Cuticular drusen
Clinical and genetic studies on a subtype of age-related macular degeneration
J.P.H. van de Ven
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J.P.H. van de Ven

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Cover image: A starry sky at night, resembling the “stars-in-the-sky” appearance of cuticular drusen on a fluorescein angiograph.

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Cuticular drusen

Clinical and genetic studies on a subtype of age-related macular degeneration

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“To my father”
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List of abbreviations

AOPE    apolipoprotein E (gene)
ART     automatic real-time
aHUS    atypical hemolytic uremic syndrome
AMD     age-related macular degeneration
ARMS2   age-related maculopathy susceptibility 2 (gene)
BMI     body-mass index
C2      complement component 2 (gene)
C3      complement component 3 (gene)
CD      cuticular drusen
CFB     complement factor B (gene)
CFH     complement factor H (gene)
CFI     complement factor I (gene)
CI      confidence interval
CIRCL   Cologne Image Reading Center and Laboratory
CNV     choroidal neovascularization
cSLO    confocal scanning laser ophthalmoscope
DNA     deoxyribonucleic acid
eGFR    estimated glomerular filtration rate
ETDRS   early treatment diabetic retinopathy study
EUGENDA European Genetic Database
FA      fluorescein angiography
FB      factor B (protein)
FD-OCT  Fourier-Domain optical coherence tomography
FH      factor H (protein)
FI      factor I (protein)
FIMAC   factor I membrane attack complex domain
GWAS    genome-wide association study
LDLR    low-density lipoprotein receptor
MCP     membrane co-factor protein (protein)
MO      morpholino
mRNA    messenger ribonucleic acid
MPGN    membranoproliferative glomerulonephritis
OCT     optical coherence tomography
OD      oculus dexter / right eye
OR      odds ratio
OS      oculus sinister / left eye
PCR     polymerase chain reaction
PDT     photo dynamic therapy
PED     pigment epithelial detachment
RNA     ribonucleic acid
RPE     retinal pigment epithelium
SD-OCT  Spectral-Domain optical coherence tomography
LIST OF ABBREVIATIONS

SNP  single nucleotide polymorphism
SP   serine protease
tRNA transfer ribonucleic acid
VEGF vascular endothelial growth factor
Chapter One
General introduction
1.1 Anatomy of the eye

1.1.1 Visual pathway
Vision is arguably the most used of the five senses and is one of the primary means that we use to gather information from our surroundings. When we look at an object, visible light waves reflected by that object enter the eye through the cornea to finally be absorbed by the photoreceptors of the retina (Figure 1.1). The retina is the light-sensitive tissue lining the inner surface of the eye. One could argue that vision starts here when the photoreceptors convert light into electrical impulses, which are signaled to the brain via the optic nerve.

![Figure 1.1. Anatomy of the human eye](image)

1.1.2 Retina

Anatomy
During embryonic development, the retina and the optic nerve originate as outgrowths of the developing brain. The retina is a thin transparent membrane with an area of about 266 mm² and is the only part of the central nervous system that can be visualized non-invasively.

Histologically, the retina has several distinct layers of neurons interconnected by synapses and supporting cells (Figure 1.2). The deepest retinal layer is the retinal pigment epithelium (RPE). This layer as well as the retinal vascular endothelium possess a well-developed junctional complex that includes both adherence and tight junctions, called the blood-retina barrier. The physiological function of the RPE layer is multifaceted. Besides the active transport of nutrients and growth factors by controlling solute and fluid permeability between the choroidal vessels and
subretinal space, the RPE layer also plays a key role in the conversion and storage of retinoids, the phagocytosis of shed photoreceptor outer segment tips, and the absorption of scattered light.\textsuperscript{1}

The remaining nine layers compose the neurosensory retina. This part of the retina is responsible for converting light into electrical impulses, initial neuronal processing, and subsequently transmission to the optic nerve. The order of the layers from the outer to inner neuroretina are 2) the photoreceptor layer, containing the outer and inner segments of the rods and cones, 3) the external limiting membrane, composed of the closely apposed processes of Müller cells from the fifth layer, 4) the outer nuclear layer, containing the cell bodies of the photoreceptor cells, 5) the outer plexiform layer, the zone of synapses between the interneurons and the photoreceptors, 6) the inner nuclear layer, composed of the cell bodies of the interneurons and Müller cells, 7) the inner plexiform layer, the zone of synapses between the interneurons and the ganglion cells, 8) the ganglion cell layer, containing the cell bodies of the ganglion cells, 9) the nerve fiber layer, composed of the unmyelated axons of the ganglion cells that course towards the optic disc, and 10) the internal limiting membrane, like the external limiting membrane formed by the apposed processes of the same Müller cells. This layered pattern is similar across the entire human retina, with the fovea (see below) as the only exception.

**Macula lutea**

The central area of the posterior retina is defined as the macula lutea, or yellow spot (Figure 1.3). The conventional boundary of the macula lutea, as defined histologically, is the area that contains two or more layers of ganglion cells, spans 5 to 6 mm in diameter, and is centered vertically between the temporal vascular arcades.
The yellow color is attributed to macular pigment, consisting of the xanthophyll carotenoids lutein, zeaxanthin and meso-Z, which are powerful antioxidants and act as a filter of actinic short-wavelength blue light, thus limiting (photo-) oxidative damage to retinal cells.2-5

The central 1.5 mm of the macula lutea is called the fovea centralis (or fovea), which is anatomically characterized by a depression in the inner retinal surface. Its floor in the very center is called the foveola, an area of 0.35 mm in diameter. The fovea is dominated by cone photoreceptors, which are concentrated at maximum density (~199,000/mm²) in the foveola, in the absence of rod photoreceptors. Moreover, retinal capillaries are absent and the axons of the photoreceptors almost immediately bend away from the photoreceptor bodies, nearly parallel to the retinal surface, thereby minimizing light scatter. It is this anatomy and photoreceptor composition that makes the fovea specialized for high spatial acuity and color vision. Although the fovea comprises only 0.5% of the retina, approximately 50% of the nerve fibers in the optic nerve carry information from the fovea, due to its dense cone photoreceptor concentration.

**Photoreceptors**

Light striking the retina must travel through the full thickness of the human retina to reach the rods and cones. These photoreceptors convert light into electrical impulses by means of the phototransduction cascade.6,7 The density and distribution of the rod and cone photoreceptor cells vary with topographic location within the retina (Figure 1.4). The fovea contains a densely packed arrangement of cone photoreceptors with a peak density of about 199,000 cones/mm². The number of cone photoreceptors falls steeply with increasing eccentricity away from the...
foveal center; the periphery contains a minimum of cone photoreceptors. The rod photoreceptors have their greatest density in a zone lying about 20° from the foveal center, where they reach a peak density of about 160,000 rods/mm². Although the rod photoreceptor density is high, the visual acuity in this region is limited because of summation of multiple rod photoreceptor responses in each receptive field. This offers sensitivity at the cost of image resolution. The density of rod photoreceptors decreases towards the periphery.

The rod photoreceptors contain the visual pigment rhodopsin and are sensitive to blue-green light with a peak sensitivity around 500 nm wavelength of light. In contrast to cones, rod photoreceptors are sensitive enough to respond to a single photon of light, thus enabling vision in dim light (scotopic vision). Rods are essential for detection of contrast, brightness, as well as motion. Cone photoreceptors, on the other hand, contain cone opsins as visual pigment and, depending on the exact structure of the opsin molecule, are sensitive to either long wavelengths of light (L-cones), medium wavelengths of light (M-cones) or short wavelengths of light (S-cones). L-cones are known to be maximally sensitive to wavelengths peaking at 564 nm (red light), M-cones at 533 nm (green light) and S-cones at 437 nm (blue light), respectively. The cone photoreceptors function at bright light (photopic vision) and because of their ability to detect different wavelengths of light are essential for color vision and high spatial acuity.

Apart from the capture of light in the rod and cone photoreceptors, a subset of ganglion cells is also photosensitive, expressing an opsin-like protein called melanopsin. 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. These non-image forming, adaptive ocular photoresponses are thus important in circadian rhythm entrainment.
1.1.3 Bruch’s membrane

Bruch’s membrane is an unique pentalaminar structure, which acts as a semi-permeable filter for the reciprocal exchange of biomolecules between the retina and the choroid. Starting with the innermost layer, the five layers of Bruch’s membrane are 1) the basement membrane of the RPE, 2) the inner loose collagenous layer, 3) the middle layer of elastic fibers, 4) the outer loose collagenous layer, and 5) the basement membrane of the endothelium of the choriocapillaris. The thickness of Bruch’s membrane increases with age and almost doubles in size during life. The principal part of Bruch’s membrane thickening occurs in the inner loose collagenous layer, followed by the outer loose collagenous layer. This process starts in the retinal periphery, where RPE gene expression of most structural components of Bruch’s membrane appears to be higher than in the macular area.

In general, the thickening of Bruch’s membrane is caused by increased deposition and cross-linking of (less soluble) collagen fibers and increased deposition of lipids and other substances such as complement components. This thickening eventually disturbs Bruch’s membrane integrity and causes a sharp reduction in trans-RPE fluid and nutrient transport, especially in the macula, where Bruch’s membrane appears to be thinnest. The potential correlation with neovascular ingrowth agrees well with the notion that thickening of Bruch’s membrane renders the membrane more brittle and more susceptible to breaks, allowing for neovascularization as some sort of tissue repair mechanism. Subretinal deposits, such as basal laminar deposits, basal linear deposits and drusen – the precursors of age-related macular degeneration (AMD) – preferentially develop between the basement membrane of the RPE and the inner loose collagenous layer of Bruch’s membrane. Although it is currently insufficiently understood why drusen develop mainly in the macular area, a combination of specific structural, molecular and functional properties predispose the macula to develop drusen. First, the high metabolism associated with the extremely high density of photoreceptors, particularly in the perifoveal ring, may contribute to drusen formation. Local phagocytosis of photoreceptor outer segments by the RPE causes a highly focused and localized peak of oxidative stress, and focal build up of membranous waste products. Secondly, significant differences in functional RPE properties, as annotated by differential gene expression profiles, may play a role. Third and last, the thinner and more open maze of the elastic layer of Bruch’s membrane compared to the periphery may predispose the macula to drusen formation. Most likely, oxidized molecules may get physically or chemically trapped easier, thereby triggering the first events of drusen formation in the macular area.

