such effects may be reinforced by additional influences such as oxidative stress, which may decrease CFH expression by the RPE.

An important role for inflammatory mechanisms in drusen pathogenesis has been hypothesized by Hageman and colleagues.^{174,259,260} Drusen contain a large variety of inflammation-related proteins, for instance complement factors (C1q, C3, C5 and C5b-9),^{174,261} acute phase proteins (such as CRP, vitronectin, and fibrinogen), complement regulatory proteins (such as CFH, CFB, MCP/CD46, and complement receptor 1/CR1), immune complexes, proteins involved in coagulation and fibrinolysis, as well as lipid components such as apolipoprotein E.^{174,253,262} Several of these complement- and drusen-associated molecules, including CFH, CFI, CFB, C3, and C5, may also be synthesized and secreted by the RPE and retina itself.^{253,262,305} Interestingly, a similar deposition of complement factor C3 and immunoglobulin is also characteristically seen within the glomerulus of patients with membranoproliferative MPGN.²⁶³ The C5b-9 membrane attack complex that results from complement activation, is able to effectuate cell damage, for instance to the RPE and glomerular cells.

Classical complement pathway activation. The recently described associations of AMD with variants in the SERPING1 gene,^{264,265} which encodes the C1 inhibitor, as well as with variants in the C2 gene,²⁶⁶ could also imply a role for classical complement pathway activation in AMD. Although this association was not found by another study,²⁶⁷ such a role is not illogical. After all, apart from the possible cross-talk between the classical and alternative complement pathway,^{268,269} activation of the classical pathway also leads to the formation of C5b-9, through the terminal pathway that is shared by the three complement pathways. As mentioned before, this C5b-9 membrane attack complex is a well-known constituent of drusen and is implicated in cellular damage.^{174,261,270}

Dendritic cells and toll-like receptors. Complement deposits and chemoattractant molecules secreted by the damaged RPE cells may recruit choroidal or blood-derived monocytes. These monocytes invade into Bruch's membrane and the sub-RPE space, where they mature to dendritic cells,²⁷¹ which form the core of the drusen.

Dendritic cells secrete various cytokines, including TNF- α ,²⁷² which may further reinforce the pro-inflammatory feedback loop that is thought to lie at the basis of drusen formation and growth. Dendritic cells are able to sustain and amplify the local inflammatory activation through numerous mechanisms, including complement production and activation, immune complex formation, and extracellular matrix degradation.^{174,273} Both dendritic cells and macrophages are also thought to play an important role in the stimulation of angiogenesis and the development of choroidal neovascularization in AMD.²⁷⁴⁻²⁷⁷

Recently, a specific variant in the *Toll-like receptor 3 (TLR3)* gene was found to be associated with protection against geographic atrophy,^{278,279} although results on this matter are

somewhat conflicting.²⁸⁰ Toll-like receptors are a family of (recognition) receptors involved in innate immunity, that may trigger the expression of a multitude of pro-inflammatory factors. Specifically, TLR3, which is expressed both by the RPE and dendritic cells,²⁸¹⁻²⁸³ recognizes double-stranded RNA from viruses, may induce antiviral immune responses, and may trigger cellular apoptosis in response to viral infection.^{278,282-284} Toll-like receptors may also potentiate phagocytosis by the RPE and dendritic cells.²⁸⁴⁻²⁸⁶ Intriguingly, these findings could suggest a possible influence of viral infection in AMD pathogenesis.

The retinal pigment epithelium: chief conductor in health and disease of the aging retina.

A pro-inflammatory shift in the aging macula. Aging is accompanied by a 2- to 4-fold increase in plasma levels of immune mediators such as tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and CRP.²⁸⁷ An age-related immune dysfunction, known as immunosenescence, is reflected by changes in circulating inflammatory mediators. Such increased inflammatory markers are a strong predictor of AMD,^{199-202,288} but also of mortality, independently of other risk factors and co-morbidity.²⁸⁷ Similar changes in the aging immune system are seen in the neuroretina-RPE-choroid interface,²⁸⁹ such as for example an age-related increase in CFB expression.²⁶² CFB is an activator of the complement AP, through its action as a C3 convertase in the presence of Factor D and properdin. Increased expression of CFB in the RPE is accompanied by the accumulation of complement C3 and C3a deposits at Bruch's membrane and the RPE basal membrane, which is indicative of complement activation.²⁶² Interestingly, TNF- α and IL-6 down-regulate CFH synthesis by the RPE,²⁵³ whereas TNF- α up-regulates CFB production and C3 secretion by the RPE.^{262,290} An age-related increase of these inflammatory cytokines could therefore contribute to the shift towards increased complement AP activation and inflammation in the macular microenvironment. Complement AP activation appears to be critical in the formation of choroidal neovascularization.²⁹¹⁻²⁹³ Therefore, it is plausible that specific AMD-associated variants in the CFB and/or CFH gene, and possibly other genes involved in the complement pathway, may alter the degree of macular complement activation, inflammation, drusen formation, and the development of choroidal neovascularization.

Pro-inflammatory consequences of lipofuscin formation, oxidative stress, and amyloid β deposition in the retinal pigment epithelium and drusen. Constituents of the age-related pigment lipofuscin, such as photooxidation products of A2E and bisretinoids, are able to activate the complement AP.^{294,295} In general, oxidative stress decreases CFH expression and leads to subretinal C3 accumulation.^{254,296} Oxidative damage to the RPE, damaging for instance its mitochondrial DNA,^{54,297} can increase the basal exocytosis of RPE cellular debris.^{290,298,299} These RPE waste products presumably contain intracellular markers, including A2E, that are subsequently coated with complement.^{290,300}

Drusen also contain amyloid β ,^{270,301} a pro-inflammatory component that is also characteristically seen in Alzheimer's disease plaques.^{302,303} Interestingly, amyloid β colocalizes with complement activation, not only in Alzheimer's disease plaques,^{302,303} but also in drusen, making it another candidate activator of complement in drusen.^{270,301}

The amyloid β in drusen most likely originates from degenerate RPE cells.³⁰¹ The concentration of amyloid β is highest at the edges of geographic atrophy, the region that is at high risk for further degeneration.³⁰⁴ However, it is unclear if the amyloid β deposition in drusen contributes to or is the result of AMD pathogenesis. Recent evidence suggests that direct binding of amyloid β to CFH and CFI may activate the complement AP within drusen by blocking the normal function of CFI, which is essential together with CFH for the cleavage and inactivation of C3b.³⁰⁵ In addition, amyloid β increases the production of monocyte chemoattractant protein-1 (MCP-1), and may thus attract and activate macrophages and microglia. These inflammatory cells may subsequently increase the expression of the complement AP activator CFB by the RPE, through the production of TNF- α and interleukin-1 β .³⁰⁶ Therefore, amyloid β could play an important role in the initiation and/or propagation of local complement AP pathway activation and drusen formation, both through a decreased inhibition of the complement AP and through the indirect upregulation of an important activator, CFB. The apparently shared pathogenetic characteristics between AMD and Alzheimer's disease are also supported by epidemiologic associations.³⁰⁷

Complement coating may be beneficial for the clearance of RPE and Bruch's membrane debris and retinal homeostasis.³⁰⁸ The preservation of a not-too-high complement activity may therefore prove to be important in therapeutic trials for AMD and other complement-related diseases that intend to use complement inhibitory therapies. However, in persons with an unfavorable genetic and environmental predisposition, the resultant exaggerated complement AP activation around sub-RPE cellular fragments and other waste products can form the core of drusen formation. This could be the start of a vicious circle of complement activation and damage, recruitment of monocytes by the anaphylatoxins C3a and C5a,^{309,310} that mature to antigen-presenting dendritic cells and macrophages.^{174,311} Apart from leukocyte recruitment, C3a and C5a may also directly induce expression of vascular endothelial growth factor (VEGF),³¹² one of the most prominent stimulators of angiogenesis and the formation choroidal neovascularization through the damaged Bruch's membrane.^{313,314} It is therefore possible that induction of VEGF expression and leukocyte recruitment, as a consequence of oxidative damage and complement activation, may lead to choroidal neovascularization in AMD.

3.6.2. Environmental factors and their interactions in age-related macular degeneration

Being a carrier of risk variants in *CFH* is neither necessary nor sufficient to develop AMD, as many carriers of these risk variants never develop AMD. Therefore, a complex interplay of genetic susceptibility factors must be implicated. These genetic susceptibility factors may interact with environmental influences, such as smoking, dietary habits, light exposure, infectious triggers, as well as structural factors in the anatomical microenvironment of the macula, such as aging changes in Bruch's membrane.³¹⁴

Cigarette smoking. Smoking, an important source of oxidative stress, is a major environmental risk factor for the development of AMD, increasing the risk of AMD 2- to 7-fold in current smokers compared to non-smokers.^{205,315-319} On theoretical grounds, the

negative influence of smoking on the development of AMD can certainly be explained. There are several possible links between smoking and complement activation. In vitro studies show that cigarette smoking alters the ability of CFH to bind C3.³²⁰ In addition, smoking is known to lower plasma CFH levels.³²¹ Several studies indicate that cigarette smoking may work in synergy with the genetic background in conferring an increased risk of AMD development.^{199,322,323} For instance, the odds ratio of late AMD increases from 11 in homozygous carriers of the *CFH* p.Tyr402His AMD risk variant, to 34 if these same homozygotes are current smokers,¹⁹⁹ although another study does not support such an additive effect.³²⁴ As mentioned previously, synthesis of CFH by RPE cells is down-regulated by oxidative stress and oxidized photoreceptor outer segments.^{253,254} Cigarette smoking may therefore be able to increase the level of activation of the complement AP, especially in subjects with specific CFH variants such as p.Tyr402His.^{199,325}

Besides an influence on complement AP activation, there are several other detrimental influences of tobacco smoke that could explain its strong association with AMD. Smoking damages the mitochondrial DNA,³²⁶ which is thought to play a significant role in AMD pathogenesis.²⁹⁷ In this respect, it is worth noting that the predicted *LOC387715/ARMS2* gene may encode a mitochondrial protein,³²⁷⁻³²⁹ and that smoking and carrying the p.Ala69Ser variant in this gene have a multiplicative effect.^{322,330} Smokers also have less protective macular pigment than non-smokers.^{322,331} In addition, plasma levels of VEGF are increased in smokers.³³²

Dietary influences. Other important environmental factors that modify the risk of AMD are dietary habits. An increased risk of AMD appears to be associated with high vegetable fat intake,^{333,334} low dietary intake of antioxidants and zinc,^{335,336} and obesity.^{315,337,338} Conversely, high dietary intake of zinc,^{335,339,340} the macular pigments lutein and zeaxanthin,^{340,341} as well as omega-3 poly-unsaturated fatty acids and fish may decrease the risk of the development of late AMD.^{205,334,342-345} Omega-3 poly-unsaturated fatty acids, that are found in high amounts in fat fish, appear to have an anti-angiogenic and neuroprotective effect,³⁴⁶⁻³⁴⁸ and the effect of dietary measures such as fish intake may depend on the underlying *CFH* genotype.³²⁵ The macular pigments lutein and zeaxanthin may also have a neuroprotective effect, besides their photoprotective function.^{99,349,350} The role of zinc and its supplementation in reducing the risk of AMD deserves special attention, as it illustrates the possible interactions of dietary measures with the underlying genotype and the resulting phenotype, as well as the difficulty to unravel these causal relationships. A large epidemiological study indicates that zinc supplementation not only results in a reduction of the risk of AMD progression, but this risk reduction probably differs strikingly when taking carriership of the *CFH* p.Tyr402His risk allele into account.³³⁹ Homozygous carriers of this risk allele taking zinc supplements had an 11% reduction of the risk of AMD progression, whereas individuals with the homozygous low-risk genotype had a 68% risk reduction of AMD progression when taking zinc supplementation.³³⁹

This effect may be explained by the possible role(s) of zinc in the complement system. Normally, binding of CFH to C3b leads to an acceleration of the decay of the complement

AP C3 convertase C3b,Bb and therefore inhibits complement AP activation. Zinc appears to have the same effect, and when present simultaneously at sufficient concentrations, CFH and zinc appear to show a cumulative effect.³⁵¹ It is possible that this inhibitory effect of zinc on complement activation is more pronounced in the presence of the p.Tyr402 non-risk variant in CFH. However, the precise mechanism for this interaction between zinc and the *CFH* genotype is far from clear. As a matter of fact, some studies indicate that zinc is actually involved in the initiation of AMD in the zinc-rich retina.³⁵²⁻³⁵⁵ Abnormally high zinc concentrations in the macula could initiate the oligomerization and precipitation of CFH proteins.³⁵⁴⁻³⁵⁶ CFH containing the p.Tyr402His risk variant shows a higher propensity to oligomerize.³⁵⁷ The effect of such oligomerization on CFH function is unclear, but some studies indicate a decreased complement AP regulatory effect, which would be directly contradictory to the aforementioned epidemiological findings.

Whatever the mechanism may be, the aforementioned epidemiologic findings clearly point to a protective effect of zinc on AMD progression, that appears dependent on the underlying *CFH* genotype. These findings may therefore have huge implications for the development of preventive strategies. However, replication of these epidemiologic results is required and clarification of the underlying mechanism desired. To date, there is insufficient evidence that the general population should take antioxidant vitamin and mineral supplements to prevent or delay the onset of AMD.³⁵⁸ The effectiveness of antioxidant vitamin and mineral supplementation in halting the progression of AMD in patients that already have AMD comes mainly from one large trial.³⁵⁹

Light exposure. Lipofuscin accumulation in the RPE, as well as FAF abnormalities, are not only found in monogenic retinal dystrophies, but are also seen in AMD.^{51,102,360} However, it is at present unknown if lipofuscin has a direct pathogenic role in the development of AMD.³⁶⁰ There are several lines of evidence that support such a role. Exposure to light, especially from the short, “blue” wavelength, not only promotes the formation of lipofuscin and toxic lipofuscin-derived byproducts of the visual cycle such as A2E, but it also stimulates the formation of basal laminar deposits.^{51,361} These findings appear to correspond with the increased incidence of AMD in persons with above-average exposure to sunlight,³⁶²⁻³⁶⁵ although other studies did not confirm this association.^{366,367} In a mouse model for *ABCA4*-mediated retinal dystrophies, light exposure, as well as vitamin A supplementation, accelerated lipofuscin and A2E accumulation in the RPE.^{127,368,369} Large case-control studies suggest that approximately 3% of AMD cases are associated with specific variants in the *ABCA4* gene,^{126,370-373} the gene that causes STGD1 when both alleles are mutated, although this association with AMD is probably mild and not undisputed.³⁷⁴⁻³⁷⁷ It could be envisioned, however, that mild variants in the *ABCA4* gene may modify the amount of A2E accumulation in AMD. With or without this link to *ABCA4* variants, the finding of increased FAF and lipofuscin accumulation in AMD is interesting, as photooxidation products of A2E are able to activate the complement cascade and inflammation.^{294,295} Moreover, complement AP activation may be involved in light-induced photoreceptor degeneration.³⁷⁸ These findings therefore closely

approximate several factors that are associated with AMD: light exposure, RPE lipofuscin, oxidative damage, inflammation by complement activation, and drusen formation.