1.1.4 Choroid

The choroid, also known as the choroidea, is the vascular layer of the eye positioned between Bruch’s membrane and the sclera. The choroidal blood flow is the highest rate of all body tissues and supplies oxygen and nourishment to the RPE and the...
outer retina. More than 90% of the oxygen provided by the choroid is consumed by the photoreceptors. A second function of the choroidal blood flow is to protect the retina from heat generated during exposure to bright lights by acting as a heat sink for exogenous thermal radiation. In addition to its vascular functions, the choroid contains secretory cells, involved in modulation of vascularization and in growth of the sclera.

Histologically, the choroid can be dissected in four layers. Starting from the Bruch’s membrane side, these are 1) the choriocapillaris, 2) Haller’s vascular layer, 3) Sattler’s vascular layer, and 4) the suprachoroidea. The human choroid has a maximal thickness (220 µm) in the central region, while it progressively narrows to 10 µm in the periphery. In the posterior pole, the choriocapillaris consists of a mosaic of vascular lobes (Figure 1.5). Towards the periphery, the capillary arrangement 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 blood flow in the macula, a result of the more efficient lobular pattern in this area. With age, the thickness of the choriocapillaris and the capillary lumen diameters decrease. It is this degeneration of the choriocapillaris that seems to play a role in the pathogenesis of AMD.

1.2 Clinical evaluation of retinal anatomy and function

The clinical evaluation of the retina requires a combination of conventional examinations and more recently developed specialized imaging techniques. Results of visual acuity measurements indicate the degree of foveal involvement and are important to determine disease progression in follow-up examinations. Evaluating the aspect of the retina is essential to get more information about the nature of the disease. This section provides an overview of the most frequently used imaging techniques in the clinical characterization of AMD, the topic of this thesis.
1.2.1 Ophthalmoscopy
The simplest examination technique is direct ophthalmoscopy, which provides a monocular, high-magnification (15x) image of the retina. However, the instrument’s lack of stereopsis, small field of view, and poor view of the retinal periphery limits its use. These shortcomings are overcome by using a monocular or binocular indirect ophthalmoscope in combination with a (20, 28 or 30 diopter) handheld lens that dramatically increases the field of view with lower magnification (2-3x). To evaluate subtle retinal changes, slit-lamp biomicroscopy is preferred. A wide variety of (contact) lenses are used for viewing the retina with the slit lamp, all with their own specific advantages. For better comparison of follow-up examinations, color fundus photographs may be taken at regular intervals.

1.2.2 Fluorescein angiography
Fluorescein angiography (FA) is a technique for examining the circulation of the retina and choroid using the dye tracing method. After the administration of intravenous or oral sodium fluorescein, which under normal circumstances does not pass the blood-retina barriers of the RPE and retinal vessels, an angiogram is obtained by photographing fluorescence emitted by the dye after illumination with blue light at a wavelength of 490 nanometers. Lesions on FA show variable degrees of hyperfluorescence or hypofluorescence, depending on the nature and site of the lesion relative to the normal anatomical structures. FA can also aid the detection of a particular subtype of drusen called cuticular drusen, which typically appear as “stars-in-the-sky”.

1.2.3 Optical coherence tomography
Optical coherence tomography (OCT) is a noninvasive, noncontact imaging technique that provides real-time images of the retina in micrometer-resolution. This high resolution is achieved through the principle of low-coherence interferometry, which is based on measurements of reflected or backscattered light of different internal tissue microstructures in a way that is analogous to ultrasonic pulse-echo imaging. From the resulting measurements, one can derive the reflectivity profile along the beam axis. This one-dimensional depth scan is called the A-scan. The OCT scan performs many adjacent A-scans in order to create two- or three-dimensional images of the retina. In Chapter 5 of this thesis, we demonstrate the potential of this imaging modality by evaluating the short-term changes of cuticular drusen.

1.2.4 Scanning laser ophthalmoscopy
The confocal scanning laser ophthalmoscope (cSLO) provides retinal images by using a laser beam with a specific wavelength that rapidly scans the retina in a raster fashion. The reflected light is detected by a confocal photodetector that is conjugate to the retinal plane, and the digitized signal can either be recorded on
videotape or fed to a frame-grabber interfaced to a computer. The possibility to image with several different wavelengths offers the possibility to produce retinal images with or without dyes such as fluorescein or indocyanine green, and permits selective examination of different tissue depths. By this means, cSLO is capable of imaging retinal structures poorly seen by ordinary fundus cameras and does so using low levels of light exposure and improved contrast.

1.3 Introduction to molecular genetics

It has been known for centuries that certain diseases, including a wide variety of retinal disorders, run in families. In order to understand how, at the molecular level, such a diversity may arise and why some diseases run in the family, a basic knowledge of heredity is required. This section provides an overview of the structure and function of deoxyribonucleic acid (DNA) and a basic knowledge of the structural alterations that may occur.

1.3.1 DNA structure and function

DNA is an information macromolecule containing the genetic instructions used in the development and functioning of all known living organisms. These DNA molecules consist of two long polymers of four different repeating chemical bases (adenine, A; thymine, T; guanine, G; and cytosine, C) with a linear backbone composed of a 5-carbon sugar to which are attached one or more phosphate groups (Figure 1.6). A sugar with an attached base and phosphate group therefore constitutes the basic repeat unit of a DNA strand, a nucleotide.

The DNA’s genetic information is encoded by the linear sequence of nucleotides in its strands. Regions that spell out the exact instructions required to create a particular protein are called the genes. In humans, the DNA encodes approximately 20,000 to 25,000 genes. Genes contain an open reading frame that can be transcribed into a protein, as well as regulatory sequences such as promoters and enhancers, which control the transcription of the open reading frame. Only about 1.5% of the human DNA consists of protein-coding genes. However, DNA sequences that do not code proteins may still encode functional molecules, which are involved in the regulation of gene expression.40-44

During the process of transcription, the information stored in a gene’s DNA is transferred to a similar molecule called ribonucleic acid (RNA) in the cell nucleus. Both RNA and DNA are made up of a chain of nucleotides, but they have slightly different chemical properties. The type of RNA that contains the information for making a protein is called messenger RNA (mRNA) because it carries the information, or message, from the DNA out of the nucleus into the cell’s cytoplasm. The second step in translating the genetic code of a gene into a protein, takes place in the cytoplasm. The mRNA interacts with a specialized complex called a ribosome, which “reads” the sequence of the mRNA nucleotides. Each sequence of
Figure 1.6. Schematic representation of the DNA structure. DNA is made of four types of nucleotides, which are linked covalently into a polynucleotide chain (a DNA strand) with a sugar-phosphate backbone from which the bases (A, C, G, and T) extend. A DNA molecule is composed of two DNA strands held together by hydrogen bonds between the paired nucleotides. The arrowheads at the ends of the DNA strands indicate the polarities of the two strands, which run anti-parallel to each other in the DNA molecule. In the diagram at the bottom left of the figure, the DNA molecule is shown straightened out; in reality, it is twisted into a double helix, as shown on the right.
three nucleotides, called a codon, usually codes for one particular amino acid. These amino acids are the building blocks of proteins. A type of RNA called transfer RNA (tRNA) assembles the protein, one amino acid at a time. Protein assembly continues until the ribosome encounters a “stop” codon, a sequence of three nucleotides (TAG, TAA, and TGA) that does not code for an amino acid, but terminates the process of translation instead.

1.3.2 Mutations, single nucleotide polymorphisms, and pathogenicity

Human DNA is not a static entity. Instead, it is subject to a variety of different types of heritable change. Large-scale abnormalities involve loss or gain of large parts of DNA (chromosomes). These chromosomal abnormalities can cause different types of syndromes, like Down syndrome (which is caused by three copies of chromosome 21, rather than two). Smaller scale abnormalities, which most often have smaller effects, can be categorized into two different classes: Single nucleotide polymorphisms (SNPs) and mutations.

**Single nucleotide polymorphisms**

SNPs are DNA sequence variations that occur when a single nucleotide is altered. An example of a SNP is the alteration of the DNA sequence ACG GTT AGT to ACG TTT AGT, where the second “G” is replaced by a “T”. For such a variation to be considered a SNP it must occur in at least 1% of the population. On average, SNPs occur every 250 to 300 nucleotides along the 3-billion-nucleotide human DNA, contributing to about 90% of all human genetic variation. Since only 1.5% of the human DNA consists of protein-coding exons, most SNPs are found between these coding regions. When SNPs occur within a protein-coding part of a gene or in a regulatory region near a gene, they may play a role in disease by affecting the gene’s function or expression.

Although individual SNPs do not usually cause disease, many SNPs have proven to be associated with an individual’s response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing particular diseases like AMD (Chapter 2). By this means, genetic association studies of SNPs can lead to the first insights into the pathophysiology of a disease.

**Mutations**

Mutations are rare (occurring in <1% of the population) alterations in the DNA sequence that usually have a large effect on the protein that is encoded by the mutated gene. According to the specific molecular changes at the DNA level, mutations can be classified as: 1) single nucleotide substitutions, 2) insertions, or 3) deletions. A single nucleotide substitution is, similar to a SNP, the replacement of a single nucleotide by another. An insertion involves the addition of one or more nucleotides. If an insertion occurs in a coding sequence and involves one, two or more nucleotides which are not a multiple of three (the sequence unit that encodes an amino acid), it will disrupt the reading frame. A deletion involves the loss of one
or more nucleotides. Again, if it occurs in coding sequences and involves one, two or more nucleotides which are not a multiple of three, it will disrupt the reading frame.

Based on their structural effects, mutations can be classified into synonymous and non-synonymous variations. The first is a DNA sequence alteration that does not alter the amino acid sequence of the gene. The second, a non-synonymous mutation, leads to an alteration in the encoded protein and can occur in one of the following ways: 1) missense, 2) nonsense, or 3) frameshift (Figure 1.7). When a single nucleotide substitution results in coding a different amino acid and the synthesis of an altered protein, it is termed missense mutation. If the substitution leads to the generation of one of the stop codons (TAG, TAA, and TGA), which results in premature termination of the protein, it is termed nonsense mutation. If a mutation involves a deletion or insertion of nucleotides which are not a multiple of three, it will disrupt the reading frame what is known as a frameshift mutation. The amino acids downstream of the mutation bear no resemblance to the normal sequence and will most likely seriously affect protein function. In addition, it is not unusual for a frameshift mutation to also result in a stop codon downstream of the mutation.
Apart from the mutations in coding DNA, mutations in non-coding DNA would not be expected to have an effect on protein function/expression unless they occur in the DNA sequences involved in gene regulation. Mutations in regulatory elements can affect the level of gene expression, while mutations in splice junctions can result in coding sequences being removed from, or non-coding sequences being added to, the mRNA molecule.