Infectious triggers. Another intriguing finding is the association between *Chlamydia pneumoniae* exposure and AMD.^{379,380} In homozygous p.Tyr402His CFH risk variant carriers, the risk of AMD progression increases from almost 2.5-fold to approximately 12-fold when these subjects also have high antibody titers to *Chlamydia pneumoniae*.³⁸¹ The possible association between *Chlamydia pneumoniae* infection and AMD does not appear to be related to the underlying *CFH* genotype, although *Chlamydia pneumoniae* is able to activate the complement AP.³⁸² In addition, the association between a variant in the *Toll-like receptor 3 (TLR3)* gene and atrophic AMD opens the possibility of a role for viral infection in AMD pathogenesis,²⁷⁸ as TLR 3 is involved in the host defense against viruses.²⁸² Although these possible associations with infection are far from firmly established, they are not that far-fetched, as the complement system plays a pivotal role in innate immunity against infectious invaders.³⁸³

All in all, the aforementioned findings illustrate the complex mechanism of genetic predisposition, systemic and local immunological factors, and environmental influences that may be associated with the development of drusen and its related retinal diseases. The aging macula is subject to a constant attack of factors that promote the activation of the immune system. It is therefore in great need of the scarce protective factors that are at its disposition, such as the complement inhibitor CFH. A hereditary sensitivity to an imbalance of these factors could have disastrous consequences for the macula in the long term. The balance in the macular immunological microenvironment could be further disturbed and accelerated by environmental factors such as smoking, light exposure, and adverse dietary habits. The propensity of lesions to evolve towards either chorioretinal atrophy, choroidal neovascularization, or both, could depend on variations in the delicate balance between these many influencing factors.

4. Anatomical location and multifocality of lesions in retinal disease

The preferentially affected retinal area in hereditary retinal disease may be highly variable. Gene mutations that mainly affect rod physiology are of course more likely to cause phenotypes that affect the rod-dominated peripheral retina, such as retinitis pigmentosa. Conversely, some mutations may be more detrimental to the cones that densely populate the central macula, leading to central cone dystrophies such as central areolar choroidal dystrophy. The resultant phenotype is to a certain degree age-dependent. For instance, older affected individuals are more likely to show a larger and/or higher numbers of lesions, that are more often atrophic. However, the intrafamilial and intraindividual variation of lesions in individuals carrying an identical mutation can not be explained purely on the basis of these age differences. Apart from the previously

discussed modifier genes that may be involved, the following factors also likely contribute to such conspicuous variability.

Gene expression of the various structures of the photoreceptor-RPE-Bruch's membrane-choriocapillaris complex shows considerable topographical variation.³⁸⁴⁻³⁸⁷ Moreover, the spatial expression of retinal genes may vary with age.^{386,388,389} There are clear age-dependent differences in the physiology and spatial distribution between cone and rod photoreceptors throughout the retina: for instance, cones show a prolonged survival as compared to the parafoveal rods.^{96,390,391} Like the photoreceptors, RPE cell shape and density also shows topographical variation.³⁹² Functional RPE cell properties, such as enzyme activity, also vary with the retinal location.³⁹³ As expected, macular RPE cells show the highest enzyme activity.³⁹³ Since the RPE cells in the macula have to perform at the highest level, as they are serving the highest density of photoreceptors, this most likely accounts for the largest amount of RPE lipofuscin and A2E in this part of the retina.^{51,394,395} Apart from lipofuscin and A2E, the macula contains higher amounts of melanin and complex granules such as melanolipofuscin and melanolysosomes compared to the extramacular region.^{392,396,397} Again, the relative proportion of these substances changes with age. Bruch's membrane and the choriocapillaris also show marked differences between macula and peripheral retina.³⁹⁸⁻⁴⁰⁰ Environmental influences that may play an additional modulating role, depending on an individual's susceptibility, include factors such as dietary habits,^{341,348,401} sunlight exposure,^{142,362,363} and smoking.⁴⁰² These factors may all be involved in the different propensity mutations to affect different regions of the retina.

Stargardt disease/STGD1, multifocal pattern dystrophy simulating STGD1, multifocal vitelliform dystrophy, as well as drusen in BLD and AMD all share a remarkable feature: multifocality of lesions, that are interspersed by areas of normal-appearing retina. It may seem puzzling why some retinal areas show little abnormalities, whereas other areas are obviously affected. However, there are many underlying variables that may account for this observation.⁴⁰³

The density and distribution of specific photoreceptors, for example, shows considerable variation. Such variation is not only seen between individuals, but also in the same retina, and even between two adjacent RPE cells.^{95,404} The relative amount of S, M, and L cones per RPE cell is also highly variable.^{404,405} A genetic defect that preferentially affects a specific cone subset may therefore cause dysfunction of (groups of) RPE cells that serve a large population of this cone subtype. In a similar manner, local variations in Müller cells could contribute to multifocality of lesions.^{101,406} Focal degenerative processes may thus depend on the random variation of local cell populations. The RPE itself also exhibits striking cell-cell variability in for instance melanin and lipofuscin content, as well as the expression of several proteins.

Another explanation of multifocality and interindividual phenotypic variation may be found in genetic and epigenetic mosaicism and modification of the photoreceptors and RPE.⁴⁰⁷ The term genetic mosaicism refers to differences between groups of cells and even individual cells due to differential gene expression.⁴⁰⁸ Epigenetic modification

refers to mechanisms of genetic modification that are heritable but do not involve direct alteration of the DNA sequence.⁴⁰⁹ This can be accomplished through mechanisms such as hypermethylation of gene promoters and covalent modification of histones. As a result, gene expression may be altered not only on the panretinal level, but also on the focal level, leading to multifocality of lesions.

These findings illustrate the many possible interindividual, intraindividual, intraretinal, and even intercellular differences between the numerous retinal structures that are important for retinal function. In this sense, uniformity of a clinical picture would be more surprising than the variability that is often observed.

5. Future perspectives

There is a great need for therapies that can actually stop the progression of the degenerative process in inherited retinal disease, rather than trying to control end-stage complications like choroidal neovascularization. Information about the genetic background gives a valuable insight into the pathophysiological mechanisms that underly a hereditary retinal disease. This, in turn, will undoubtedly result in future therapeutic regimens aimed at preventing or stopping the disease progression.⁴¹⁰⁻⁴¹³

The development of therapeutic strategies. With regard to these future treatment options, there are many difficult questions that need to be answered, concerning who should be treated, and how and when treatment should be started. Ideally, the identification of disease-associated genetic and environmental factors will elucidate the pathophysiological mechanisms to such an extent that preventive measurements can be implemented safely and in an optimal time frame.

However, not all interventions require revolutionary techniques like gene therapy. Behavioral changes, for instance refraining from smoking and adapting optimal dietary habits, already appear to be realistic and effective goals in lowering the risk of AMD. The effectiveness of these measures has, to date, not been firmly established in “monogenic” hereditary retinal dystrophies.

In retinal dystrophies associated with for example *peripherin/RDS* and *BEST1* mutations, but even more in AMD, the feasibility of additional interventions such as gene therapy is complicated by incomplete gene penetrance, a relatively low magnitude of risk and possible interactions with environmental factors.^{242,410} In these cases, it may be a challenging task to develop proper cost-benefit analyses of treatments that concurrently have the lowest probability of side-effects. Treatments such as gene therapy and cell transplantation will first be developed for the retinal dystrophies that are relatively frequent and have the highest degree of visual impairment.^{414,415} After all, the benefit of treatment will more easily outweigh the possible risk of intervention in these cases. However, as more experience is gained with these and other gene therapeutic strategies, the number of patients that are eligible for a certain form of gene therapy will undoubtedly expand. It could be envisioned that, in the future, the type of underlying genetic defect

may ultimately determine the choice of treatment for a given retinal dystrophy, and an individual's response to such treatments.

Especially in the highly variable *peripherin/RDS*- and *BEST1*-associated phenotypes described in this thesis, careful patient selection and proper outcome parameters are of the utmost importance in the design of clinical trials.⁴¹⁶ For the right patient selection, it is essential to have sufficient knowledge about the natural history and clinical characteristics of the disease. The confounding variables should be identified, such as the influence of modifier genes, systemic diseases, as well as dietary and environmental influences. The choice of suitable outcome parameters is a delicate issue. Measurement of visual acuity, static and kinetic perimetry, FAF, OCT and (multifocal) ERG may all prove to be useful parameters. Which tests are eligible to be used as an outcome parameter, depends on which clinical parameters have to be included to test the treatment efficacy, and how sensitive these tests are to reliably record significant differences.

The aforementioned lack of predictability of the phenotypic consequences of *peripherin/RDS* and *BEST1* mutations makes it particularly difficult to provide adequate genetic counseling to the patient. This applies not only to clinically affected individuals, but even more to asymptomatic carriers of genetic risk factors. In this regard, the possible psychological burden of knowing to be at risk for a certain disease should not be underestimated.

Suitable animal models, large-scale epidemiological studies and randomized-controlled trials are all important for the development of appropriate preventive and therapeutic strategies. In the case of retinal dystrophies such as those associated with *peripherin/RDS* and *BEST1* mutations, as well as retinal disease such as BLD caused by compound heterozygous *CFH* gene variants, one gene is predominantly responsible for the pathophysiological sequence. In these retinal diseases, it may seem a little less difficult to disentangle the web of causality than in AMD. However, large epidemiological studies are hardly feasible because these phenotypes are relatively rare. The development and improvement of proper animal models is invaluable in these cases, for instance to develop accurate gene therapeutic strategies.^{141,417-421}

Pharmacogenetics and complement inhibition. Having a specific genetic profile may modify ones likelihood of response to specific therapies or dietary measurements. For example, individuals with the low-risk AMD *CFH* genotype (p.Tyr402/p.Tyr402) may benefit more from specific nutritional supplements such as zinc than those with the high-risk genotype (p.His402/p.His402).³³⁹ Preliminary studies indicate that patients who are homozygous for the *CFH* p.Tyr402His AMD risk allele respond less favorably to treatment of neovascular AMD with intravitreal injection of bevacizumab.^{422,423} On the other hand, patients with neovascular AMD who carry two "normal" p.Tyr402 alleles in *CFH* appear to fare worse after treatment with photodynamic therapy than patients with at least one p.Tyr402His AMD risk allele.⁴²⁴ Ideally, one might envision a personalized "pharmacogenetic" approach, using a preventive and therapeutic strategy tailored to the individual patient with his or her specific genetic profile and lifestyle.⁴²⁵ The genetic profile would then considerably influence decision making in the choice of a specific

treatment and the advice on specific dietary and lifestyle changes.

As AMD and BLD are tightly linked to complement activation, inhibition of such complement activation could be an attractive preventive and/or therapeutic approach. Several possible agents can be used for this purpose.^{426,427} Compstatin, for instance, is a potent and selective inhibitor of C3, the central component in the complement cascade.⁴²⁸⁻⁴³⁰ Another potential therapeutic candidate for complement inhibition in AMD and BLD is eculizumab. Eculizumab is a humanized monoclonal antibody directed against C5, that blocks the proinflammatory and cytolytic effects of terminal complement activation.⁴³¹ In a clinical context, eculizumab has already been approved for the treatment of paroxysmal nocturnal hemoglobinuria.⁴³¹ CD59, which blocks the formation of the C5b-9 membrane attack complex, is another potential therapeutic agent that was shown to inhibit the formation of choroidal neovascularization in mice.²⁹³ The supplementation with normal CFH or the upregulation of normal *CFH* is yet another possible future therapeutic route.

But how and when should these complement inhibitors be administered? Systemic administration of such inhibitors does not seem an attractive approach in AMD and BLD, as the complement system is an essential part of innate immunity. Systemic complement inhibition therapy would at least have to restore the required delicate balance of complement activation in the complement pathways. This becomes even more complicated given the fact that marked inter- and intraindividual variations of complement concentrations are observed. Thus, intraocular delivery of complement inhibitors appears a safer and more feasible approach. Such topical complement inhibition may be achieved through intravitreal injections, an intravitreal long-term delivery device, or - most elegantly - eye drops. The first two options are more invasive, but probably more powerful in reaching their target, the retina, as eye drops face the problem of limited penetration to the posterior segment of the eye.

Even when complement inhibitors are administered locally in the eye, there are several potential drawbacks of such therapy. When chronically exposed to complement inhibitors, the eye may also become increasingly susceptible to inflammation and infections. In addition, one should bear in mind that the formation of drusen and its complications in AMD and BLD most likely is the result of many years of local complement activation and other processes in the web of inflammatory events. Administration of complement inhibitors in eyes with advanced, confluent drusen, choroidal neovascularization, or geographic atrophy therefore seems pointless because the time of intervention is probably too late in these cases. Ideally, such therapy should thus commence in the earlier drusen stages. However, only a small minority of such lesions eventually evolve to advanced AMD, and this process often takes several decades.^{103,104} Early invasive therapeutic interventions such as intravitreal injections or implants therefore appear unreasonable. Decision making on who should be treated will become much easier with solid information on a patient's individual genetic and environmental risk profile.^{191,198}

The aforementioned studies illustrate that promising therapeutic perspectives become more and more realistic. Nevertheless, numerous unknown variables and

difficulties still have to be cleared up, such as unknown gene-gene interactions, gene-environment interactions, epigenetic variations, as well different treatment options and variables. Again, one should definitely have regard for the psychological consequences for a patient of knowing to be “at risk” for a certain disease. Therefore, diagnostic and therapeutic decisions on these complex issues in individual patients should primarily be made in dialogue with the patient.

The impact of retinal disease and visual handicap. The psychological and social impact of suffering from macular degeneration or a retinal dystrophy should not be underestimated.⁴³²⁻⁴³⁶ In patients with AMD, for instance, the rate of depressive disorder is twice that found generally among elderly people living in the community.⁴³² Visually impaired people are overrepresented in residential care homes,⁴³⁷ where visual impairment is associated with a higher prevalence of depression.⁴³⁸ In this regard, increased public awareness of retinal disease and its impact is needed, as less than 10% of the population is aware of conditions such as AMD.⁴³⁹ Better information and support of the patient and his or her environment, tailored rehabilitation measures, and continuous evaluation of interventions may not only increase visual abilities, but also improve patient satisfaction and psychological well-being.