1.4 Age-related macular degeneration

Age-related macular degeneration (AMD) is the most common cause of visual impairment and blindness among the elderly in industrialized nations, affecting worldwide 30 to 50 million individuals.46-51 This retinal disease, characterized by a loss of central, color and sharp vision due to a progressive deterioration of the macula, has a tremendous impact on the ability of patients to perform critical activities of daily living such as reading and driving (Figure 1.8). As such, the impact of AMD on the independence and well-being of those affected can be devastating.52

1.4.1 Early age-related macular degeneration and drusen

Early AMD is present when a spectrum of changes is observed in the ageing macula before the onset of overt loss of vision.53 Among these changes, drusen are considered as the most characteristic and earliest physical signs of AMD.54,55 Drusen usually appear after the age of 50 years and are seen on ophthalmoscopy as focal subretinal deposits ranging in color from white to yellow, sometimes with a crystalline glittering aspect. At histological examination, drusen consist of amorphous extracellular material lying between the inner collagenous zone of Bruch’s membrane and the basal lamina of the RPE.22,25,56 Although their exact origin remains unproven, a profile of the molecular composition of drusen indicates that they are the result of a chronic inflammatory stimulus that exacerbates the effect of

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**Fig. 1.8. Visual distortion by age-related macular degeneration (AMD).** (A) Normal vision. (B) The same scene as viewed by a person suffering from advanced AMD.
Fig. 1.9. Drusen phenotypes in age-related macular degeneration. Color fundus photographs (1A, 2A, 3A, 4A, 5A), fluorescein angiographs (1B, 2B, 3B, 4B, 5B), and optical coherence tomographs (1C, 2C, 3C, 4C, 5C) of several eyes showing hard drusen, soft drusen, crystalline drusen, reticular drusen or cuticular drusen. Hard drusen are visible as small yellow pinpoint lesions (1A; white arrowheads) with corresponding hyperfluorescent spots in the fluorescein angiogram (1B). A representative OCT scan shows the dome-shaped deposition of material under the retinal pigment epithelium (RPE) (1C). Soft drusen are identical to hard drusen though they are typically larger (2A, 2B, 2C). A crystalline druse is visible as a glistening, bright yellow lesion (3A; white arrowhead) with a corresponding hyperfluorescent spot in the fluorescein angiograph (3B). The OCT scan shows the development of RPE atrophy in the area corresponding to the crystalline druse (3C). Reticular drusen are visible as grayish spots (4A) with corresponding hypofluorescent lesions in the fluorescein angiogram (4B). The OCT shows relatively flat aggregates between the boundary of the inner and outer segments and the underlying RPE (4C). Cuticular drusen are visible as numerous hard drusen scattered through the fundus (5A). Fluorescein angiography reveals the pathognomic “stars-in-the-sky” appearance (5B). The OCT scan shows multiple small RPE elevations (5C).
primary pathogenic stimuli, which are believed to be cellular remnants and debris derived from degenerated RPE cells.\textsuperscript{57}

Various drusen classification systems have been described, reviewed and/or proposed by a number of investigators in an attempt to estimate the contribution of different drusen phenotypes to the development of advanced AMD.\textsuperscript{53, 58-60} The terminology most commonly used to distinguish drusen phenotypes are “hard”, “soft”, “crystalline”, “reticular”, and “cuticular” drusen (Figure 1.9). Hard drusen are defined as small (<63 µm), round hemispherical structures with well-defined edges. In small numbers, hard drusen are not considered as risk factors for developing advanced AMD, which is defined as geographic atrophy or choroidal neovascularization.\textsuperscript{61-64} Individuals with large numbers of hard drusen have a 0.5% to 1.8% chance to develop advanced AMD within a follow-up period of 15 years.\textsuperscript{65} Soft drusen, as compared to hard drusen, are typically larger in diameter (≥63 µm), more irregular in shape and have poorly demarcated boundaries which gives them a softer appearance. This drusen phenotype is formed by enlargement and fusion of a cluster of hard drusen, and therefore represents late-stage drusen progression.\textsuperscript{20} In accordance with this advanced stage of drusen progression, eyes with soft drusen have an increased risk of 2.7% to 39.3% to develop advanced AMD within a follow-up period of 15 years.\textsuperscript{65} Both hard drusen and soft drusen can undergo calcification (crystalline drusen), thus giving the druse a glistening appearance. Calcification of drusen usually presage drusen regression and the development of RPE atrophy (advanced AMD).\textsuperscript{66}

In contrast to the previous terms that describe a single druse, reticular drusen and cuticular drusen are drusen phenotypes defined by drusen patterns. Reticular drusen are defined as multiple “drusen” that form ill-defined networks of broad interlacing ribbons.\textsuperscript{67} They are identified easily with blue-channel examination, near-infrared photography, red-free photography, autofluorescence imaging and OCT.\textsuperscript{67-69} A unique histological feature of reticular drusen is that they are located internal instead of external to the RPE.\textsuperscript{69} In this respect, the use of the term “drusen” is not entirely correct. However, similar to other drusen types, reticular drusen increase the risk on developing advanced AMD significantly.\textsuperscript{67, 70-73} It is estimated that individuals with reticular drusen are at a 26% risk of developing advanced AMD during a follow-up period of 5 years.\textsuperscript{68} The last drusen phenotype, cuticular drusen, will be discussed into greater detail in the next section.

**Cuticular drusen**

The first report on cuticular drusen was published in 1977, when Donald Gass described a “new” clinical entity characterized by fundoscopic findings that included “innumerable, small (25 to 75 µm), round, uniformly sized, slightly raised, yellow, subretinal nodules, randomly scattered throughout the fundus”.\textsuperscript{74} In a follow-up study published eight years later,\textsuperscript{75} Gass and his associates referred to this entity as “basal laminar drusen” or “cuticular drusen” based on their interpretation of the data,
which suggested that the small subretinal lesions in this clinical entity were focalized, nodular thickenings of the basal lamina of the RPE. Recent studies, however, have revealed that these deposits are both ultrastructurally and compositionally identical to the drusen that are associated with AMD. Anatomically, these deposits are located between the basal lamina of the RPE and the inner collagenous layer of Bruch’s membrane, which was described previously by Müller as the inner cuticular zone. Based on these histopathological features, the preferred term for this particular drusen phenotype is “cuticular drusen”.

Commonly appearing in early adulthood, this drusen phenotype is best appreciated on fluorescein angiography, which reveals the pathognomic “stars-in the-sky” or “milky-way” appearance in the fundus. These hyperfluorescent lesions start to fluoresce in the early arteriovenous phase of the fluorescein angiogram and typically outnumber the drusen discernable on ophthalmoscopy. Although the risk of developing advanced AMD in patients with cuticular drusen is currently unknown, the onset of advanced AMD seems to be on average a few years earlier as compared to the onset of advanced AMD in other drusen phenotypes.

1.4.2 Advanced age-related macular degeneration

Advanced AMD can be classified into geographic atrophy and choroidal neovascularization (CNV). Geographic atrophy (“dry” AMD), which accounts for approximately 85% of all cases with advanced AMD, is characterized by the development of one or more sharply delineated areas of chorioretinal atrophy that usually start to develop in the region near the fovea without involving the foveal center. However, because these atrophic lesions tend to enlarge and coalesce steadily over a period of years, most patients will lose central vision in the late stages when the atrophy expands into the foveal center.

The alternative form of advanced AMD, CNV (“wet” AMD), is characterized by the formation of abnormal new blood vessels from the choriocapillaris that grow through the Bruch’s membrane into the subretinal space and the retina. These fenestrated and friable new vessels leak serous fluid, lipids, and blood beneath and into the neural retina, with subsequent fibrous scarring. This process of neovascularization and fibrovascular scar formation can cause a rapid and severe loss of central vision in a matter of weeks or months. Whereas CNV accounts for only 15% of all cases of advanced AMD, more than 90% of AMD patients with severe loss of central vision manifest CNV.
1.4.3 Risk factors

Environmental risk factors
Although advanced age is the major risk factor associated with AMD, environmental and lifestyle factors may significantly alter individual risk. Cigarette smoking is the best characterized modifiable factor that has been consistently associated with a two- to three-fold increased risk for developing AMD. Oxidative stress and antioxidant depletion have been implicated in retinal damage from smoking, although the precise mechanism remains unclear. Other factors that have been reported to influence risk for AMD include obesity, sunlight exposure and a history of cardiovascular disease (e.g., hypertension). Regarding diet, omega 3 polyunsaturated fatty acids, the carotenoids lutein and zeaxanthin, and antioxidants such as zinc, and vitamins A and E may protect against the development of AMD. Differences in the prevalence of AMD between the two sexes, with a prevalence among females being greater than that among men, could reflect environmental as well as genetic risk factors.

Genetic risk factors
The first evidence for the influence of the genetic make-up on AMD came from family and twin studies. First-degree relatives of patients with AMD are at increased risk for the disorder, compared to first-degree relatives in families without AMD. In addition, they are affected at a younger age, and have an increased lifetime risk of an advanced form of AMD. In more recent efforts to reveal the underlying genetic modifiers in AMD, a number of high-risk SNPs have been identified in genes that encode components of the complement system, a part of the innate immune system. The most prominent is the Y402H SNP in the complement factor H (CFH) gene, producing a nearly three-fold increased risk of disease in individuals who harbor one copy of the Y402H SNP. Other genes of the complement system harboring confirmed risk alleles are complement component 2 (C2), complement factor B (CFB), complement component 3 (C3), complement factor I (CFI) and age-related maculopathy susceptibility 2 (ARMS2). Although the latter is not known to be part of the complement pathway itself, there is growing evidence that ARMS2 is associated with complement activation.

Other biological pathways that harbor AMD-associated SNPs are the HDL cholesterol pathway, which includes the APOE, LIPC, CETP, ABCA1, FADS1_3, and LPL genes, and the extracellular matrix involving the COL8A1 and COL10A1 genes, and the TIMP3 gene.

1.4.4 Pathogenesis
The pathogenesis of AMD is not completely understood. Many theories exist, however, a common theme shared by most of these theories is the central role of inflammation and the immune system. Immune mechanisms and cellular interactions in AMD are similar to those seen in other diseases characterized by the accumulation of extracellular deposits such as atherosclerosis and Alzheimer’s disease. Evidence
is growing that the complement system plays a significant role in the pathogenesis of AMD. Inflammatory and immune-mediated events involving complement proteins have been implicated in the biogenesis of drusen. Consistent with this histopathological link is the genetic association between AMD and several variants in genes encoding complement components.

**Complement system**

The complement system is one of the first lines of defense in innate immunity and is important for microbial killing, immune complex handling, apoptotic cell clearance, tissue homeostasis and modifying the adaptive immune response. Critical to these functions is the triggering of a series of cascades that result in the formation of metastable protease complexes which ultimately form the membrane attack complex, also known as the terminal complement complex. In sequential order, this series of cascades can be divided in four main steps: initiation of complement activation, C3 convertase activation, C5 convertase activation, and terminal pathway activity with the assembly of the membrane attack complex (**Figure 1.10**).