Since the first description of AMD, BVMD, and STGD1, more than a century ago, huge progress has been made in the clinical and genetic characterization of these and related retinal degenerative disorders, as well as in the understanding of their pathogenesis, especially in the last decade. The first effective treatments for neovascular AMD have emerged and constitute a true revolution for ophthalmologists and, more importantly, the affected patients. To date, treatment options of patients with retinal dystrophies and atrophic AMD are very limited, although the exciting first steps in these areas are encouraging.^{141,163,440-445} We sincerely hope that the findings described in this thesis will contribute to the development of effective preventive and therapeutic interventions in the near future.

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Chapter 7

Summary / Samenvatting

Summary

More than four decades after it was postulated that different retinal dystrophies are determined by different genes, this hypothesis has been proven to be unmistakably true. However, mutations in one gene do not simply cause one disease. This thesis shows that a single gene mutation can be associated with a broad range of retinal phenotypes. Moreover, a single mutation in the same family may be associated with strikingly different retinal phenotypes. Such broad phenotypic variation can only be explained by the additional influence of modifying genes and environmental factors. Conversely, a particular phenotype, such as multifocal vitelliform dystrophy or basal laminar drusen, can be genetically heterogeneous. Some phenotypes can be remarkably similar. Central areolar choroidal dystrophy and basal laminar drusen, for instance, may closely mimic age-related macular degeneration. Multifocal pattern dystrophy simulating Stargardt disease/fundus flavimaculatus can be easily confused with Stargardt disease (STGD1). In this thesis, the clinical characteristics and molecular genetic background of several of these phenotypes were studied elaborately, which enables a better comparison and differentiation of such conditions.

Chapter 1 serves as a general introduction on retinal anatomy and function. The basic principles of molecular genetics are addressed, as well as the general clinical and genetic aspects of retinal dystrophies and age-related macular degeneration.

Chapter 2 offers an introduction on the theoretical and practical background of fundus autofluorescence (FAF). FAF imaging is able to visualize lipofuscin and its precursors in the retinal pigment epithelium (RPE). Lipofuscin is a mixture of substances that contain autofluorescent fluorophores. These fluorophores chiefly originate from the photoreceptor outer segments. Various retinal dystrophies demonstrate abnormalities in the accumulation of these fluorophores. Consequently, a broad range of characteristic FAF patterns may be observed in these retinal dystrophies. FAF imaging appears a useful additive tool in the diagnosis and follow-up of various retinal degenerative disorders, including those described in this thesis.

Chapter 3 is dedicated to the phenotypes caused by mutations in the *BEST1* gene. Section 3.1 reviews the *BEST1* gene and the associated ocular phenotypes caused by *BEST1* mutations. The *BEST1* gene encodes the bestrophin-1 protein, which is localized in the RPE. Bestrophin-1 presumably functions as a volume-sensitive Ca^{2+} -dependent Cl^- channel that regulates ion flow across the RPE. In addition, it influences intracellular Ca^{2+} concentrations by regulating voltage-dependent Ca^{2+} channels. Mutations in the *BEST1* gene have been found in Best vitelliform macular dystrophy, adult-onset foveomacular vitelliform dystrophy, autosomal dominant vitreoretinochoroidopathy, the microcornea, rod-cone dystrophy, cataract, posterior staphyloma (MRCS) syndrome, and autosomal recessive bestrophinopathy. The latter three phenotypes are associated with ocular developmental abnormalities that extend well beyond the retina. This points to a role for bestrophin-1 in normal ocular development, in addition to its aforementioned roles in ion homeostasis. To a certain extent, *BEST1* mutations and the associated phenotypes

comply with a genotype-phenotype correlation model.

Section 3.2 is a detailed clinical and molecular genetic analysis of 20 patients with Best vitelliform macular dystrophy from 15 different families, who all carried a mutation in the *BEST1* gene. Eight different *BEST1* mutations were found, including two novel mutations. A broad phenotypic variability was observed, even in association with a single *BEST1* mutation. As much as 60% of the macular lesions could not be classified as a typical stage of Best vitelliform macular dystrophy. These findings complicated the establishment of distinct genotype-phenotype correlations. FAF and optical coherence tomography, especially when used in combination, proved to be very useful non-invasive imaging methods for the phenotyping and follow-up of Best vitelliform macular dystrophy patients. These imaging techniques are able to visualize abnormalities within vitelliform lesions that are not seen on ophthalmoscopy and fluorescein angiography. As such, FAF and optical coherence tomography also provide a valuable insight into the pathogenesis of Best vitelliform macular dystrophy.

Section 3.3 describes the clinical and genetic findings in multifocal vitelliform dystrophy. Multifocal vitelliform dystrophy is shown to be both clinically and genetically heterogeneous. Fifteen patients with multifocal vitelliform lesions were studied, as well as their affected family members. Nine of these 15 patients (60%) carried a mutation in the *BEST1* gene. Seven different *BEST1* mutations were identified, including 4 novel mutations. The electro-oculogram was abnormal in all patients with a *BEST1* mutation. The age at onset of visual loss was highly variable, as was the number and size of the vitelliform lesions outside the macula. However, the appearance of the lesions outside the macula was quite similar to the central vitelliform lesion on ophthalmoscopy, FAF, and optical coherence tomography, despite the fact that they were smaller. The findings in this study indicate that a multifocal vitelliform response is associated with, but not exclusive to, mutations in the *BEST1* gene. Multifocal vitelliform dystrophy in patients with a *BEST1* mutation and an abnormal electro-oculogram can be considered a multifocal variant of Best vitelliform macular dystrophy.

In **Chapter 4**, section 4.1 serves as a review of the *peripherin/RDS* gene and the broad spectrum of retinal dystrophies caused by mutations in this gene. The *peripherin/RDS* protein is a structural protein that plays an important role in the morphogenesis of the photoreceptor outer segments. Mutations in the *peripherin/RDS* gene may first of all cause various autosomal dominant macular dystrophies. These include three phenotypes that have been classified as pattern dystrophies: butterfly-shaped pigment dystrophy, adult-onset foveomacular vitelliform dystrophy, and multifocal pattern dystrophy simulating Stargardt disease/fundus flavimaculatus. Other *peripherin/RDS*-related macular dystrophies are central areolar choroidal dystrophy and age-related macular degeneration (AMD)-like late-onset macular dystrophy. Apart from these macular dystrophies, *peripherin/RDS* mutations may also cause cone-rod dystrophy, which shares ophthalmoscopic and FAF features with central areolar choroidal dystrophy. In addition, mutations in *peripherin/RDS* are among the most frequently identified mutations in autosomal dominant retinitis pigmentosa. Finally, a specific *peripherin/RDS* mutation causes digenic retinitis

pigmentosa, when co-inherited with a mutation in the ROM1 gene. A single *peripherin/RDS* mutation may cause an intriguingly broad range of phenotypes, even within a single family, which makes it difficult to recognize consistent genotype-phenotype correlations in *peripherin/RDS*-related retinal dystrophies.

Section 4.2 is the largest clinical and genetic study that has thus far been published on central areolar choroidal dystrophy (CACD), describing a group of 103 CACD patients. Follow-up data were available for 42% of the patients, with a follow-up period up to 35 years. This specific macular dystrophy was shown to be caused by autosomal dominant inheritance of a p.Arg142Trp *peripherin/RDS* mutation in 95% of the patients in our study. This high percentage of p.Arg142Trp mutations is most likely due to the fact that *peripherin/RDS* p.Arg142Trp is a relatively frequent founder mutation in the southeast region of the Netherlands. The remaining CACD patients, who were members of the same family, carried a p.Arg172Gln mutation in *peripherin/RDS*. Lesions corresponded to typical CACD stages in virtually all patients. *Peripherin/RDS* p.Arg142Trp-associated CACD was shown to be a central cone dystrophy phenotype. A remarkable variability in disease severity was observed, and non-penetrance was seen up to the age of 64, in up to 21% of mutation carriers. The overlapping age at onset and similar clinical features of CACD and atrophic age-related macular degeneration, together with the decreased penetrance of the p.Arg142Trp *peripherin/RDS* mutation, can make the differential diagnosis between these conditions challenging.

Section 4.3 is the first study that specifically analyzed multifocal pattern dystrophy simulating Stargardt disease/fundus flavimaculatus (MPD), an autosomal dominant pattern dystrophy of the retina. Mutations in the *peripherin/RDS* gene were found to be the major cause of this phenotype. We describe nine different *peripherin/RDS* mutations, including six novel mutations, that were found in 10 different MPD families. All patients with *peripherin/RDS*-related MPD showed a retinal dystrophy characterized by irregular yellow-white flecks in the posterior pole. These flecks were highly similar to those observed in the fundus flavimaculatus subtype of Stargardt disease, which is caused by autosomal recessive mutations in the ABCA4 gene. Clinical characteristics of MPD that may help to distinguish MPD from Stargardt disease are the autosomal dominant inheritance pattern, the relatively late age at onset of visual loss, and the absence of a “dark choroid” on fluorescein angiography. However, the decreased penetrance and markedly variable expressivity of several of these *peripherin/RDS* mutations may complicate the differentiation between MPD and Stargardt disease. In these cases, analysis of the ABCA4 and *peripherin/RDS* genes is especially helpful.

Chapter 5 discusses the clinical and molecular genetic findings in phenotypes associated with variants in the complement factor H (CFH) gene, with an emphasis on the phenotypes associated with drusen, the hallmark lesions in age-related macular degeneration. Drusen are yellow-white deposits between the RPE and Bruch’s membrane. The CFH protein is a multifunctional protein, that primarily plays a role in the inhibition of excessive activation of the alternative pathway of the complement cascade. The complement cascade is an essential part of innate immunity.

Section 5.1 extensively reviews the spectrum of phenotypes associated with variants in the *CFH* gene. This phenotypic spectrum includes renal phenotypes, such as membranoproliferative glomerulonephritis and atypical hemolytic uremic syndrome, as well as ocular phenotypes, including basal laminar drusen and AMD. In addition, several overlapping clinical entities associated with *CFH* gene variants are discussed. An interesting common feature of age-related macular degeneration, basal laminar drusen, and membranoproliferative glomerulonephritis, is the presence of drusen, although these drusen appear with a different age at onset. This common feature of drusen may be explained by a partially similar pathogenetic background, involving an abnormally active alternative complement pathway.

The phenotypic consequences of *CFH* variants depend on their differential impact on the regulatory function of plasma- and surface-bound CFH. Therefore, distinct genotype-phenotype correlations can be observed. In this thesis, we discuss these correlations, and we propose a genotype-phenotype correlation model for *CFH*-related diseases.

In section 5.2, the role of the *CFH* gene was evaluated in 30 patients from different families with early-onset basal laminar drusen. The phenotype of basal laminar drusen is characterized by an innumerable amount of small drusen in the macula, and often scattered throughout the entire fundus. These drusen correspond to a characteristic “stars-in-the-sky” picture on the fluorescein angiogram. We show that basal laminar drusen is a genetically heterogeneous phenotype, as we found four different *CFH* gene mutations in five basal laminar drusen families. Our findings strongly support a recessive disease model in this subgroup of patients with basal laminar drusen. In these families, individuals develop early-onset basal laminar drusen when they carry a *CFH* mutation on one allele and the *CFH* p.Tyr402His risk variant on the other allele. The presence of a *CFH* mutation in the absence of the p.Tyr402His risk variant may contribute to the development of age-related macular degeneration at a later age. Thus, basal laminar drusen and age-related macular degeneration appear to belong to a spectrum of diseases, characterized by drusen, that are associated with either monogenic or multifactorial inheritance of variants in the *CFH* gene.

Chapter 6 is a general discussion of the findings in this thesis. Similarities and differences between similar phenotypes are discussed, based on their clinical, genetic, and pathophysiological characteristics. The proposed genotype-phenotype correlation models are discussed. This general discussion also attempts to shed a light on the phenomena of phenotypic variability and non-penetrance that were regularly observed with the genes in this thesis, by discussing possible genetic and environmental modifying factors. Age-related macular degeneration is the example par excellence of a multifactorial retinal disease associated with complement activation and drusen. Therefore, it is also discussed in the light of the important contributing genetic and environmental factors, and their pathophysiological consequences. Finally, future perspectives on gene therapy and other possible therapeutic approaches are discussed.

A profound knowledge on the clinical, genetic, and pathophysiologic characteristics of the hereditary retinal diseases described in this thesis is important. After all, such

knowledge enables optimal patient information and genetic counseling. In addition, it may facilitate the application and evaluation of future therapeutic strategies in these diseases. A thorough insight in the genetic and phenotypic characteristics of hereditary retinal disease may determine which patients are most eligible for treatments such as anti-angiogenic and gene therapy.

Samenvatting

Veertig jaar nadat werd gepostuleerd dat verschillende retinale dystrofieën worden veroorzaakt door verschillende genen, blijkt deze hypothese onomstotelijk waar. Het is echter niet zo dat mutaties in één gen ook altijd simpelweg één ziekte veroorzaken. Dit proefschrift laat zien dat mutaties in een specific gen geassocieerd kunnen zijn met een breed scala aan klinische beelden. Sterker nog, een specifieke mutatie in een familie kan geassocieerd zijn met opvallend verschillende retinale dystrofieën. Een dergelijke fenotypische variatie kan alleen verklaard worden door de invloed van modificerende genen en omgevingsfactoren.

Omgekeerd kan een specifiek fenotype, zoals bijvoorbeeld multifocale vitelliforme dystrofie of basal laminar drusen, genetisch heteroogeen zijn. Sommige klinische beelden kunnen opvallende gelijkenissen vertonen. Centrale areolaire chorioidea dystrofie en basal laminar drusen, bijvoorbeeld, kunnen sterk lijken op leeftijdsgebonden maculadegeneratie. Multifocale patroondystrofie met kenmerken van de ziekte van Stargardt/fundus flavimaculatus kan gemakkelijk verward worden met de ziekte van Stargardt (STGD1). In dit proefschrift werden de klinische kenmerken en de moleculair genetische achtergrond van een aantal van deze fenotypes uitgebreid onderzocht. Op die manier kan een betere vergelijking en onderscheid worden gemaakt tussen deze klinische beelden.

Hoofdstuk 1 is een algemene inleiding over de anatomie en functie van de retina, de basisprincipes van de moleculaire genetica, evenals de algemene klinische en genetische aspecten van retinale dystrofieën en leeftijdsgebonden maculadegeneratie.

Hoofdstuk 2 begint met het bespreken van de theoretische en praktische achtergronden van fundus autofluorescentie (FAF). Beeldvorming met FAF maakt het mogelijk om de opstapeling van lipofuscine en lipofuscine-gerelateerde stoffen te visualiseren, met name in het retinale pigment epithel (RPE). Lipofuscine is een mengsel van afvalstoffen dat autofluorescente fluoroforen bevat. Deze fluoroforen vinden hun oorsprong met name in de buitensegmenten van de fotoreceptoren. Verschillende netvliesdystrofieën vertonen afwijkingen in de opstapeling van deze fluoroforen. Derhalve kunnen allerhande typische FAF patronen worden waargenomen bij deze retinale aandoeningen. Beeldvorming van de retina met FAF lijkt een nuttig aanvullend middel te zijn in de diagnose en follow-up van verscheidene retinale dystrofieën, waaronder degene die beschreven worden in dit proefschrift.

Hoofdstuk 3 is gewijd aan oogziekten die worden veroorzaakt door mutaties in het *BEST1* gen. In deel 3.1 wordt een overzicht gegeven van het *BEST1* gen, en de oculaire fenotypes die worden veroorzaakt door mutaties in dit gen. Het *BEST1* gen codeert het bestrophin-1 eiwit, dat zich bevindt in het RPE. Bestrophin-1 is waarschijnlijk een volume-sensitief Ca^{2+} -afhankelijk Cl^- kanaal, dat de ion-huishouding ter hoogte van het RPE reguleert. Daarnaast beïnvloedt bestrophin-1 intracellulaire Ca^{2+} concentraties door regulatie van voltage-afhankelijke Ca^{2+} kanalen. Mutaties in het *BEST1* gen zijn gevonden in Best vitelliforme maculadystrofie, adult-onset foveomaculaire vitelliforme dystrofie,

autosomaal dominante vitreoretinochoroidopathie (ADVIRC), het microcornea, staaf-kegeldystrofie, cataract, posterior stafyloma (MRCS) syndroom, en autosomaal recessieve bestrophinopathie. De drie laatstgenoemde fenotypes zijn geassocieerd met afwijkingen in de oculaire ontwikkeling die verder reiken dan de retina. Dergelijke ontwikkelingsanomalieën van het oog wijzen op een rol van bestrophin-1 in de normale ontwikkeling van het oog, naast zijn rol in ion homeostase. Tot op zekere hoogte voldoen mutaties in het *BEST1* gen en de geassocieerde fenotypes aan een genotype-fenotype correlatie model.

Deel 3.2 is een gedetailleerde klinische en moleculair genetische analyse van 20 patiënten met Best vitelliforme maculadystrofie uit 15 verschillende families. Alle patiënten droegen een mutatie in het *BEST1* gen en hadden een abnormaal electro-oculogram. Acht verschillende *BEST1* mutaties werden gevonden, inclusief twee niet eerder beschreven mutaties. Een opvallende fenotypische variabiliteit werd waargenomen, zelfs in associatie met één enkele *BEST1* mutatie. Maar liefst 60% van de vitelliforme laesies kon niet worden geklassificeerd als een typisch stadium van Best vitelliforme maculadystrofie. Deze bevindingen bemoeilijkten het herkennen van duidelijke genotype-fenotype correlaties bij Best vitelliforme maculadystrofie. Beeldvorming met FAF en optische coherentie tomografie (OCT) bleken, met name wanneer ze in combinatie werden gebruikt, zeer nuttige niet-invasieve beeldvormingsmethoden voor de fenotypering en follow-up van patiënten met Best vitelliforme maculadystrofie. Deze beeldvormingstechnieken kunnen afwijkingen in vitelliforme laesies detecteren die niet zichtbaar zijn met funduscopie en fluorescentie angiografie. Op die manier vergroten FAF en OCT het inzicht in de pathogenese van Best vitelliforme maculadystrofie.

Deel 3.3. beschrijft de klinische en moleculair genetische bevindingen in multifocale vitelliforme dystrofie. Multifocale vitelliforme dystrofie blijkt zowel klinisch als genetisch heterogeen te zijn. Vijftien patiënten met multifocale vitelliforme laesies werden bestudeerd, evenals hun aangedane familieleden. Negen van deze 15 patiënten (60%) bleken een mutatie in het *BEST1* gen te dragen. Zeven verschillende *BEST1* mutaties werden gevonden, inclusief vier niet eerder beschreven mutaties. Het electro-oculogram was abnormaal in alle patiënten met multifocale vitelliforme dystrofie en een *BEST1* mutatie. De leeftijd waarop de visusklachten begonnen was zeer variabel, net als het aantal en de grootte van de vitelliforme laesies buiten de macula. Het aspect van deze extramaculaire laesies kwam, ondanks hun kleinere afmetingen, echter behoorlijk overeen met de centrale vitelliforme laesie, zowel op funduscopie, FAF, als op OCT. De bevindingen in deze studie wijzen erop dat het beeld van multifocale vitelliforme dystrofie geassocieerd is met, maar niet exclusief is voor, mutaties in het *BEST1* gen. Daarom kan multifocale vitelliforme dystrofie bij patiënten met een *BEST1* mutatie en een abnormaal electro-oculogram beschouwd worden als een multifocale variant van Best vitelliforme maculadystrofie.

Hoofdstuk 4 bespreekt het scala aan retinale dystrofieën dat veroorzaakt wordt door mutaties in het *peripherin/RDS* gen. Deel 4.1 dient als een overzicht van het *peripherin/RDS* gen en het intrigerend brede spectrum van doorgaans autosomaal dominant

overervende retinale dystrofieën, die worden veroorzaakt worden door mutaties in dit gen. Het *peripherin/RDS* proteïne is een structureel eiwit, dat een belangrijke rol speelt in de vorming van de schijfvormige buitensegmenten van de fotoreceptoren. Ten eerste kunnen mutaties in het *peripherin/RDS* gen verscheidene dystrofieën van de macula veroorzaken, inclusief drie fenotypes die geklassificeerd zijn als “patroondystrofieën”: butterfly-shaped (vlindervormige) pigment dystrofie, adult-onset foveomaculaire vitelliforme dystrofie, en multifocale patroondystrofie met kenmerken van de ziekte van Stargardt/fundus flavimaculatus. Andere *peripherin/RDS*-geassocieerde maculadystrofieën zijn centrale areolaire chorioidea dystrofie en leeftijdsgebonden maculadegeneratie-achtige late-onset maculadystrofie (AMD-like late-onset macular dystrophy). Naast deze maculadystrofieën kunnen *peripherin/RDS* mutaties ook kegel-staaf dystrofie veroorzaken, een beeld dat in dit geval zowel funduscopisch als op FAF kan lijken op centrale areolaire chorioidea dystrofie. Mutaties in *peripherin/RDS* behoren bovendien tot de meest frequente veroorzakers van autosomaal dominante retinitis pigmentosa. Tenslotte kan een specifieke *peripherin/RDS* mutatie, in samenwerking met een mutatie in het *ROM1* gen, digene retinitis pigmentosa veroorzaken. Eén enkele *peripherin/RDS* mutatie kan een opvallend breed scala aan retinale fenotypes veroorzaken, zelfs bij leden van dezelfde familie. Dit bemoeilijkt het herkennen van consistente genotype-fenotype correlaties in *peripherin/RDS*-gerelateerde retinale dystrofieën.

Deel 4.2 is de grootste klinische en genetische studie die tot op heden is gepubliceerd over centrale areolaire chorioidea dystrofie (CACD) en beschrijft de kenmerken van een groep van 103 CACD patiënten. Follow-up data waren beschikbaar van 42% van de patiënten, met een follow-up periode tot 35 jaar. Deze specifieke maculadystrofie bleek in 95% van de gevallen te worden veroorzaakt door autosomaal dominante overerving van een p.Arg142Trp mutatie in het *peripherin/RDS* gen. Dit hoge percentage p.Arg142Trp mutaties is hoogstwaarschijnlijk te wijten aan het feit dat *peripherin/RDS* p.Arg142Trp een relatief frequente founder mutatie is, afkomstig van een gemeenschappelijke voorouder, in de zuidoostelijke regio van Nederland. De overige CACD patiënten, afkomstig uit één familie, droegen een p.Arg172Gln mutatie in *peripherin/RDS*. In vrijwel alle patiënten kwamen de maculaire laesies overeen met een typisch CACD stadium. *Peripherin/RDS* p.Arg142Trp-geassocieerde CACD bleek een centrale kegeldystrofie fenotype te zijn. Een opvallende variabiliteit in de ernst van het ziektebeeld evenals non-penetrantie werd waargenomen tot en met de leeftijd van 64 jaar, in tot wel 21% van de dragers van de p.Arg142Trp mutatie. De differentiaal diagnose tussen CACD en atrofische leeftijdsgebonden maculadegeneratie kan uitdagend zijn, vanwege de gelijkaardige klinische kenmerken, de overlap in de leeftijd waarop deze aandoeningen symptomatisch worden, alsmede de verminderde penetrantie van de p.Arg142Trp mutatie in het *peripherin/RDS* gen.

Deel 4.3 is de eerste studie die specifiek gericht is op de klinische en genetische analyse van multifocale patroondystrofie met kenmerken van de ziekte van Stargardt/fundus flavimaculatus (MPD), een autosomaal dominante patroondystrofie van de retina. Mutaties in het *peripherin/RDS* gen bleken de belangrijkste oorzaak te zijn van dit

fenotype. Wij beschrijven negen verschillende *peripherin/RDS* mutaties in 10 verschillende families, inclusief zes niet eerder beschreven mutaties. Alle patiënten met *peripherin/RDS*-geassocieerde MPD presenteerden zich met onregelmatige, geelwitte vlekjes in de achterpool. Deze vlekjes leken erg op de geelwitte laesies die worden gezien in het fundus flavimaculatus-subtype van de ziekte van Stargardt, veroorzaakt door autosomaal recessieve mutaties in het *ABCA4* gen. Verschillende klinische kenmerken van MPD kunnen het maken van een onderscheid tussen MPD en de ziekte van Stargardt vergemakkelijken. Tot deze differentiaal diagnostische kenmerken behoren de autosomaal dominante overervingswijze, de relatief late leeftijd waarop het visusverlies doorgaans optreedt, evenals de afwezigheid van een “dark choroid” on fluorescentie angiografie. De verminderde penetrantie en de opvallend variabele expressiviteit van meerdere van deze *peripherin/RDS* mutaties kan het onderscheid tussen MPD en de ziekte van Stargardt echter complicerken. In dergelijke gevallen is een analyse van het *ABCA4* gen en het *peripherin/RDS* gen aangewezen.

Hoofdstuk 5 beschrijft de klinische en moleculair genetische bevindingen in fenotypes die geassocieerd zijn met varianten in het *Complement factor H* (*CFH*) gen. Hierbij wordt de nadruk gelegd op de fenotypes die geassocieerd zijn met drusen, de kenmerkende laesies in leeftijdsgebonden maculadegeneratie. Drusen zijn ronde, geelwitte deposities tussen het RPE en de membraan van Bruch. Het *CFH* eiwit is een multifunctioneel proteïne, dat hoofdzakelijk een rol speelt in het afremmen van overmatige stimulatie van de alternatieve route van de complementcascade. De complementcascade is een essentieel onderdeel van de aangeboren immuniteit.

Deel 5.1 is een uitgebreid overzicht van het spectrum van fenotypes die geassocieerd zijn met varianten in het *CFH* gen. Dit klinische spectrum omvat nierziekten zoals membranoproliferatieve glomerulonefritis en het atypisch hemolytisch uremisch syndroom, maar ook oogziekten, zoals basal laminar drusen en leeftijdsgebonden maculadegeneratie. Daarnaast worden verschillende overlappende klinische beelden besproken die geassocieerd zijn met *CFH* varianten. Een interessant gemeenschappelijk kenmerk van leeftijdsgebonden maculadegeneratie, basal laminar drusen, en membranoproliferatieve glomerulonefritis, is de aanwezigheid van drusen, hoewel de leeftijd waarop deze drusen zichtbaar worden verschilt tussen deze aandoeningen. Het gemeenschappelijke kenmerk van drusen bij deze ziekten kan worden verklaard door een deels gelijkaardige pathogenese, die gerelateerd is aan een abnormaal verhoogde activatie van de alternatieve complementcascade. De klinische consequenties van varianten in het *CFH* gen hangen af van hun impact op de functie van plasma- en celoppervlak-gebonden *CFH*. Als gevolg daarvan kunnen specifieke genotype-fenotype correlaties worden waargenomen. In dit proefschrift worden deze correlaties besproken en stellen we een genotype-fenotype correlatiemodel voor *CFH*-geassocieerde ziekten voor.

In deel 5.2 wordt de rol van het *CFH* gen geëvalueerd in 30 patiënten uit verschillende families met early-onset basal laminar drusen. Het ziektebeeld basal laminar drusen wordt gekenmerkt door een ontelbaar aantal kleine drusen in de macula, en vaak ook verspreid over de hele fundus. Deze drusen vertonen een karakteristiek “stars-in-the-sky”

(sterrenhemel-)beeld op fluorescentie angiografie. Wij tonen aan dat basal laminar drusen een genetisch heterogeen fenotype is, aangezien we vier verschillende *CFH* varianten in vijf basal laminar drusen families vonden. Onze bevindingen wijzen sterk op een recessief overervingsmodel in deze subgroep van patiënten met basal laminar drusen. In deze families ontwikkelt men early-onset basal laminar drusen als men een *CFH* mutatie op het ene allel draagt, en de p.Tyr402His variant in *CFH* op het andere allel. Het dragen van een *CFH* mutatie in de afwezigheid van de p.Tyr402His variant kan bijdragen aan de ontwikkeling van leeftijdsgebonden maculadegeneratie op latere leeftijd. Zo lijken basal laminar drusen en leeftijdsgebonden maculadegeneratie dus deel uit te maken van een spectrum van ziektebeelden gekenmerkt door drusen, die geassocieerd zijn met monogene dan wel multifactoriële overerving van varianten in het *CFH* gen.

Hoofdstuk 6 is een algemene discussie van de bevindingen in dit proefschrift. De overeenkomsten en verschillen tussen gelijkaardige fenotypes worden besproken, op basis van hun klinische, genetische, en pathofysiologische kenmerken. De voorgestelde genotype-fenotype correlatiemodellen worden besproken. Daarnaast wordt in deze algemene discussie gepoogd om een licht te werpen op de fenomenen van fenotypische variabiliteit en non-penetrantie, die regelmatig werden geobserveerd in combinatie met de in dit proefschrift besproken genen. Mogelijke modificerende genetische- en omgevingsfactoren van deze erfelijke netvliesaandoeningen worden besproken. Leeftijdsgebonden maculadegeneratie, het voorbeeld bij uitstek van een multifactoriële netvliesaandoening geassocieerd met complementactivatie en drusen, wordt bediscussieerd in het licht van de belangrijke bijdragende genetische- en omgevingsfactoren, en hun pathofysiologische consequenties. Tenslotte worden de toekomstperspectieven wat betreft gentherapie en andere mogelijke therapeutische benaderingen besproken.