Initiation of complement activation proceeds via either of three distinct pathways: the classical pathway, the lectin pathway and the alternative pathway. Current evidence suggests that the alternative complement pathway is most closely related to the pathogenesis of AMD. The initiation of this complement pathway depends on the constant low-grade activation by spontaneous hydrolysis of the internal thioester bond of C3, which results in the formation of the C3b-like molecule C3(H2O). This initially generated hydrolyzed C3 then binds directly via its labile thioester to activating foreign or self-surfaces in the body. When C3(H2O) is bound to these surfaces, it interacts with complement factor B (FB), which is cleaved by complement factor D into two factors, Ba and Bb. The latter remains bound to C3(H2O) to form the initial fluid-phase C3 proconvertase C3(H2O)Bb. This C3 proconvertase constantly cleaves more C3 molecules generating C3b and is referred to as the “tick over” of the alternative pathway. The generated C3b then interacts with FB to form the more active alternative pathway C3 convertase (C3bBb). It is this self-propagation that results in an exponential amplification of the alternative complement pathway, necessitating tight control by either membrane-bound or soluble regulators.

Enzymatic inactivation of C3b into iC3b is the main mechanism of control of the alternative pathway. This step is mediated by four factors: circulating complement factor I (FI), a regulatory enzyme of the complement system, the plasma protein complement factor H (FH), and two cell-membrane proteins, complement receptor type 1 and membrane co-factor protein. FH is the main fluid-phase regulator, and in its absence the regulation of alternative pathway complement activation is severely disturbed. The FH protein is a potent inhibitor of the alternative complement pathway because it works through three mechanisms of action: it effectuates the decay of C3 convertase (C3bBb), it competes with FB in binding to C3b, and finally FH is a cofactor for the serine protease FI to allow the cleavage of C3b into the
inactive form iC3b. Mutations in the genes encoding these complement regulators are associated with the AMD subtype of cuticular drusen and will be discussed in Chapters 3 and 4 of this thesis.

Independent of the initiation of complement activation, all three complement pathways ultimately converge with the formation of C3 convertase (C4b2a or C3bBb). These C3 convertases cleave more C3 to C3a and C3b, allowing the binding of an additional C3b molecule to the C3 convertase complexes, leading to the formation of C3bBbb, the C5 convertase of the alternative pathway, or C4b2b3b, the C5 convertase for the classical and lectin pathways. These C5 convertases then initiate the assembly of the membrane attack complex by cleavage of C5 to C5a and C5b. The newly formed C5b forms a tri-molecular complex by binding C6 and C7. After inserting into a cell membrane, this complex binds C8 and multiple C9 molecules. This results in the completion of the pore-forming membrane attack complex (C5b-9) and ultimately in cell lysis.

Fig. 1.10. Schematic representation of the complement system. (A) Complement is initiated by three major pathways: 1) the alternative pathway, 2) the classical pathway and 3) the lectin pathway. (B) The three pathways initiate the formation of component 3 (C3) convertases. These enzymes cleave the central complement component C3 and generate the anaphylactic and antimicrobial peptide C3a and the opsonin C3b, which can be deposited onto any nearby surface. This activation is followed by an amplification reaction that generates additional C3 convertases and deposits more C3b at the local site. Subsequently, C3b is inactivated and sequentially degraded and the various degradation products mediate important effector functions. For example, C3b deposition on a surface results in opsonization, which allows for the interaction of C3b with specific host C3 receptors expressed on the surface of immune effector cells. (C) If activation progresses, a new enzyme, the C5 convertase is generated. C5 convertase cleaves C5, releases the potent anaphylactic peptide C5a and generates C5b. (D) C5b can initiate the terminal pathway, which recruits the components C6, C7, C8 and C9 to the surface of the target and inserts the C9 complex as a into the membrane. The activated complement system generates multiple effector compounds that drive and orchestrate further immune reactions. (Figure derived from Zipfel et al., 2009).
1.4.5 Present and future therapeutic options for AMD

Preventive interventions

The prevention of AMD is mainly focused on diminishing risk factors that exacerbate oxidative stress. Cessation of cigarette smoking is recommended since its adverse association with advanced AMD is unequivocal. Supplementation with antioxidants, on the other hand, aims at counteracting the reactive oxygen species involved in oxidative stress. Well-known supplements used to prevent AMD progression are zinc and the macular carotenoids lutein and zeaxanthin.\(^{134}\)

The evidence as to whether diets rich in antioxidant micronutrients will prevent the development of AMD is less consistent. In 1997, the AREDS trial reported that supplementation with high doses of beta-carotene, vitamins C and E, and zinc reduced the relative risk of progression from early to advanced disease by about 20%.\(^{135}\) However, high doses of dietary supplements, particularly beta-carotene, proved to be harmful to smokers and is not without risk.\(^{136}\) The benefits and harms of taking supplements need to be assessed for the individual patient. Accumulating evidence from longitudinal observational studies supports the view that retinal function may be improved or better maintained after supplementation with carotenoids and anti-oxidants.\(^{137}\)

Palliative interventions

For patients with AMD who develop a CNV, monotherapy with an anti-vascular endothelial growth factor (anti-VEGF) drug is the current standard of care. Use of these biologics in the management of neovascular AMD has led to the abandonment of laser-based treatments that were the standard treatment in the previous decades, and involved the ablation of the area of neovascularization with thermal laser or the induction of vascular thrombosis by photodynamic therapy (PDT). Although laser treatments were beneficial compared with the natural history of AMD, they did not generally result in vision gain on the long term.\(^{138,139}\) The subsequent introduction of PDT proved beneficial in the prevention of vision loss, but with the arrival of anti-VEGF drugs, such as ranibizumab and bevacizumab, it became possible to regain visual acuity in patients with exudative AMD.\(^{140,141}\)

For the dry form of AMD, characterized by geographic atrophy, there is currently no effective therapy. Advances in gene therapy and stem cell therapy have the potential to alleviate vision loss and the development of this end-stage AMD lesion in the near future. However, AMD is a complex disease and a thorough appreciation of the pathogenic mechanisms underlying its different clinical subtypes will be necessary to improve the current therapeutic options.
1.5 Aims and outline of this thesis

The main objective of this thesis was to improve our clinical and genetic understanding of the cuticular drusen subtype of AMD. These insights can be used to improve the recognition of this subtype and to counsel patients and their families. We envision that the gained understanding of the pathogenic mechanisms will aid the identification of new therapeutic avenues for cuticular drusen and AMD as a whole.

Chapter 2 evaluates the AMD risk factors in the cuticular drusen subtype of AMD and compares the effect size of the risk factors to AMD in general.

Chapter 3 focuses on the clinical and genetic studies performed in 3 families with cuticular drusen. It describes the identification of novel mutations in the \textit{CFH} gene, followed by a detailed description of the clinical features of individuals carrying these mutations.

Chapter 4 presents the identification of rare missense mutations in the \textit{CFI} gene as a novel cause of AMD, followed by functional studies to investigate the impact of the mutations on protein expression and function. In addition, zebrafish models are used to evaluate the impact of the mutation on vascular architecture \textit{in vivo}.

Chapter 5 concerns an evaluation of drusen morphology over a period of 4 months in patients with cuticular drusen. Drusen that show a spontaneous change in volume over time are further analyzed according to a set of morphologic parameters to determine whether initial drusen morphology can predict the future course of drusen development.

In Chapter 6, the studies described in this thesis are further discussed and placed in a broader perspective. A summary of the findings is provided in Chapter 7.
References


CHAPTER ONE


Chapter Two
Association analysis of genetic and environmental risk factors in the cuticular drusen subtype of age-related macular degeneration

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Objectives: To assess the association of gender, cigarette smoking, body-mass index, and nine genetic risk variants with cuticular drusen (CD), a well recognized subtype of age-related macular degeneration (AMD).

Methods: A total of 757 patients with AMD, including 217 patients with CD, and 553 control individuals were interviewed with a questionnaire and underwent an ophthalmic examination. Venous blood samples were obtained for genomic DNA extraction, and genotyping was performed of single nucleotide polymorphisms previously associated with AMD. Odds ratios were calculated for patients with CD, using unaffected control individuals as a reference. Furthermore, odds ratios in patients with CD were compared to those in patients with “non-CD” AMD.

Results: The CD subtype of AMD was significantly associated with current smoking as well as variants in the complement factor H (CFH), age-related maculopathy susceptibility 2 (ARMS2), complement factor B/complement component 2 (CFB/C2), complement component 3 (C3) and apolipoprotein E (APOE) genes. In patients with CD, the association with the CFH Y402H risk allele was significantly higher ($P = 0.022$), whereas the association with current smoking was significantly lower ($P < 0.001$) than in the heterogeneous group of patients with “non-CD” AMD.

Conclusions: The AMD subtype of CD was associated with previously identified genetic AMD risk factors. However, the association with the CFH Y402H risk allele appeared to be stronger, whereas the association with smoking was less pronounced when compared to AMD as a whole. This study suggests a more important role for genetic factors than environmental factors in the development of this well-defined subtype of AMD. These findings stress the importance of detailed phenotyping in AMD to identify homogeneous AMD subtypes, which may be associated with different risk factors and disease mechanisms. Such studies will improve the accuracy of predictive models and the effectiveness of preventive and therapeutic options in AMD.
Introduction

Age-related macular degeneration (AMD) is the most common cause of irreversible and progressive visual loss among the elderly in the Western world.\textsuperscript{1,2} The abnormalities of this disorder range from discrete drusen deposits and pigmentary changes in early AMD to geographic atrophy and/or choroidal neovascularization (CNV) in the advanced forms.