Een grondige kennis van de klinische, genetische, en pathofysiologische kenmerken van erfelijke netvliesaandoeningen is belangrijk. Op die manier wordt immers een optimale informatievoorziening en genetisch advies aan de patiënt mogelijk. Daarnaast kunnen deze gegevens de toepassing en evaluatie van toekomstige therapeutische strategieën voor deze ziekten vergemakkelijken. Dergelijke inzichten kunnen immers bepalend zijn in de besluitvorming over welke patiënten het meest in aanmerking komen voor behandelingen met bijvoorbeeld vaatgroeiremmers en gentherapie.

Publications related to this thesis:

Clinical and genetic heterogeneity in multifocal vitelliform dystrophy.

C.J.F. Boon, B.J. Klevering, A.I. den Hollander, M.N. Zonneveld, T. Theelen, F.P.M. Cremers, C.B. Hoyng.

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Mutations in the *peripherin/RDS* gene are an important cause of multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus.

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C.J.F. Boon, N.C. van de Kar, B.J. Klevering, J.E.E. Keunen, F.P.M. Cremers, C.C.W. Klaver, C.B. Hoyng, M.R.

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Invest Ophthalmol Vis Sci (Letter, in press)

Color figures

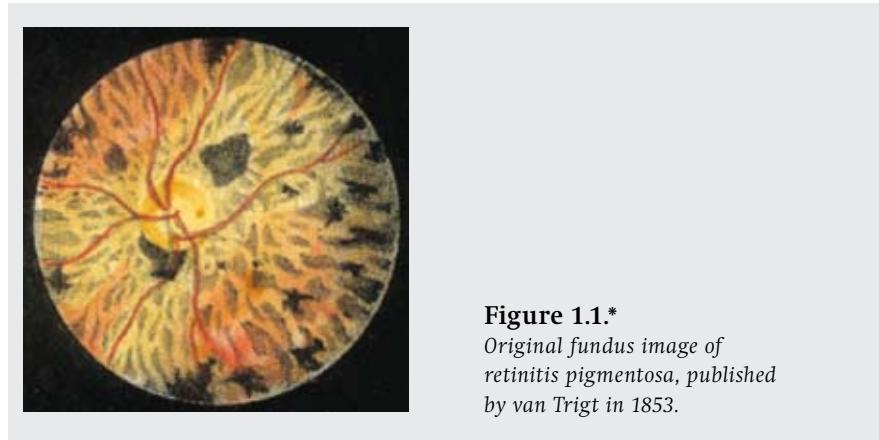


Figure 1.1.*
Original fundus image of
retinitis pigmentosa, published
by van Trigt in 1853.

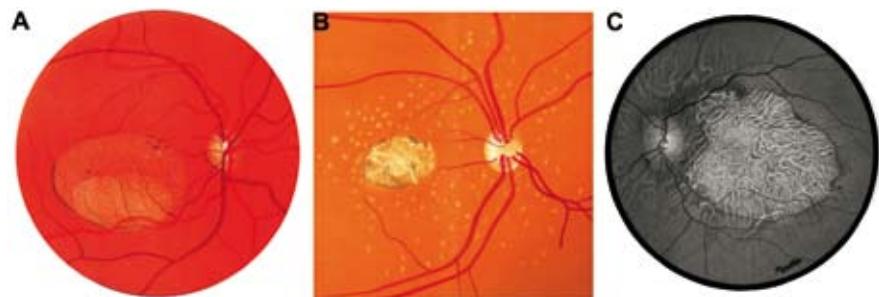


Figure 1.3.*
Original figures published by Best, Stargardt, and Sorsby. A. Best vitelliform macular dystrophy, drawing published by Friedrich Best in 1905. B. Stargardt disease, drawing published by Karl Stargardt in 1909. C. Central areolar choroidal dystrophy, drawing published by Arnold Sorsby in 1939.



Figure 1.13.*
Age-related macular degeneration (AMD). A. Soft drusen in the macula, that merge to a larger pigment epithelial detachment with hyperpigmentation. B. Advanced atrophic AMD, showing profound chorioretinal, "geographic" atrophy of the macula, surrounded by large drusen. C. Neovascular AMD, associated with hemorrhages within the lesion.

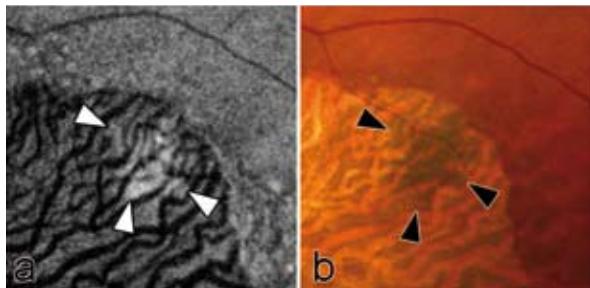


Figure 2.2.*

Pigmented choroidal naevi (black arrowheads), corresponding to focally increased near-infrared fundus autofluorescence (NIR-FAF, white arrowheads). The overlying retinal pigment epithelium (RPE) in atrophic in this patient with central areolar choroidal dystrophy. This RPE atrophy corresponds to decreased NIR-FAF, bordered by flecks of mildly increased NIR-FAF.

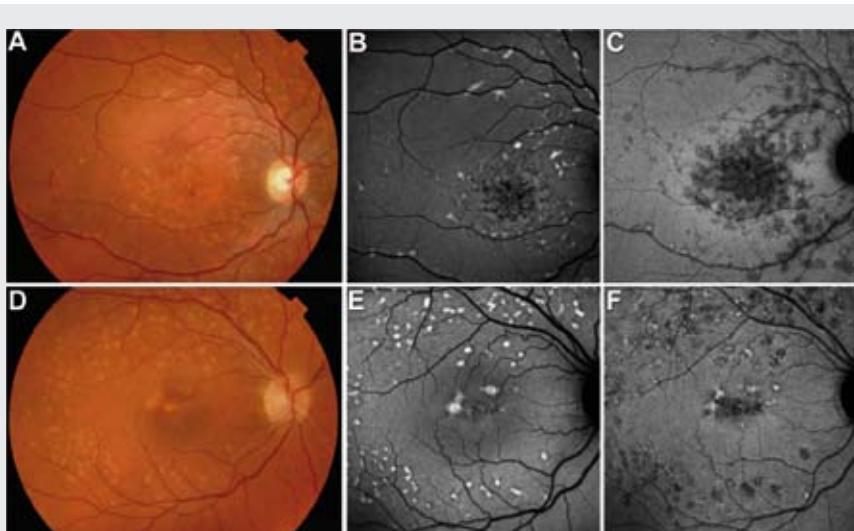


Figure 2.3.*

(**A-C**) Stargardt disease (STGD1)/fundus flavimaculatus, caused by autosomal recessive ABCA4 gene mutations. **A.** Fundus photograph of STGD1, showing multiple yellowish, irregular flecks throughout the fundus, as well as a “beaten bronze” chorioretinal atrophy of the macula. **B.** Short-wavelength fundus autofluorescence (FAF) shows increased FAF of the flecks, and mottled areas of decreased FAF in the macula. **C.** On near-infrared fundus autofluorescence (NIR-FAF), the lesions are clearly larger and more numerous. On NIR-FAF, most lesions have a decreased intensity. (**D-F**) Multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus, caused by an autosomal dominant mutation in the peripherin/RDS gene. The irregular yellowish flecks are highly similar to those in STGD1, on ophthalmoscopy (**D**), short-wavelength FAF (**E**), as well as NIR-FAF (**F**). The macular lesion in this patient, however, appears smaller and less atrophic.

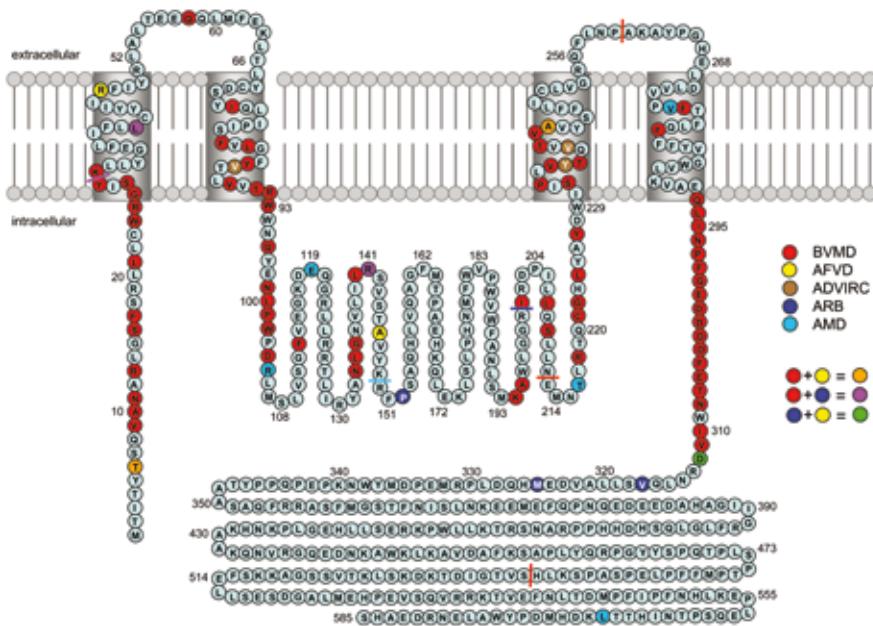
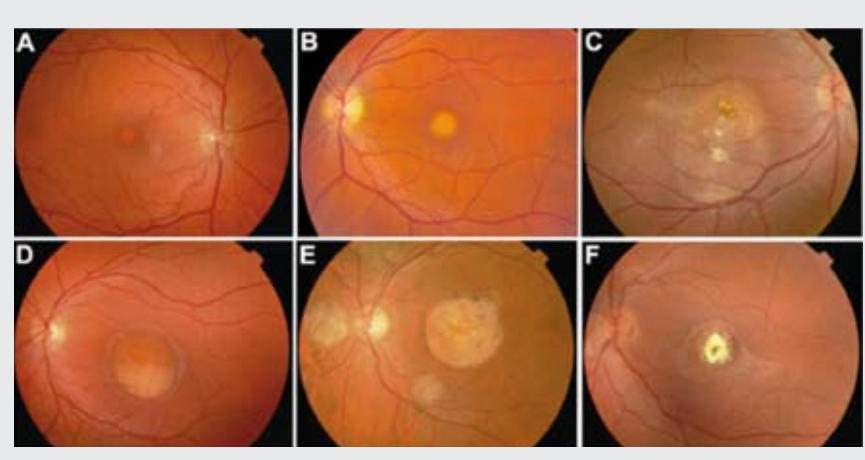
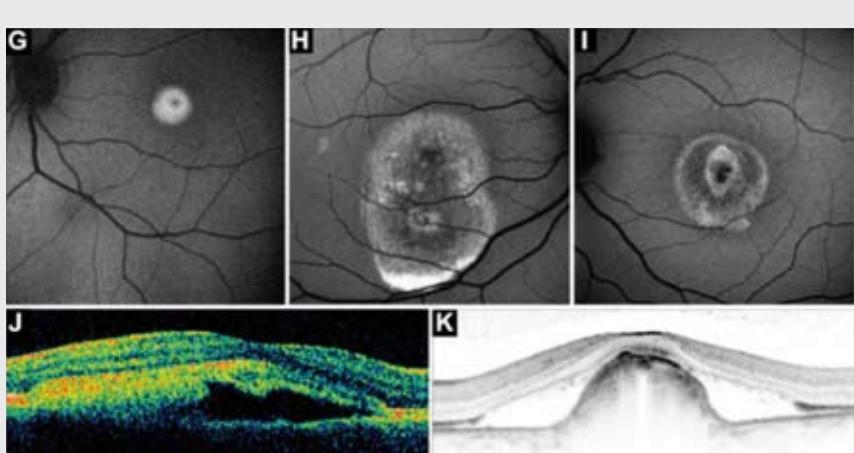


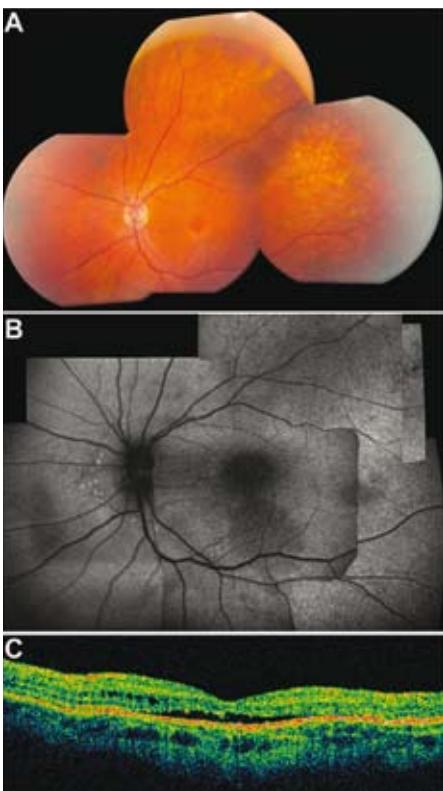
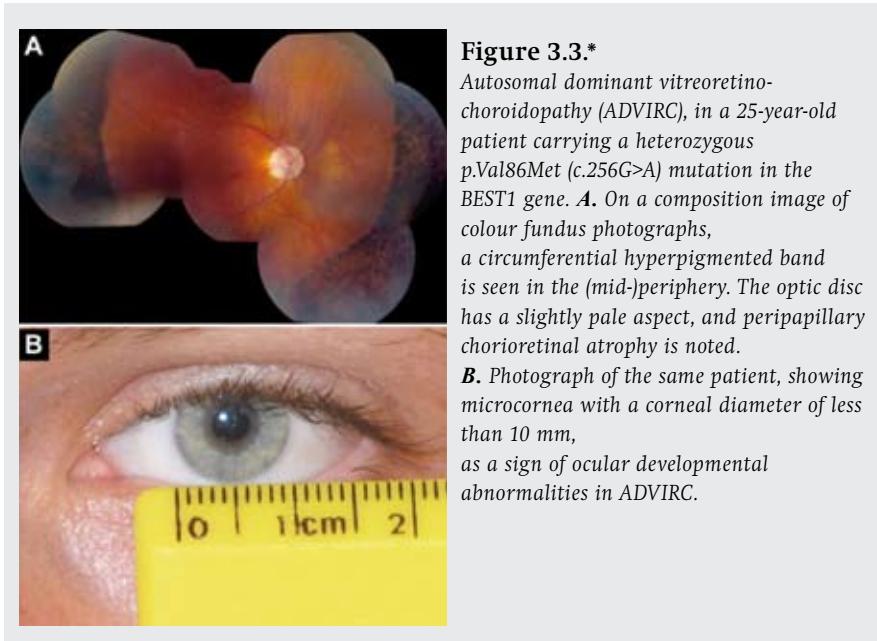
Figure 3.1.*

Protein model of bestrophin-1 (adapted from Milenovic). The model of Milenovic and colleagues was used, as their experiments have shown that only the four domains depicted in the figure are located in the cell membrane. The known human disease-associated mutations are indicated with colours. The protein variants found in age-related macular degeneration are also shown, although functional studies question their significance in disease pathogenesis. Coloured residue: missense mutation or in-frame deletion. Coloured bar: nonsense or frameshift mutation. Abbreviations: BVMD, Best vitelliform macular dystrophy; AFVD, adult-onset foveomacular vitelliform dystrophy; ADVIRC, autosomal dominant vitreoretinochoroidopathy; ARB, autosomal recessive bestrophinopathy; AMD, age-related macular degeneration.