AMD is a clear example of a multifactorial disease, and a wide variety of risk factors have been associated with the development and progression of AMD. Advanced age, female gender, cigarette smoking, and a high body-mass index (BMI > 30) have been reported as the most consistently reproducible demographic and environmental risk factors in AMD.\textsuperscript{3-7} Familial aggregation analyses and twin studies have provided clear evidence of heritability, and more recently strong associations were found with the Y402H (rs1061170) polymorphism in the complement factor H (\textit{CFH}) gene and with the A69S (rs10490924) polymorphism in the age-related maculopathy susceptibility 2 (\textit{ARMS2}) gene.\textsuperscript{8-12} These two allelic variants contribute to late AMD in more than 80% of cases.\textsuperscript{13,14} Other genes that harbor established risk variants for AMD include the complement factor B (\textit{CFB}), complement component 2 (\textit{C2}), complement component 3 (\textit{C3}), complement factor I (\textit{CFI}), and the apolipoprotein E (\textit{APOE}) genes.\textsuperscript{15-22}

The fact that AMD is highly heterogeneous in its clinical presentation is well recognized. Nevertheless, most studies reporting on the influence of environmental and genetic risk factors analyzed the AMD phenotype as a whole, without attempting to determine these risk factors in more homogeneous subtypes of the disorder. Based on the clinically observed abnormalities, several subtypes of AMD may be recognized, including polypoidal choroidal vasculopathy, retinal angiomatous proliferation, and cuticular drusen (CD).\textsuperscript{23-29} The latter, also known as basal laminar drusen,\textsuperscript{30} is characterized by the fundoscopic findings of innumerable, small (25 \textmu m to 75 \textmu m), uniformly sized, round drusen.\textsuperscript{31} Most commonly appearing in early adulthood, these drusen are easy visualized with fluorescein angiography (FA). In more advanced stages, the multitude of drusen produce a typical “stars-in-the-sky” appearance in early phases of the angiogram.\textsuperscript{32} Researchers have estimated that the CD phenotype comprises approximately 10% of the AMD spectrum.\textsuperscript{29}

In the present study, we investigated whether the AMD subtype of CD displays different environmental and genetic risk factors than AMD as a whole.

Methods

Subjects
In this study, we evaluated a total of 757 unrelated patients with AMD, including 217 patients with CD, and 553 control individuals. All subjects were retrieved from the European Genetic Database (EUGENDA), a multicenter database for clinical and molecular analysis of AMD. In the current study, only Caucasian participants from the Nijmegen (the Netherlands) area participated.

Before being enrolled in EUGENDA, all subjects were interviewed with a questionnaire to document their medical history and lifestyle habits, such as BMI and smoking status. Subjects who reported a kidney disease were excluded from the study to preclude including patients with membranoproliferative glomerulonephritis (MPGN) type II. Pupillary dilatation was achieved with topical 1.0% tropicamide and 2.5% phenylephrine before retinal imaging. Digital non-stereoscopic 30° color fundus photography was performed with a Topcon TRC 50IX (Topcon Corporation, Tokyo, Japan). In addition, patients with AMD received FA and high-resolution Fourier-domain optical coherence tomography (FD-OCT), performed with a combined confocal scanning laser ophthalmoscope/FD-OCT device (SPECTRALIS, Heidelberg Engineering, Heidelberg, Germany).

Color fundus photographs of both eyes of all cases were evaluated by two independent reading center graders according to the standard protocol of the Cologne Image Reading Center and Laboratory (CIRCL). AMD was defined by using international standards as described previously. Individuals of similar age as the AMD cases and who exhibited no signs of AMD in either eye were collected as controls. The CD subtype was defined as a symmetric distributed pattern between both eyes of at least 50 scattered, uniformly-sized, small (25 μm to 75 μm) hyperfluorescent drusen on FA in each eye, of which a minimum of 20 drusen are located outside the Wisconsin age-related maculopathy grading template. After the grading was completed, the AMD cohort was divided into a cohort of patients with the AMD subtype of CD and a group of patients with “non-CD” AMD.

This study was reviewed and approved by the local institutional review boards and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all individuals before they participated in the study.

Single nucleotide polymorphism genotyping
Genomic DNA was isolated from peripheral blood leukocytes using standard techniques and stored at −20 °C. Genotyping of single nucleotide polymorphisms (SNPs) in the CFH (rs1410996), ARMS2 (rs10490924), CFB (rs4151667), C2 (rs9332739), C3 (rs2230199), CFI (rs10033900), and APOE (E2 allele; rs7412 and E4 allele; rs429358) genes in the “non-CD” AMD, CD, and control cohorts were performed as
previously described. The CFH variant Y402H (rs1061170) was analyzed with direct sequencing of PCR products using forward primer 5’-TCA TTG TTA TGG TCC TTA GG-3’ and reverse primer 5’-AAA GAC ATG AAC ATG CTA GG-3’. These nine SNPs were selected because they were previously associated with AMD. Fourteen percent of the genotypes were done in duplicate, resulting in a concordance of ≥ 99.9%.

Statistics
Genotype frequencies in the control individuals were tested for Hardy–Weinberg equilibrium. Baseline and clinical characteristics were analyzed with standard descriptive statistics, and differences in gender, smoking status, and BMI were analyzed with a multivariate logistic regression analysis to adjust for the covariates age, gender, BMI, and smoking status where applicable. Subsequently, to study the associations of allele frequencies for AMD-associated SNPs among the “non-CD” AMD cohort, the CD cohort, and the controls, a multivariate logistic regression analysis was performed to adjust for the covariates age, gender, smoking status, and BMI. The differences between the three cohorts are presented as odds ratios (ORs) with 95% confidence intervals (95% CIs).

Data analysis was performed using SPSS software, version 18.0 (SPSS Inc., Chicago, IL). The reported P values are two-sided, and a value of < 0.05 was considered statistically significant.

Results
Baseline demographics and risk allele frequencies of the “non-CD” AMD (n = 540), CD (n = 217), and control (n = 553) cohorts are depicted in Table 2.1 and Table 2.2. The mean age was 76.7 years (range 55–94; standard deviation [SD] 7.4) in the “non-CD” AMD cohort, 69.3 years (range 50–91; SD 10.4) in the CD cohort, and 73.1 years (range 55–92; SD 6.3) in the controls.

Current smoking showed an association with CD (P = 0.032; OR: 2.06; 95% CI: 1.07–4.00), and this association was significantly lower (P < 0.001; OR: 0.32; 95% CI: 0.17–0.58) compared to the “non-CD” AMD cohort. Female gender showed a trend (P = 0.086), and no association with BMI was found for CD.

All genotype frequencies conformed to Hardy–Weinberg equilibrium in the control cohort. The risk allele frequency of the CFH Y402H (rs1061170) variant was 64.1% in the CD cohort, which closely approximates the prevalence reported previously in patients extensively affected with CD. A significantly higher CFH Y402H risk allele frequency was found in the CD cohort when compared with the control cohort (P < 0.001; OR: 2.88; 95% CI: 2.23–3.73), and when compared to the “non-CD” AMD cohort (P = 0.022; OR: 1.35; 95% CI: 1.04–1.74).
### Table 2.1. Demographics in “non-CD” AMD, CD and control individuals

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<th></th>
<th>Controls</th>
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<th>CD</th>
<th>P-value OR (95-C.I.)</th>
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<tr>
<td>Mean age (SD)</td>
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<td>76.7 (7.42)</td>
<td>69.3 (10.40)</td>
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<td>Male (%)</td>
<td>242 (43.8%)</td>
<td>210 (38.9%)</td>
<td>77 (35.5%)</td>
<td>0.040* 1.33 (1.01-1.75)*</td>
<td>0.086* 1.36 (0.96-1.92)*</td>
<td>0.232* 1.27 (0.86-1.88)*</td>
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<td>Female (%)</td>
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<td>92 (42.4%)</td>
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<td>0.616† 0.92 (0.65-1.30)†</td>
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<td>&gt;30 (%)</td>
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<td>81 (15.0%)</td>
<td>30 (13.8%)</td>
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<td>Never (%)</td>
<td>257 (46.5%)</td>
<td>230 (42.6%)</td>
<td>99 (45.6%)</td>
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<td>Past (%)</td>
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<td>230 (42.6%)</td>
<td>96 (44.2%)</td>
<td>0.685† 1.06 (0.80-1.40)†</td>
<td>0.977† 1.01 (0.71-1.43)†</td>
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<td>80 (14.8%)</td>
<td>22 (10.1%)</td>
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<td>2.06 (1.07-4.00)</td>
<td>2.1×10^{-41} 0.32 (0.17-0.58)</td>
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</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CD, cuticular drusen; OR, odds ratio; CI, confidence interval; BMI, body-mass index; Ref., reference group.

* Adjusted for age, body-mass index and smoking status.

† Adjusted for age, gender and smoking status.

‡ Adjusted for age, gender and body-mass index.
Table 2.2. Risk allele frequencies in “non-CD” AMD, CD and control individuals

<table>
<thead>
<tr>
<th></th>
<th>Controls (%)</th>
<th>“non-CD” AMD (%)</th>
<th>P-value</th>
<th>OR (95-C.I.)</th>
<th>CD (%)</th>
<th>P-value</th>
<th>OR (95-C.I.)</th>
<th>P-value</th>
<th>OR (95-C.I.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs10490924/ARMS2</td>
<td>21.9</td>
<td>42.9</td>
<td>2.0x10^{-21}</td>
<td>2.94 (2.35-3.67)</td>
<td>38.3</td>
<td>1.7x10^{-9}</td>
<td>2.25 (1.73-2.93)</td>
<td>0.140</td>
<td>0.83 (0.64-1.06)</td>
</tr>
<tr>
<td>rs1061170/CFH</td>
<td>38.1</td>
<td>57.7</td>
<td>1.9x10^{-14}</td>
<td>2.17 (1.78-2.65)</td>
<td>64.1</td>
<td>8.6x10^{-16}</td>
<td>2.88 (2.23-3.73)</td>
<td>0.022</td>
<td>1.35 (1.04-1.74)</td>
</tr>
<tr>
<td>rs1410996/CFH</td>
<td>56.9</td>
<td>74.8</td>
<td>9.7x10^{-15}</td>
<td>2.25 (1.83-2.76)</td>
<td>76.8</td>
<td>3.0x10^{-11}</td>
<td>2.53 (1.93-3.33)</td>
<td>0.209</td>
<td>1.20 (0.90-1.61)</td>
</tr>
<tr>
<td>rs9332739/C2</td>
<td>4.7</td>
<td>2.5</td>
<td>0.004</td>
<td>0.47 (0.28-0.79)</td>
<td>1.9</td>
<td>0.016</td>
<td>0.38 (0.17-0.83)</td>
<td>0.744</td>
<td>0.86 (0.34-2.15)</td>
</tr>
<tr>
<td>rs2230199/C3</td>
<td>20.8</td>
<td>27.4</td>
<td>0.001</td>
<td>1.46 (1.18-1.80)</td>
<td>27.9</td>
<td>0.013</td>
<td>1.42 (1.08-1.88)</td>
<td>0.965</td>
<td>1.01 (0.77-1.32)</td>
</tr>
<tr>
<td>rs4151667/CFB</td>
<td>4.9</td>
<td>2.8</td>
<td>0.006</td>
<td>0.49 (0.29-0.82)</td>
<td>2.3</td>
<td>0.027</td>
<td>0.42 (0.19-0.91)</td>
<td>0.750</td>
<td>0.86 (0.35-2.14)</td>
</tr>
<tr>
<td>rs10033900/CFI</td>
<td>48.2</td>
<td>51.8</td>
<td>0.101</td>
<td>1.17 (0.97-1.42)</td>
<td>51.5</td>
<td>0.232</td>
<td>1.16 (0.91-1.49)</td>
<td>0.809</td>
<td>1.03 (0.49-1.35)</td>
</tr>
<tr>
<td>rs7412/APOE2</td>
<td>8.0</td>
<td>10.7</td>
<td>0.115</td>
<td>1.29 (0.94-1.77)</td>
<td>12.1</td>
<td>0.011</td>
<td>1.65 (1.12-2.42)</td>
<td>0.131</td>
<td>1.37 (0.91-2.05)</td>
</tr>
<tr>
<td>rs429358/APOE4</td>
<td>14.4</td>
<td>10.2</td>
<td>0.058</td>
<td>0.74 (0.54-1.01)</td>
<td>11.1</td>
<td>0.047</td>
<td>0.68 (0.46-1.00)</td>
<td>0.660</td>
<td>0.91 (0.59-1.40)</td>
</tr>
</tbody>
</table>

Abbreviations: AMD, age-related macular degeneration; CD, cuticular drusen; OR, odds ratio; CI, confidence interval; ARMS2, age-related maculopathy susceptibility 2; CFH, complement factor H; C2, complement component 2; C3, complement component 3; CFB, complement factor B; CFI, complement factor I; APOE, apolipoprotein E.

Missings in genotypes are <15%.

Data are adjusted for age, gender, body-mass index and smoking status.
The risk allele frequencies of the ARMS2 (rs10490924), CFH (rs1410996), C3 (rs2230199), and APOE E2 (rs7412) variants were significantly higher in the CD cohort compared to the control cohort, and the protective allele frequencies of the C2 (rs9332739), CFB (rs4151667), and APOE E4 (rs429358) variants were significantly lower in the CD cohort compared to the control cohort. These odds ratios were comparable with the “non-CD” AMD cohort, and no significant differences were observed between the CD and “non-CD” AMD cohort for these SNPs. No association with the CFI (rs10033900) risk allele was found in the CD cohort.

Discussion

The clinical spectrum of AMD is broad, and this clinical heterogeneity will influence the results of association studies on demographic, environmental, and genetic risk factors. Improved phenotyping will increase the power of association studies in predictive models for AMD, and will lead to a better understanding of the pathogenesis of the different AMD subtypes.

In the present study, we focused on CD, a well-defined subtype of AMD. The relatively early onset of CD, as well as the observation that the CD phenotype is often clustered in families, implies a greater contribution of the genetic constitution when compared to AMD in general. This is further supported by our observation that one of the most important environmental risk factors, current smoking, showed a significantly lower association with CD than with “non-CD” AMD. The latter may also imply that the general advice to patients with AMD for cessation of smoking could be of limited effect in individuals with the CD subtype of AMD. However, this does certainly not mean that cessation of smoking should not be encouraged in patients with CD as current smoking could worsen the natural history of the disease.

Genetic evaluation of our CD cohort showed significant associations between this AMD subtype and variants in the CFH, ARMS2, CFB, C2, C3, and APOE genes. Risk alleles of the rs1410996 (CFH), rs10490924 (ARMS2), rs4151667 (CFB), rs9332739 (C2), rs2230199 (C3), rs7412 (APOE E2), and rs429358 (APOE E4) SNPs were significantly associated with CD. However, no significant differences for the previously mentioned risk alleles were observed between patients with CD and AMD in general. This suggests that there is a shared genetic background between AMD in general and CD, which has also been described for other AMD subtypes such as polypoidal choroidal vasculopathy and retinal angiomatous proliferation. A lack of association between the rs10033900 (CFI) risk allele and CD could be due to insufficient power in our study to detect small effects. However, the debate over whether this variant is associated with AMD continues as conflicting results have been observed. Additional studies are needed to clarify the nature of the association between AMD and this particular variant near the CFI gene.
A previous study of Caucasian patients who were severely affected with CD demonstrated a strong association with the Y402H (rs1061170) variant in the \textit{CFH} gene.\textsuperscript{25} Our study shows that, in spite of the various stages of the CD phenotype included in our cohort, patients with CD are 1.35 times more likely to carry the \textit{CFH} Y402H risk allele compared to patients with “non-CD” AMD. This higher allele frequency of the \textit{CFH} Y402H risk allele in patients with CD suggests that activation of the alternative pathway of the complement system may play a larger role in the pathogenesis of the CD phenotype than in the remainder of the AMD phenotypes.\textsuperscript{40,41} This is supported by our previous studies that identified rare pathogenic \textit{CFH} mutations in a subset of families with CD.\textsuperscript{23,29} These mutations have not been found in patients with AMD who did not display the CD phenotype. In patients with MPGN type II, or dense deposit disease, \textit{CFH} mutations and disturbed serum complement activation levels have also been demonstrated.\textsuperscript{42} Remarkably, almost 70% of individuals with MPGN type II develop extensive drusen in a pattern matching that of extensive CD during their second decade of life.\textsuperscript{43} In approximately 10% of these patients, CNV and/or central geographic atrophy may develop at a relatively young age.\textsuperscript{44-46} These alterations of the complement system may contribute to the relatively early onset of CD.

The increased insights into the mechanisms underlying AMD have led to possible therapeutic options that have recently entered phase 1 clinical trials.\textsuperscript{47} One option may be the use of specific anti-inflammatory molecules that block complement activation.\textsuperscript{47} These complement inhibitors may especially benefit individuals with the CD subtype of AMD, where complement activation appears more fundamental to the disease process compared to AMD in general.

In conclusion, the analysis of a large cohort of the CD subtype of AMD has revealed that genetic risk factors affecting the complement system are especially prevalent in these patients. In addition, the environmental risk factor of smoking appears less influential than in AMD in general. These findings stress the importance of detailed phenotyping in AMD to identify homogeneous AMD subgroups, which may be associated with different risk factors and disease mechanisms. Such studies may improve the accuracy of predictive models and the effectiveness of preventive and therapeutic options in AMD.


**References**


Chapter Three
Clinical evaluation of 3 families with cuticular drusen caused by novel mutations in the complement factor H gene

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Objectives: To identify novel complement factor H (CFH) gene mutations and to specify the clinical characteristics in patients with cuticular drusen (CD), a clinical subtype of age-related macular degeneration.

Methods: Twenty-one probands with CD were included in this study. The ophthalmic examination included nonstereoscopic 30° color fundus photography, fluorescein angiography, and high-resolution spectral-domain optical coherence tomography. Renal function was tested by measurement of serum creatinine and urea nitrogen levels. Venous blood samples were drawn for genomic DNA, and all coding exons and splice junctions of the CFH gene were analyzed by direct sequencing.

Results: In 3 families, we identified novel heterozygous mutations in the CFH gene: p.Ile184fsX, p.Lys204fsX, and c.1697-17_-8del. Ten of 13 mutation carriers displayed the CD phenotype with a wide variety in clinical presentation, ranging from limited macular drusen to extensive drusen in the posterior pole as well as the peripheral retina. Two patients with CD developed endstage kidney disease as a result of membranoproliferative glomerulonephritis type II.

Conclusions: The early-onset CD phenotype can be caused by heterozygous mutations in the CFH gene. Because some patients with CD are at risk to develop membranoproliferative glomerulonephritis type II, we recommend that patients with extensive CD undergo screening for renal dysfunction.
Introduction

Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the Western world among people 65 years or older, with a prevalence of advanced AMD of 12% after 80 years of age. Several risk factors for the development of AMD have been recognized, including modifiable risk factors, such as smoking, higher body mass index, and low dietary intake of antioxidants and zinc. Nonmodifiable risk factors include advancing age, female sex, white race, and a great variety of genetic factors.

Genes involved in the complement system have received heightened attention because single-nucleotide polymorphisms in the genes encoding complement factor H (CFH), complement factor B (CFB), complement factor I (CFI), component 2 (C2), and component 3 (C3) are associated with increased risk of AMD. A particularly strong association has been reported by many studies for a nonsynonymous single-nucleotide polymorphism in CFH that encodes a tyrosine-to-histidine missense variant at amino acid 402 (p.Tyr402His). Carriership of this variant increases the risk for AMD with an odds ratio ranging from 2.45 to 7.40 and may account for more than 50% of the attributable risk of AMD.

The complement factor H (FH) protein inhibits the alternative pathway by competing with complement factor B in binding to C3b, accelerating the decay of the alternative pathway C3 convertase and acting as a cofactor for the factor I–mediated proteolytic inactivation of C3b. By this mechanism, FH is essential to maintain complement homeostasis in plasma and to restrict complement activation on complement activating self-surfaces such as the retinal pigment epithelium.

Age-related macular degeneration is characterized by multiple heterogeneous subtypes, with drusen as the hallmark lesions and usually the first clinical finding. Cuticular drusen (CD), also termed basal laminar drusen or early adult onset, grouped drusen, is one of the subtypes in the AMD spectrum. The CD phenotype shows characteristic innumerable, small, subretinal, raised yellow drusen that are hyperfluorescent on fluorescein angiography, resulting in a typical “stars-in-the-sky” appearance. The CD phenotype is also associated with the p.Tyr402His variant in the CFH gene, with a risk allele frequency up to 70% vs 55% in “typical” AMD-affected individuals. Boon and colleagues found an association of compound heterozygous variants in the CFH gene with CD. Specific mutations and variants in the CFH gene are associated with a broad range of phenotypes, from early-onset renal diseases with high mortality rates to disorders limited to the eye, such as AMD. Some patients have concurrent renal and retinal abnormalities. It has been postulated that the type, onset, and severity of renal and/or retinal abnormalities show a considerable degree of genotype-phenotype correlation.

The purposes of this study were to identify novel CFH gene mutations and to specify the clinical characteristics in patients with CD.
Methods

In this study, we included 21 probands diagnosed as having AMD who were noted on initial examination to have CD on fluorescein angiography and 192 ethnically matched control subjects of similar age who showed no signs of maculopathy. Informed consent was obtained from all subjects after explanation of the nature and possible consequences of the study. We conducted the study in accordance with the tenets of the Declaration of Helsinki, and it was approved by the Committee on Research Involving Human Subjects at the Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands.