**Figure 3.2.***

Best vitelliform macular dystrophy (BVMD). All patients in this figure had an abnormal electro-oculogram and a positive family history for BVMD. **A.** Previtelliform or carrier stage in a 40-year-old patient carrying a p.Tyr227Asn mutation in the BEST1 gene. The visual acuity (VA) was 20/16. Hypopigmented, slightly atrophic retinal pigment epithelial changes are seen in the fovea, which remained stationary during a follow-up period of 30 years, starting at the age of 10. **B.** Vitelliform stage in a 41-year-old patient carrying a p.Arg25Trp mutation in BEST1. The lesion is entirely filled with yellowish material. **C.** Vitelliruptive or “scrambled-egg” lesion in a 12-year-old patient with a VA of 20/25. This patient carried a p.Lys194_Ala195insVal mutation in BEST1. Scattered yellow-white vitelliform deposits are observed throughout the lesion. **D.** Pseudohypopyon stage in a 11-year-old patient carrying a p.Thr6Pro BEST1 mutation. The VA was 20/25. **E.** Atrophic stage in a 60-year-old patient who carried a p.Thr6Pro mutation in BEST1. Note the multifocal atrophic lesions besides the central, large atrophic lesion in this patient, who had a VA of 10/100. **F.** Cicatricial stage in a 17-year-old patient, who also carried a p.Thr6Pro mutation in the BEST1 gene, with a VA of 20/100. **G.** Fundus autofluorescence (FAF) image of the vitelliform lesion in the patient described with image (B), showing an intensely increased FAF signal. **H.** FAF image of the vitelliruptive lesion of the same patient as on image (C), showing a dispersion of the material of increased FAF. In addition, a small pseudohypopyon with increased FAF can be seen in the inferior part of the lesion. **I.** FAF image of the same cicatricial stage lesion as on image (F), showing decreased to absent FAF in the center of the scar, and increased FAF at the edge of the scar, as well as at the edge of the lesion. **J.** Vertical optical coherence tomography (OCT) section of the pseudohypopyon lesion as depicted on image (D). The vitelliform material in the inferior part of the lesion is hyperreflective on OCT. Deposition of hyperreflective material is also seen on the outer retinal surface under the fovea. The transparent fluid in the upper part of the lesion corresponds with hyporeflectivity on the OCT image. **K.** High-resolution spectral domain-OCT image of the same cicatricial lesion as on images (F) and (I), revealing an elevation of the macula with a highly reflective subfoveal mass, corresponding to the scar. All retinal layers and RPE seem to be preserved over the entire area. The thickened photoreceptor layer at the borders of the retinal detachment corresponds to an outer ring of increased FAF in panel (I). An area of partial photoreceptor loss around the central mass co-locates to decreased perifoveal FAF.



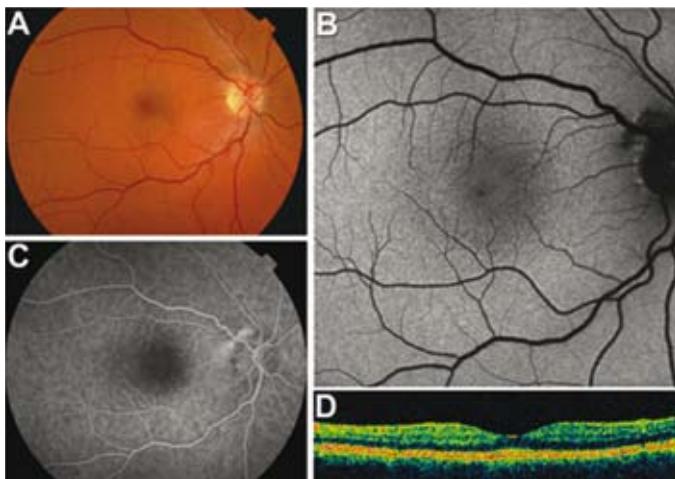


Figure 3.5.*

Carrier stage. **A.** Fundus photograph of patient 4 (p.Thr6Pro mutation in BEST1), the 41-year-old father of patient 5, does not show any abnormalities. **B.** Fluorescein angiography does not show any abnormalities of the macula. **C.** Fundus autofluorescence image, showing a normal autofluorescence pattern of the macula. **D.** Optical coherence tomography scan shows a normal aspect of the retinal layers.

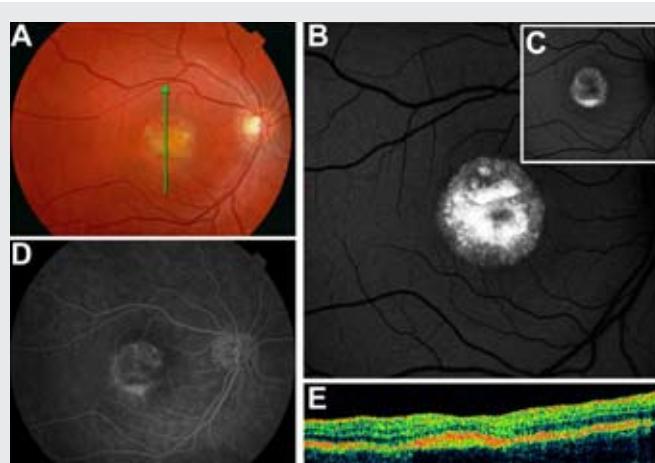
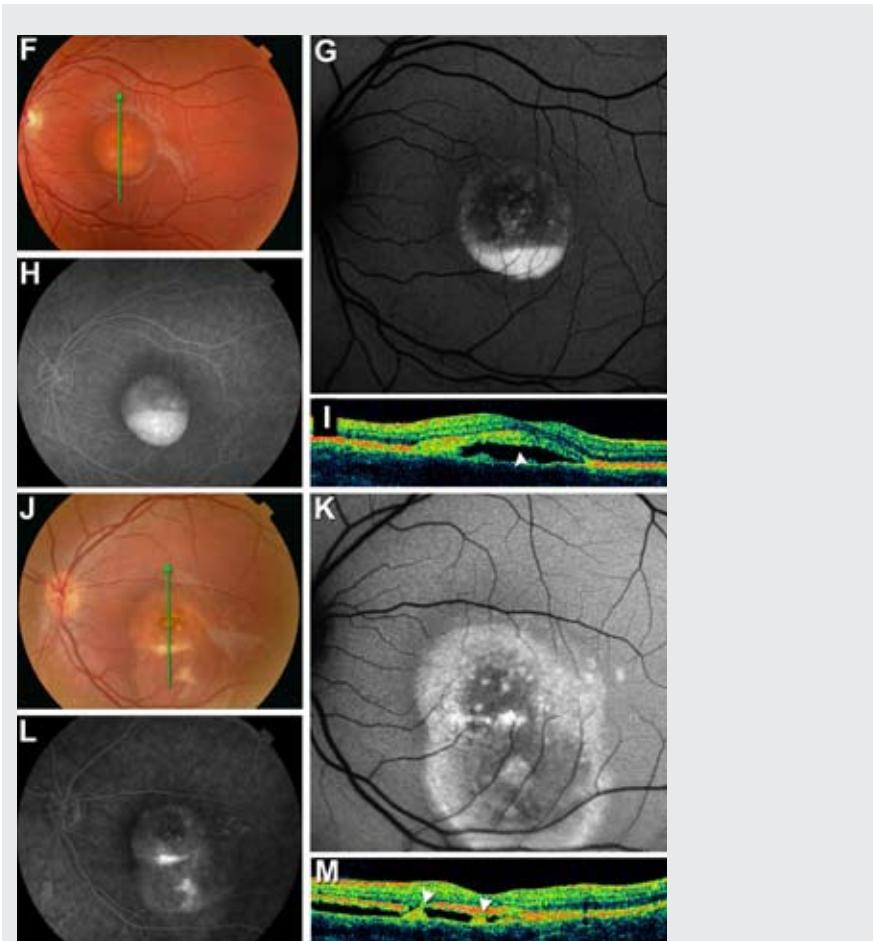


Figure 3.6.*

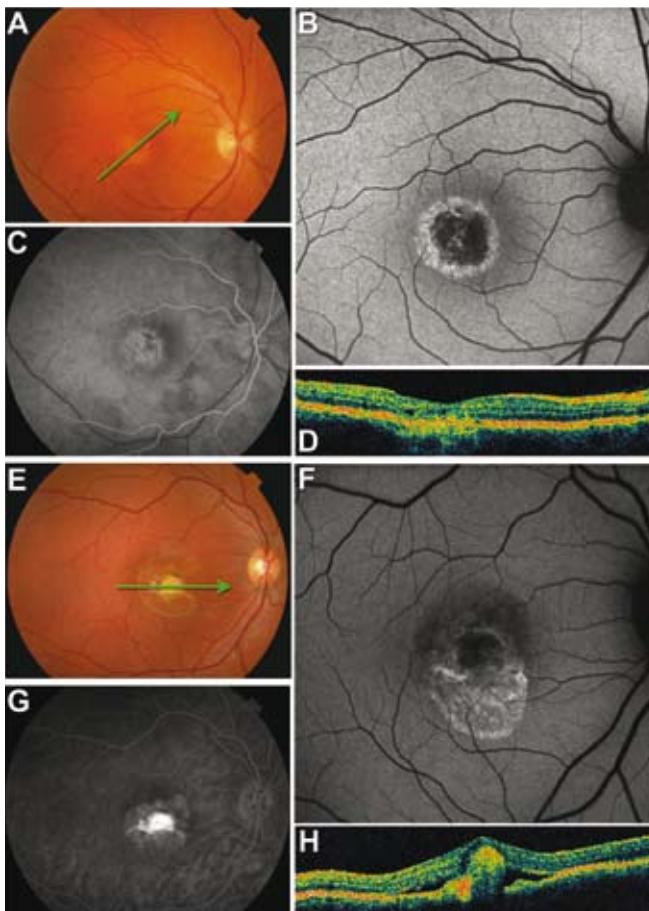
Vitelliform stage.

A. Fundus photograph of patient 3 (p.Thr6Pro mutation in BEST1), showing a yellowish lesion with some fibrosis and pigmentation at the boundaries of the lesion. **B.** Fundus autofluorescence (FAF) of the yellowish parts of the lesion is significantly increased, but

becomes less intense towards the lesions margins. Zones of increased FAF are beginning to cluster within the lesion. **C.** Seven months later, the ophthalmoscopic and FAF picture evolved to a lesion with combined characteristics of the scrambled-egg and pseudohypopyon stage. **D.** At this point in time, fundus fluorescein angiography (FFA) was performed, showing patchy hyperfluorescence in the superior part of the lesion, and a hyperfluorescent pseudohypopyon. **E.** A vertical optical coherence tomography (OCT) scan (arrow in panel A) shows a hyperreflective subretinal lesion in the vitelliform stage.



Pseudohypopyon stage. **F.** Fundus photograph of patient 7, who also carried a p.Thr6Pro mutation, showing yellowish material inferiorly with transparent fluid in the superior part of the lesion. **G.** The yellowish material of the pseudohypopyon shows substantially increased FAF, with only some small spots of increased FAF in the superior part of the lesion. **H.** The pseudohypopyon is intensely hyperfluorescent in the mid-late phase of FFA. **I.** The pseudohypopyon corresponds to subretinal material of increased reflectivity on a vertical OCT section (arrow in panel F). Additional hyperreflective material is attached to the outer retina and to the bottom of the subretinal space (arrowhead). **Vitelliruptive/Scrambled-egg stage.** **J.** Fundus photograph of patient 10 (p.Lys194_Ala195insVal mutation in the BEST1), showing a large lesion with scattered clusters of yellowish material within transparent fluid. **K.** In addition to the locations of clinically apparent yellowish material, increased FAF can be observed at the borders of the lesion where yellowish subretinal material cannot readily be visualized by ophthalmoscopy. **L.** On FFA, the lesion shows patchy hyperfluorescence. **M.** OCT scan taken vertically (arrow in panel J), showing elevation of the retina by subretinal fluid that has no reflectance. The clusters of yellow material that were seen on the fundus photograph appear as prominent hyperreflective structures (arrowheads).

**Figure 3.7.***

Atrophic stage. **A.** Fundus photograph of patient 19 (*p.Gly299Ala* mutation in BEST1) showing an atrophic lesion. **B.** The fundus autofluorescence (FAF) image shows markedly decreased FAF of the atrophic area, surrounded by a ring-shaped area of increased FAF. **C.** Fundus fluorescein angiography (FFA) shows a well-circumscribed, hyperfluorescent window defect due to atrophy of the retinal pigment epithelium. **D.** This atrophic area corresponds with retinal thinning with underlying hyperreflectivity on the optical coherence tomography (OCT) scan (arrow in panel A). Small intraretinal cystoid changes can also be discerned. Cicatricial stage. **E.** The fundus photograph of patient 2 (*p.Thr6Pro* mutation in BEST1) shows a prominent yellow-white subretinal scar with pigmented borders, surrounded by a serous retinal detachment. The visual acuity (20/25) was remarkably preserved. **F.** FAF imaging reveals significantly decreased FAF of the central scar, surrounded by small amounts of material of increased FAF intensity scattered throughout the rest of the lesion. This material is not evident on the fundus photograph. **G.** In the late phase of FFA, the scar is intensely hyperfluorescent due to passive leakage of fluorescein into the scar, whereas the surrounding part of the lesion shows more discrete, patchy hyperfluorescence. **H.** OCT (arrow in panel E) shows a prominent and hyperreflective structure, in contact with the overlying retina. This structure is surrounded by elevated retina with underlying spots of increased reflectivity.

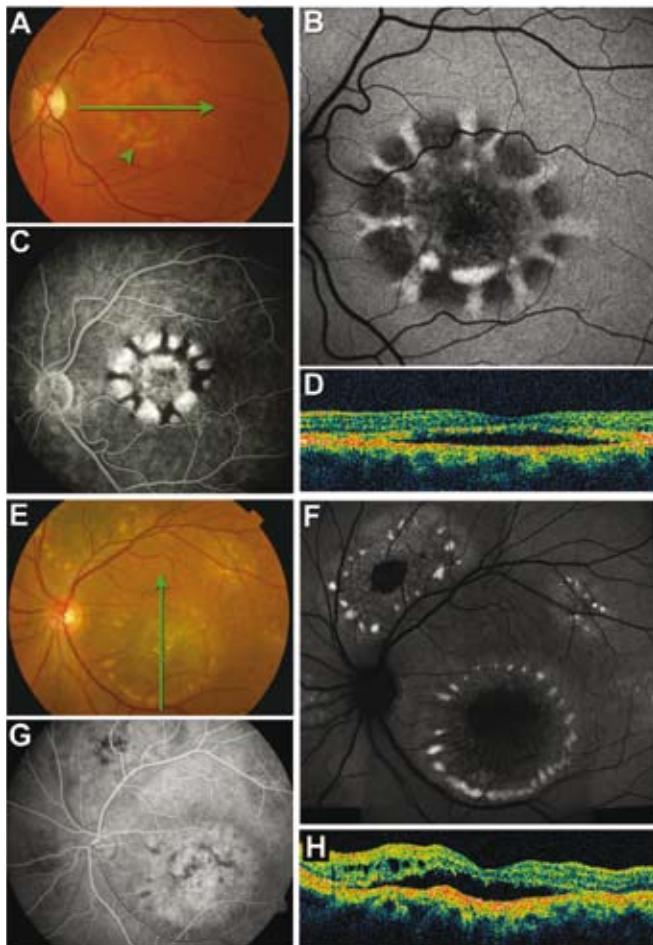
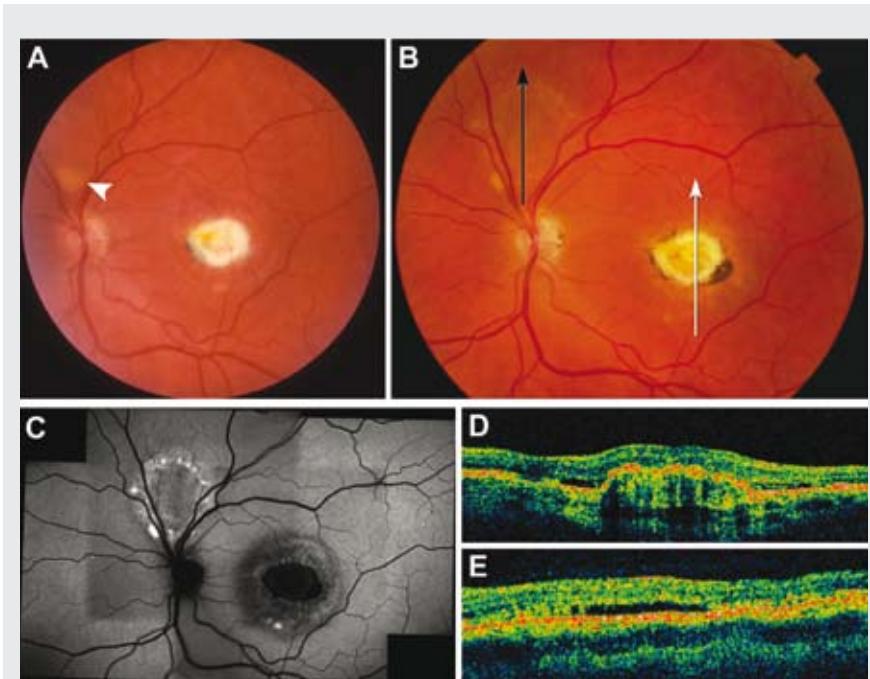


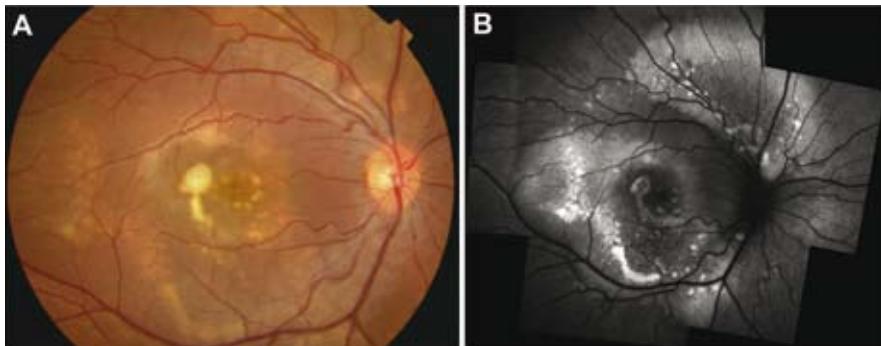
Figure 3.8.*
Atypical forms of Best vitelliform macular dystrophy. **A.** Fundus photograph of patient

14 (p.Ala243Val mutation in BEST1). A small pseudohypopyon (arrowhead) and some scattered yellow deposits are combined with a spoke-like pattern of subretinal yellowish changes, resembling a pattern dystrophy. **B.** The lesion shows increased fundus autofluorescence (FAF) of the small pseudohypopyon and a radial pattern of spoke-like extensions with increased FAF on a background of markedly decreased FAF. **C.** The spokes of increased FAF are hypofluorescent on fundus fluorescein angiography (FFA), whereas the spokes of decreased FAF are hyperfluorescent.

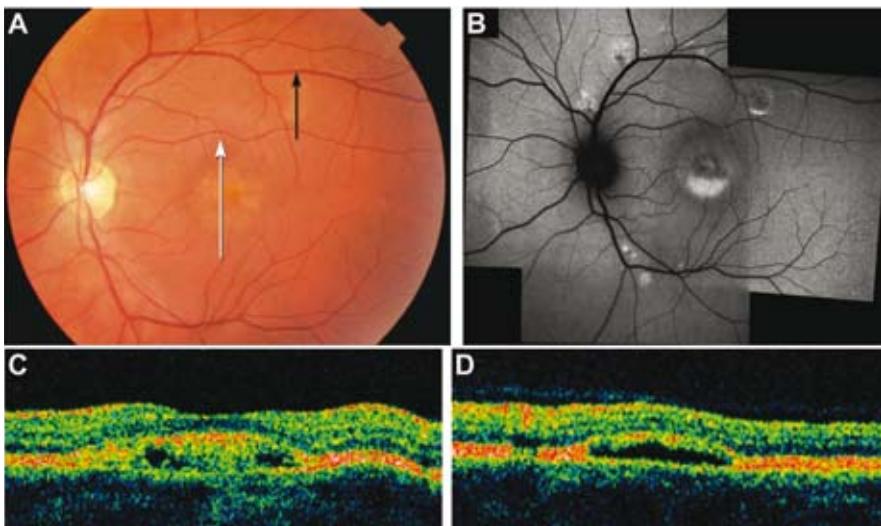
D. A horizontal optical coherence tomography (OCT) scan (arrow in panel A) shows an elevated retina with irreflective subretinal material. **E.** Fundus photograph of patient 20 (p.Gly299Ala mutation in BEST1), who presented with multifocal vitelliform lesions. The different lesions all have characteristics of the vitelliruptive stage, with circular deposition of yellowish material along the edges of the lesions. The lesions show some degree of fibrosis and atrophy as well. **F.** FAF shows that the yellowish deposits are intensely autofluorescent, whereas areas with atrophy and fibrosis show absent FAF. **G.** On FFA, lesions are predominantly hyperfluorescent. **H.** A vertical OCT scan (arrow in panel E) through the central lesion reveals a serous retinal elevation with intraretinal cystoid changes. The underlying layer shows increased reflectivity, probably corresponding with the fibrosis seen on ophthalmoscopy.

**Figure 3.9.***

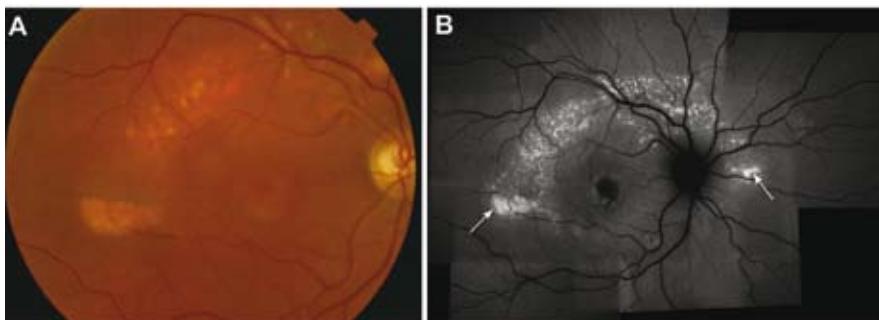
A. Fundus image of the left eye of patient 5 (*p.Asp302_Asp304del* mutation) at the age of 18 years. Besides a central cicatricial lesion, a lesion superior to the optic disc with characteristics of a pseudohypopyon is evident (arrowhead). At the age of 44, the lesion superior to the optic disc shows a “scrambled-egg” aspect (**B**). On the fundus autofluorescence (FAF) image (**C**), the central lesion shows markedly decreased FAF of the area that corresponds with the scar on ophthalmoscopy, surrounded by a zone of mottled alterations of FAF intensity. The suprapapillary lesion shows spots of increased FAF at the edges of the lesion, which correspond with the remnants of vitelliform material on ophthalmoscopy. **D.** A vertical optical coherence tomography (OCT) scan through the central lesion (white arrow in Fig. 3.9B) reveals a prominent hyperreflective structure, corresponding with the central scar seen on ophthalmoscopy. This structure is surrounded by a hyporeflective subretinal space, suggesting subretinal fluid. **E.** A vertical OCT section through the suprapapillary lesion (black arrow in Fig. 3.9B) shows a dome-shaped structure with a optically clear centre and a hyperreflective band above it, which possibly corresponds to the elevated photoreceptor layer.

**Figure 3.10.***

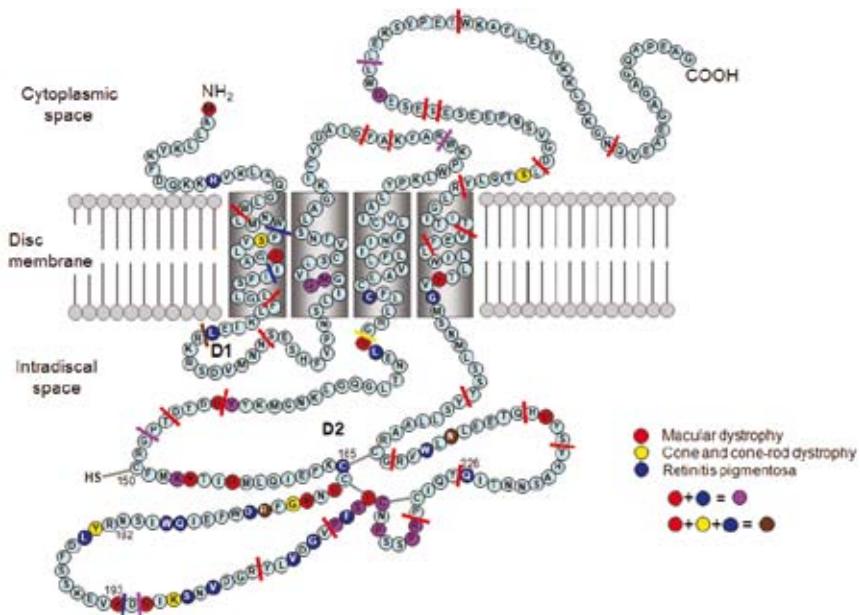
Fundus photograph and autofluorescence image of the right eye of patient 4, carrying a p.Lys194_Ala195insVal mutation in the BEST1 gene (A and B, respectively). **A.** The fundus image shows extensive lesions with scattered yellow-white deposits. **B.** These deposits are intensely autofluorescent. The lesions show a generally increased autofluorescence signal, which enables a better appreciation of the size and extent of the lesions.

**Figure 3.11.***

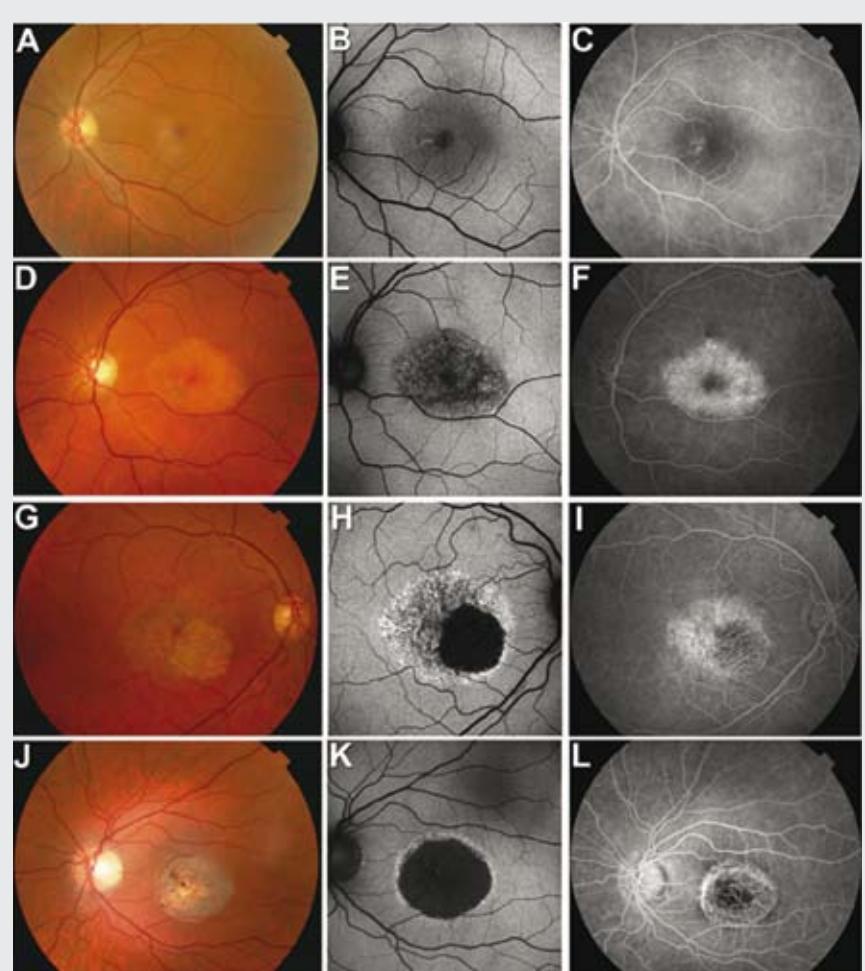
A. Fundus image of the left eye of patient 12, who did not carry a mutation in BEST1. Multiple vitelliform lesions are visible, which can be more clearly delineated with autofluorescence imaging (**B**). Both the central and superior temporal lesion show a pseudo-hypopyon-like distribution of the material of increased autofluorescence. Vertical optical coherence tomography images of the central lesion (**C**, white arrow in Fig. 3.11A) and the lesion temporal and superior to the central lesion (**D**, black arrow in Fig. 3.11A) are highly similar, although the central lesion shows more subfoveal material of increased reflectivity.