Ophthalmic examination
Ophthalmic examination of the subjects included Early Treatment Diabetic Retinopathy Study visual acuity and slitlamp biomicroscopy after pupil dilatation. Digital nonstereoscopic 30° color fundus photographs were taken with a digital fundus camera (Topcon TRC 50IX; Topcon Corporation). To confirm the diagnosis of CD, we performed fluorescein angiography and high-resolution Fourier-domain optical coherence tomography using the combined confocal scanning laser ophthalmoscope/Fourier-domain optical coherence tomography device (Spectralis; Heidelberg Engineering). In the early stages, the diagnosis was based on fluorescein angiographic confirmation of innumerable small drusen in the macula and/or peripheral retina, giving a symmetrically distributed pattern of innumerable, scattered, uniformly sized, small (25 to 75 μm) hyperfluorescent lesions in both eyes. The occurrence of confluent (soft) drusen in the macular region and the subsequent development of a drusenoid pigment epithelial detachment are considered characteristic for the later stages of this disease. A final feature is the central geographic atrophy of the retinal pigment epithelium, frequently observed after resolution of the drusenoid pigment epithelial detachment or the development of choroidal neovascularization (CNV).

Renal function
Renal function was tested by measuring serum creatinine and urea nitrogen levels. The following ranges were considered for normal kidney function: 0.68 to 1.24 mg/dL for creatinine and 7.0 to 19.6 mg/dL for urea nitrogen. (To convert creatinine to micromoles per liter, multiply by 88.4, and to convert urea nitrogen to millimoles per liter, multiply by 0.357.)

Mutation analysis
Venous blood samples were drawn for genomic DNA extraction from peripheral blood leukocytes. The DNA was analyzed for mutations in CFH (NCBI Entrez Gene NM_000186) by polymerase chain reaction amplification of the 22 coding exons and splice junctions. Reactions were performed using standard protocols. (Primer sequences and polymerase chain reaction conditions are available from the authors on request.) Amplification products were purified, quantified on a 2% agarose gel,
and diluted for direct sequencing on an automated sequencer (BigDye Terminator, version 3 on a 3730 DNA analyzer; Applied Biosystems, Inc). Sequences were assembled using proprietary software (ContigExpress, Vector NTI suite, version 10.0; InforMax, Inc). Each of the novel mutations identified was validated through an independent polymerase chain reaction and a sequencing reaction.

**Results**

In 3 of the 21 probands, we identified novel heterozygous mutations in the **CFH** gene: 2 frameshift mutations in exon 5 and 1 splice-site mutation in the splice-acceptor site of exon 12 (*Figure 3.1*). None of these **CFH** mutations were identified in 192 control subjects who had no signs of maculopathy, and no nonsense, frameshift, or splice-site mutations were identified in 369 ethnically matched controls from our in-house exome database.

The probands who carried a mutation in the **CFH** gene could not be distinguished clinically from the probands who did not carry a **CFH** mutation (*Table 3.1*). Of the 20 additional family members who underwent screening for the novel **CFH** gene mutations, 10 were shown to carry the same mutation as the proband, of whom 7 proved to be affected by CD (*Figure 3.2* and *Table 3.2*). However, only the probands of the 3 families noticed visual loss before the diagnosis of CD was established.

Of all the patients carrying a mutation in **CFH**, 5 (in families A and B) were compound heterozygous for the novel **CFH** mutation together with the AMD risk allele p.Tyr402His in the **CFH** gene. The other 8 patients (in families A, B, and C) did not carry the p.Tyr402His risk allele or carried it heterozygously on the same allele as the mutation. An overview of the clinical and genetic characteristics of the 3 families is given in *Table 3.2*.

*Figure 3.1. Sequences of heterozygous mutations detected in the CFH gene. For each CFH mutation, the chromatogram corresponding to the DNA sequence surrounding the mutation in CFH is shown. MUT indicates mutated CFH allele; WT, wild-type CFH allele.*
### Table 3.1. Clinical and genetic characteristics of the 21 evaluated probands

<table>
<thead>
<tr>
<th>Patient Code/Age</th>
<th>Age, y</th>
<th>Visual Acuity</th>
<th>Retinal Phenotype</th>
<th>CFH Mutation</th>
<th>CFH p.Tyr402His, Allele 1/Allele 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at onset, y/Sex</td>
<td>OD</td>
<td>OS</td>
<td>Both eyes: innumerable hard and confluent soft drusen in midperipheral retina; macular drusenoid PED, surrounded by crystalline drusen **</td>
<td>p.Ile184fsX</td>
</tr>
<tr>
<td>A-II:3/56/M</td>
<td>62</td>
<td>20/25</td>
<td>20/33</td>
<td>Both eyes: innumerable hard and confluent soft drusen in midperipheral retina; macular drusenoid PED, surrounded by crystalline drusen</td>
<td>p.Ile184fsX</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Both eyes: macular drusen with barely discernable (mid) peripheral drusen, clearly visualized on fluorescein angiography **</td>
<td>p.Lys204fsX</td>
</tr>
<tr>
<td>B-II:1/47/F</td>
<td>52</td>
<td>20/17</td>
<td>20/24</td>
<td>Both eyes: macular drusen with barely discernable (mid) peripheral drusen, clearly visualized on fluorescein angiography **</td>
<td>p.Lys204fsX</td>
</tr>
<tr>
<td>C-II:4/55/M</td>
<td>58</td>
<td>20/20</td>
<td>20/16</td>
<td>Both eyes: confluent soft drusen in posterior pole, surrounded by hard drusen **</td>
<td>c.1697-17_-8del</td>
</tr>
<tr>
<td>D/50/F</td>
<td>61</td>
<td>20/35</td>
<td>20/50</td>
<td>Both eyes: innumerable small drusen in posterior pole **</td>
<td>None</td>
</tr>
<tr>
<td>E/54/F</td>
<td>60</td>
<td>20/20</td>
<td>20/25</td>
<td>Both eyes: innumerable small drusen scattered throughout retina **</td>
<td>None</td>
</tr>
<tr>
<td>F/49/F</td>
<td>54</td>
<td>20/25</td>
<td>20/25</td>
<td>Both eyes: confluent macular drusen, drusenoid PED, patches of chorioretinal atrophy **</td>
<td>None</td>
</tr>
<tr>
<td>G/46/F</td>
<td>57</td>
<td>20/25</td>
<td>LP</td>
<td>OD: confluent macular drusen surrounded by small drusen, drusenoid PED</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OS: fibrotic scar **</td>
<td>None</td>
</tr>
<tr>
<td>H/55/F</td>
<td>57</td>
<td>20/33</td>
<td>20/33</td>
<td>Both eyes: confluent macular drusen surrounded by small drusen **</td>
<td>None</td>
</tr>
<tr>
<td>I/58/F</td>
<td>71</td>
<td>20/33</td>
<td>20/25</td>
<td>OD: innumerable small drusen scattered throughout retina, small classic CNV</td>
<td>None</td>
</tr>
<tr>
<td>J/68/M</td>
<td>75</td>
<td>20/25</td>
<td>20/80</td>
<td>OD: macular small drusen in posterior pole</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>OS: macular small drusen in posterior pole, large classic CNV **</td>
<td>None</td>
</tr>
</tbody>
</table>

**Abbreviations:** CNV, choroidal neovascularization; ellipses, no visual loss reported; GA, geographic atrophy; His, histidine; LP, light perception; OD, right eye; OS, left eye; PED, pigment epithelial detachment; Tyr, tyrosine.
### Table 3

<table>
<thead>
<tr>
<th>Patient Code/Age</th>
<th>Allele 1/Allele 2*</th>
<th>Visual Acuity</th>
<th>Retinal Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>K/48/M 74</td>
<td>Tyr/His</td>
<td>20/20 20/25</td>
<td>Both eyes: innumerable small drusen in posterior pole</td>
</tr>
</tbody>
</table>
| L/66/F 68        | His/His           | 20/33 20/120  | OD: confluent macular drusen surrounded by small drusen, drusenoid PED
OS: central GA surrounded by small drusen |
| M/55/M 55        | His/His           | 20/16 20/16   | Both eyes: extensive small drusen in posterior pole                               |
| N/…/F 38        | Tyr/His           | 20/20 20/20   | Both eyes: extensive soft drusen in posterior pole, numerous small peripheral drusen |
| O/67/F 71        | Tyr/His           | 20/20 20/20   | Both eyes: innumerable small drusen scattered throughout retina                   |
| P/68/F 70        | Tyr/His           | 20/20 20/40   | OD: innumerable small drusen in midperipheral retina, classic CNV
OS: innumerable small drusen in midperipheral retina |
| Q/43/F 48        | Tyr/His           | 20/25 20/100  | Both eyes: confluent macular drusen surrounded by small drusen, drusenoid PED, patches of chorioretinal atrophy |
| R/62/F 63        | Tyr/His           | 20/50 20/60   | Both eyes: central GA surrounded by small drusen                                 |
| S/…/M 43        | His/His           | 20/20 20/20   | Both eyes: small drusen scattered throughout retina                               |
| T/45/F 52        | Tyr/His           | 20/33 20/50   | Both eyes: innumerable small drusen scattered throughout retina                   |
| U/63/M 75        | Tyr/His           | 20/25 20/25   | Both eyes: central pseudovitelliform lesion surrounded by innumerable small drusen |

**Abbreviations:** CNV, choroidal neovascularization; ellipses, no visual loss reported; GA, geographic atrophy; His, histidine; LP, light perception; OD, right eye; OS, left eye; PED, pigment epithelial detachment; Tyr, tyrosine.