**Figure 3.12.***

A. Fundus image of the right eye of patient 15 (no BEST1 mutation), showing a small central vitelliform lesion and a large arcuate lesion, below the superior temporal vascular arcade. **B.** A composition of fundus autofluorescence (FAF) images shows the large eccentric lesion with scattered areas of increased FAF. At the inferior edges of this lesion, pseudohypopyon-like deposits of material of increased FAF can be observed (arrows).

**Figure 4.1.***

Protein model of peripherin/rds (adapted from Connell and Molday,³⁷ Travis et al.,³⁸ and Goldberg et al.⁵³). The mutations associated with retinal dystrophies are indicated. Colored residue: missense mutation or in-frame deletion. Colored bar: nonsense or frameshift mutation.

**Figure 4.5.***

Clinical stages of central areolar choroidal dystrophy (CACD). **A.** Color fundus photograph of stage I CACD, showing slight parafoveal hypopigmentation. **B.** This area of hypopigmentation on ophthalmoscopy corresponds with an area increased fundus autofluorescence (FAF). **C.** Fluorescein angiography shows hyperfluorescent parafoveal changes in stage I CACD. **D.** In stage II CACD, a round to oval area of hypopigmentation is seen in the macula. **E.** FAF in stage II shows a corresponding area of speckled changes of increased and decreased FAF. Initially, increased FAF may predominate, but with time, as the lesion enlarges and atrophy of the retinal pigment epithelium (RPE) progresses, decreased FAF may become more prominent. **F.** The fluorescein angiogram in stage II displays a speckled hyperfluorescence of the lesion, corresponding with partial atrophy of the RPE. **G.** Stage III CACD shows one or more patches of well-circumscribed chorioretinal atrophy, appearing outside the central fovea, within the area of slight hypopigmentation. **H.** These areas of chorioretinal atrophy correspond to severely decreased to

Figure 4.5.* continued

absent FAF. **I.** Fluorescein angiography in stage III CACD clearly visualizes the remaining choroidal vessels in the area of chorioretinal atrophy. In the later phase of the angiogram, discrete leakage of fluorescein could be observed at the edge of the lesion, corresponding with incomplete atrophy of the choriocapillaris. **J.** In stage IV CACD, the well-defined area of chorioretinal atrophy involves the fovea, with a corresponding severe decrease in visual acuity. **K.** This area corresponds with a round to oval zone of absent FAF involving the fovea in late stage IV CACD, bordered by a small residual band of increased FAF. **L.** End-stage CACD also shows a well-demarcated area of chorioretinal atrophy on the fluorescein angiogram, with enhanced visibility of the residual underlying choroidal vessels.

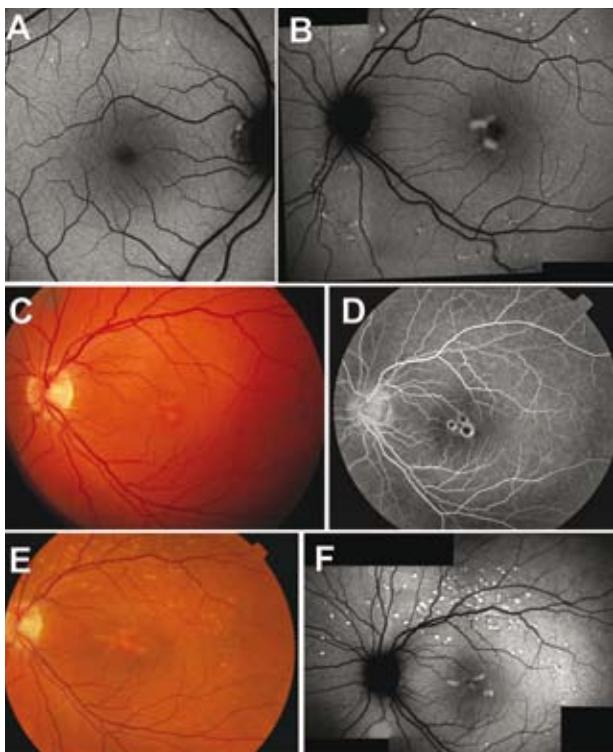
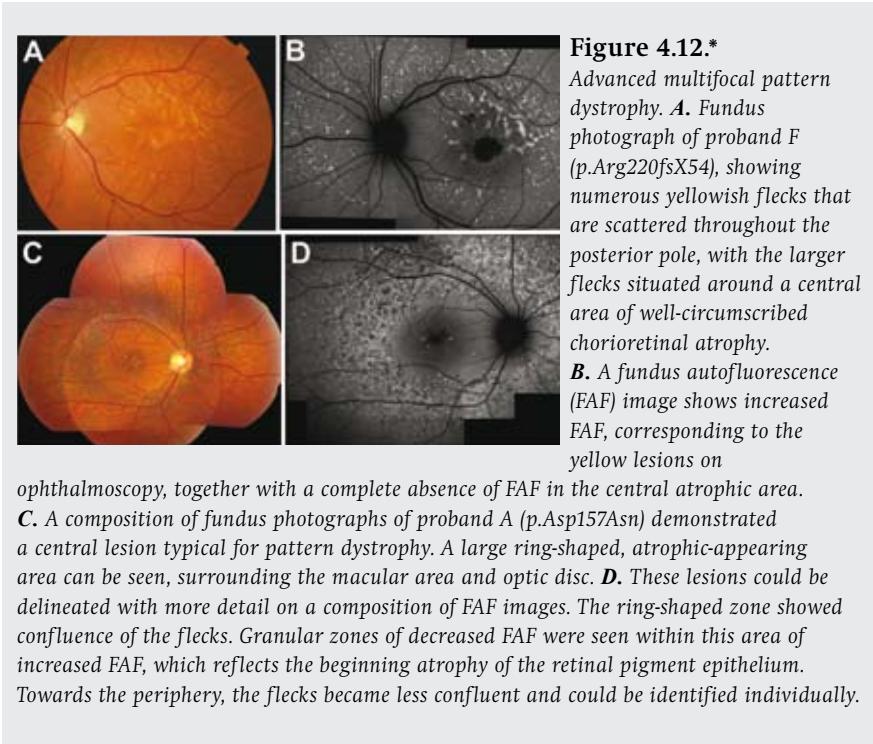


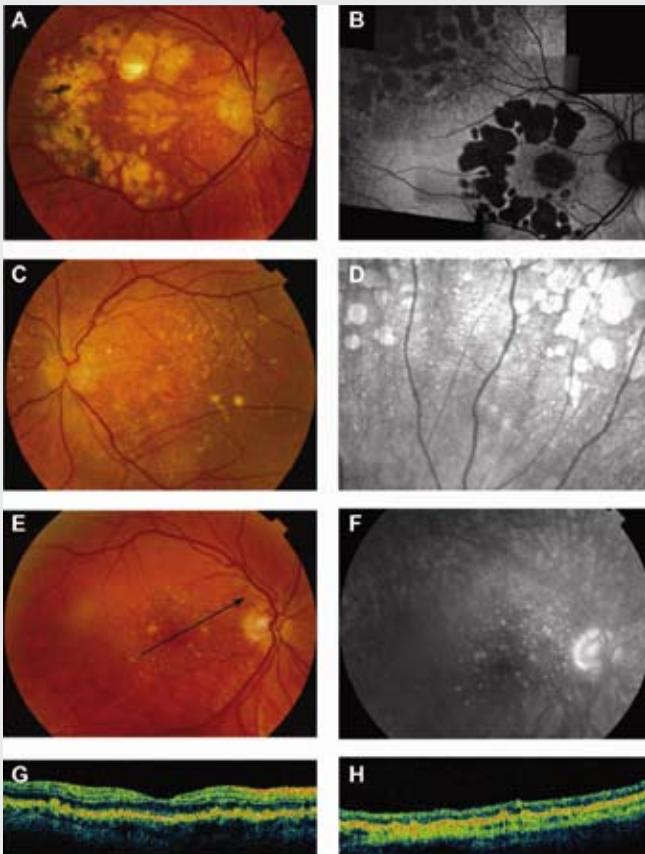
Figure 4.11.*

The development of lesions in multifocal pattern dystrophy simulating STGD1/fundus flavimaculatus.

A. Fundus autofluorescence (FAF) image of the 37-year-old daughter (E-IV:2, p. Pro146fsX4) of proband E, demonstrating small dots of increased FAF, in the absence of foveal lesions. This picture may represent an early stage of multifocal pattern dystrophy. **B.** Composition of FAF images of individual H-II:2 (p.Arg203fsX8), showing two irregular lesions with increased FAF in the macular area and small irregular flecks around the retinal vessels.

Note that most of these flecks show adjacent zones of decreased FAF. **C.** Fundus photograph of proband B (p.Asp145fsX30) taken at the age of 35, demonstrating three pigmented spots with a depigmented border in the macula. **D.** These lesions display a "dot-and-halo" aspect on the fluorescein angiogram, which was made before we had the possibility to perform FAF imaging. **E.** Six years later, the phenotype had evolved to a picture mimicking STGD1, with irregular yellow-white flecks around the vascular arcades, which showed predominantly increased FAF on the composition of FAF images (**F**). The macular lesion had a butterfly-shaped configuration both on ophthalmoscopy and on the FAF image (**E, F**).



**Figure 5.9***

Retinal phenotypes of drusen patients carrying the combination of a p.Gln408X mutation and the p.Tyr402His AMD risk variant. (A-D) Retinal phenotype of patient A-III.5.

A. Fundus photograph of the right eye, showing extensive chorioretinal atrophy of the posterior pole. At the first examination, 17 years earlier, these areas corresponded with large confluent drusen with incipient atrophy. **B.** The short-wavelength fundus autofluorescence (FAF) image shows an absence of FAF corresponding with the patches of chorioretinal atrophy, not only in the macular area, but

also in the midperipheral retina. Moreover, diffuse changes in FAF intensity can be observed. **C.** Fundus photograph of the left eye, showing large confluent drusen and mild chorioretinal atrophy. **D.** An infrared reflectance photograph clearly visualizes small, round midperipheral drusen scattered between the patches of chorioretinal atrophy. The drusen on infrared reflectance corresponded with discrete, round, yellow-white drusen with a slightly pigmented border on ophthalmoscopy. **(E-H)** Retinal phenotype of patient B-II.1. **E.** Fundus photograph showing confluent macular drusen. **F.** The macular drusen can be more easily detected on the fluorescein angiogram. **G.** Optical coherence tomography (OCT, oblique section) showing small dome-shaped elevations of the “outer red line”, corresponding with visible drusen on ophthalmoscopy (arrow). **H.** Like in patient A-III.5, small midperipheral drusen were also seen in this patient, which had a similar aspect on OCT as the macular drusen. **(I-J)** Patient B-III.2, the 22-year-old asymptomatic son of patient B-II.1 showed tiny hyperfluorescent drusen in the parafoveal area (**I**) and in the (mid-)peripheral retina (**J**) on fluorescein angiography, which were difficult to discern on ophthalmoscopy. His 25-year-old brother (B-III.1) had similar midperipheral lesions, but to a lesser extent.

Curriculum Vitae

Camiel Jan Fons Boon was born on the 15th of January in 1980, in 's-Hertogenbosch (the Netherlands). He graduated cum laude from secondary school on Gymnasium Beekvliet in Sint-Michielsgestel. After passing the entrance exam for medical students in Belgium, he studied medicine at the Faculty of Medical Sciences of the Catholic University of Leuven, where he received his M.D. degree in 2005 "with high distinction". During his medicine study, he started as a research student at the Department of Pediatrics, investigating bowel dysfunction in children. In this period, he was co-founder of the Leuvense Vereniging voor Student-Onderzoekers (LVSO), the association of medicine students doing research in Leuven. He also was the initiator and co-founder of O.L.D. Benedictus, a student society uniting Dutch and Flemish students in Leuven. His particular interest in ophthalmology arose in the third year of his medicine study, when studying the subject of neuro-anatomy. He consequently started doing research at the Department of Ophthalmology (Head: Prof. dr. W. Spileers) on the same university, on the topic "Confocal microscopy of the cornea". At this department, his preference for ophthalmology was further reinforced.

In 2005, he started with his Ph.D. research on hereditary retinal diseases, that resulted in this thesis, at the Institute of Ophthalmology (Head: Prof. dr. J.E.E. Keunen) of the Radboud University Nijmegen Medical Centre in Nijmegen. In 2007, he started with his residency in ophthalmology at the same institute.

He is a reviewer for *Ophthalmology*, *Retina*, and *Graefe's Archive for Clinical and Experimental Ophthalmology*.

Dankwoord

Het is een voorrecht om deel uit te maken van een traditie van fundamentele interesse in en gedegen onderzoek naar erfelijke netvliesaandoeningen door de afdeling oogheelkunde van het UMC St Radboud. Deze traditie was er nooit geweest zonder het pionierswerk van professor Deutman en wijlen dr. Pinckers, die een schat aan kennis en een indrukwekkend fotoarchief hebben nagelaten. Hun onderzoek op dit gebied werd voortgezet door dr. Hoyng, dr. Klevering, en meer recent mijn paranimfen, dr. van Lith-Verhoeven en dr. Go.

Dokter Hoyng en professor Cruysberg ben ik dankbaar voor het feit dat ze mijn gedrevenheid en interesse voor oogheelkunde en oogheelkundig onderzoek destijds onderkenden en vertrouwden in mijnen kunnen.

Voor het doen van promotieonderzoek moet je allereerst verschrikkelijk eigenwijs zijn. Zonder de deskundige ondersteuning en het enthousiasme van anderen was dit proefschrift echter nooit tot stand gekomen. Iedereen die hieraan heeft bijgedragen wil ik daarom uitdrukkelijk bedanken, en het is gepast om enkelen bij naam te noemen.

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