*Tyr represents the wild-type allele, and His represents the risk allele.
<table>
<thead>
<tr>
<th>Patient Code/Age</th>
<th>Visual Acuity</th>
<th>Retinal Phenotype</th>
<th>CFH Mutation</th>
<th>CFH p.Tyr402His, Allele 1/Allele 2†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-I:2/…/F</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/His</td>
</tr>
<tr>
<td>A-II:1/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-II:3 (P)/56/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/His</td>
</tr>
<tr>
<td>A-II:5/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-II:7/…/F</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-III:1/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-III:2/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-III:3/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-III:4/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-III:5/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>B-II:1 (P)/47/F</td>
<td>OD: hyperpigmented macular scar</td>
<td></td>
<td>None</td>
<td>His/His</td>
</tr>
<tr>
<td>B-II:3/…/F</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>His/His</td>
</tr>
<tr>
<td>B-II:4/…/M</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>His/His</td>
</tr>
<tr>
<td>B-II:6/…/F</td>
<td>OD: normal</td>
<td></td>
<td>None</td>
<td>His/His</td>
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<tr>
<td>Patient Code/Age</td>
<td>Age, y</td>
<td>Retinal Phenotype</td>
<td>CFH Mutation</td>
<td>Allele 1/Allele 2†</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------</td>
<td>-----------------------------------------------------------------------------------</td>
<td>--------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>A-I:2</td>
<td>83</td>
<td>NA NA Both eyes: normal</td>
<td>CFH p.Tyr402His,</td>
<td>Tyr/His</td>
</tr>
<tr>
<td>A-II:1</td>
<td>64</td>
<td>NA NA Both eyes: mostly mid-peripheral hard drusen; some hard drusen in macular area</td>
<td>CFH p.Ile184fsX,</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
<td>A-II:3 (P)</td>
<td>56</td>
<td>20/25 20/33 Both eyes: innumerable hard and confluent soft drusen in mid-peripheral retina</td>
<td>CFH p.Ile184fsX,</td>
<td>Tyr/His</td>
</tr>
<tr>
<td>A-II:5</td>
<td>61</td>
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<td>CFH p.Ile184fsX,</td>
<td>Tyr/His</td>
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<tr>
<td>A-II:7</td>
<td>60</td>
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<td>None</td>
<td>Tyr/His</td>
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<tr>
<td>A-III:1</td>
<td>31</td>
<td>20/20 20/20 Both eyes: hard drusen in macular area and innumerable hard drusen in peripheral retina</td>
<td>CFH p.Ile184fsX,</td>
<td>Tyr/His</td>
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<td>A-III:2</td>
<td>27</td>
<td>20/20 20/20 Both eyes: hard drusen in peripheral retina</td>
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<td>Tyr/His</td>
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<td>A-III:3</td>
<td>22</td>
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<td>Tyr/His</td>
</tr>
<tr>
<td>C-II:2</td>
<td>54</td>
<td>20/25 20/25 Both eyes: extensive drusen in posterior pole, innumerable hard drusen in periphery</td>
<td>CFH c.1697-17_-8del,</td>
<td>Tyr/Tyr</td>
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<tr>
<td>C-II:4 (P)/55/M</td>
<td>58</td>
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<td>CFH c.1697-17_-8del,</td>
<td>Tyr/Tyr</td>
</tr>
<tr>
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<td>20/12 20/60 (A) Both eyes: normal</td>
<td>None</td>
<td>Tyr/His</td>
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<tr>
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<td>Tyr/Tyr</td>
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<tr>
<td>C-III:2/…/M</td>
<td>25</td>
<td>20/20 20/20 Both eyes: normal</td>
<td>None</td>
<td>Tyr/Tyr</td>
</tr>
</tbody>
</table>

Abbreviations: A, amblyopia; ellipses, no visual loss reported; His, histidine; NA, no visual acuity available; OD, right eye; OS, left eye; P, Proband; PED, pigment epithelial detachment; T, old *Toxoplasma gondii* infection; Tyr, tyrosine.

* Proband are indicated parenthetically after the patient code.

† Tyr represents the wild-type allele, and His represents the risk allele.

CUTICULAR DRUSEN CAUSED BY A MUTATION IN THE CFH GENE
Family A
In family A, we identified the heterozygous c.550delA; p.Ile184fsX frameshift mutation in exon 5. This mutation occurs in the third short consensus repeat of the FH protein.

The proband of family A (A-II:3) first noticed metamorphopsia and a decrease in visual acuity in both eyes at age 56 years. Ophthalmoscopy revealed extensive small and large confluent drusen in the posterior pole with a drusenoid pigment epithelial detachment in the macula of both eyes (Figure 3.3B and H). Among the affected siblings of the proband, patient A-II:1 (aged 64 years) showed hard drusen in the

Figure 3.2. Molecular genetic analyses of the CFH gene in families affected with cuticular drusen (CD). Squares indicate men; circles, women; slashes, deceased family members; black symbols, patients with CD; shaded symbols, patients who display drusen but without CD; numbers in the pedigree symbols, current age (in years); plus signs, the wild-type allele; 402H, the CFH Y402H risk allele; and 402Y, the CFH wild-type allele. Mutations are in red, risk alleles in orange, and wild-type alleles in black. (A) All individuals affected by CD were heterozygous for the p.Ile184fsX frameshift mutation with the exception of the youngest mutation carrier, who had only some soft peripheral drusen at the time of examination. (B) All individuals carrying the p.Lys204fsX frameshift mutation were affected by CD. (C) Two carriers (C-II:2 and C-III:1) of the c.1697-17_-8del frameshift mutation did not display CD.
midperipheral retina, mostly located temporal to the fovea, whereas patient A-II:5 (aged 61 years) had dense, macular, small and soft confluent drusen (Figure 3.3A and C). Small hard drusen were seen in the peripheral retina of patient A-III:1 (aged 31 years) and A-III:2 (aged 27 years), with increasing numbers of these peripheral drusen with increasing age (Figure 3.3D, E, and G). Additional macular hard drusen were observed only in the oldest mutation carrier of the third generation (A-III:1) but to a lesser extent compared with his father (A-II:1). Patient A-III:4, the youngest mutation carrier in this family (aged 18 years) had some soft drusen in the peripheral retina but no hard drusen as were found in other family members carrying the c.550delA mutation (Figure 3.3F).

**Family B**
In family B, we identified the heterozygous c.607-610dupCCAA; p.Lys204fsX frameshift mutation in exon 5. As was the case for the c.550delA mutation in family A, this mutation also occurs in the region of the third short consensus repeat in the FH protein.

Patient B-II:1, the proband of family B, first noticed visual loss, associated metamorphopsia, and small central scotomas in both eyes at age 47 years. The proband and her affected siblings (B-II:4 and B-II:6) showed an equivalent CD phenotype of innumerable macular hard and soft drusen, with small hard drusen extending toward the peripheral retina that were symmetrical in both fundi (Figure 3.4). The hard drusen in the peripheral retina had only a thin hyperpigmented border. Three years after the initial visual complaints, the proband reported increasing metamorphopsia and a rapid decrease in visual acuity from 20/20 to 20/67 of the left eye due to classic CNV in the left eye. This neovascularization was treated successfully during a period of 3 months with 3 intravitreal injections of bevacizumab, 0.05 mL (25 mg/mL), at an interval of 4 weeks, resulting in increased visual acuity to 20/24 in that eye for 2 years as of the last examination.

**Family C**
In family C, we identified 3 individuals with the heterozygous CFH gene mutation (c.1697-17_-8del) in the splice-acceptor site. This mutation is predicted to abolish the splice-acceptor site of exon 12 of the CFH gene given that the splice prediction score is reduced from 0.62 to 0 (as calculated by the splice-site prediction program NNSPLICE, version 0.9; http://www.fruitfly.org/seq_tools/splice.html).

Only patient C-II:4 was affected with CD. He reported a rapid decrease in visual acuity from 20/20 to 20/35 and metamorphopsia of the right eye at age 55 years. Both fundi showed a pigment epithelial detachment and pigmentary changes in the macular area, together with numerous small hard drusen in the midperipheral retina, mostly located temporal to the fovea. The right eye also showed a large area of parafoveal subretinal hemorrhage (Figure 3.5A and B). In both eyes, fluorescein angiography revealed more dense and well-circumscribed hyperfluorescent CD than were seen during direct ophthalmoscopy. In addition, the angiography revealed
parafoveal occult CNV in the right eye. This patient was treated successfully with 4 intravitreal injections of bevacizumab, 0.05 mL (25 mg/mL), during a period of 6 months, resulting in an increased and stabilized visual acuity of 20/20 without metamorphopsia for 3 years to date.

A renal biopsy in patient C-II:4 at 27 years of age showed MPGN type II, resembling the findings in patient B-II:1. At age 46 years, end-stage kidney disease and subsequent renal failure necessitated a renal transplant. At the time of the most recent ophthalmic investigation, at age 58 years, there was no hematuria or proteinuria; the serum creatinine level was 1.10 mg/dL. In the 2 other carriers (C-II:2 and C-III:1) of the c.1697-17_-8del mutation, we observed no fundus abnormalities and no signs of renal failure on blood test results.

Discussion

A subgroup of approximately 10% of patients with AMD are found to have CD at the initial examination, that is, innumerable small hard drusen throughout the fundus that are hyperfluorescent on fluorescein angiography, resulting in a typical stars-in-the-sky appearance (J.P.H.V, C.J.F.B., L.H.H., B.J.K., A.I.D., and C.B.H., unpublished data, January 2012). The age at onset of CD is typically earlier than that for regular AMD, and CD are often observed in asymptomatic family members.25 The location and histopathological composition of CD appear to be identical to the drusen found in typical AMD.30 A common mechanism of drusen biogenesis is therefore likely.

An association of the p.Tyr402His variant in the CFH gene with both AMD and the subtype of CD has been previously described and confirmed by several studies.10-12,24,31 In addition, Boon and coworkers25 were the first to find pathogenic heterozygous mutations in the CFH gene in association with the CD phenotype. In their study, the development of CD in individuals who carry a CFH mutation on one allele in combination with the presence of the p.Tyr402His variant on the other allele is described. We confirm this disease-causing model by heterozygous CFH gene mutations in a subgroup of patients affected with CD. However, the mode of inheritance of these mutations was not apparent in any of the families. Our study was not consistently in accordance with the suggested disease model of compound heterozygosity with the p.Tyr402His variant on the other allele. However, we cannot exclude that heterozygous CFH mutations will cause CD and/or MPGN type II only when coinherited with as-yet unidentified variants in other genes.

The segregation of mutations in families A and B appears to be consistent with an autosomal dominant inheritance pattern. At age 18 years, the youngest member of family A (A-III:4), who carries a CFH gene mutation, showed only peripheral soft drusen without the typical hard drusen seen in patients with CD. Because
the formation of drusen is related to age, this patient may develop more drusen in the future in accordance with the CD phenotype. In family C, 1 individual of the 3 mutation carriers was affected, suggesting reduced penetrance of the CFH mutation or digenic/multigenic inheritance of variants in other genes. Alternatively, it is possible that a combination of genetic and acquired defects in the complement system may cause the disease, as has been demonstrated for MPGN.32,33

Figure 3.3. Retinal phenotypes of patients carrying the CFH p.Ile184fsX frameshift mutation. Fundus photography of the right eyes showed extensive hard drusen in midperipheral retina, mostly located temporally in patient A-II:1 (A); extensive soft, hard, and crystalline drusen scattered throughout the fundus in patient A-II:3 (B); and macular hard and soft drusen in patient A-II:5 (C). The green line indicates the optical coherence tomography section. Clustered groups of hard drusen (white arrowheads) were seen in the peripheral retina of patient A-III:1 (D) and patient A-III:2 (E) by fundus photography. In patient A-III:4, fundus photography showed soft drusen in the peripheral retina (F). Fluorescein angiography of the right eye of patient A-III:1 revealed more tiny hyperfluorescent drusen (G) than the number seen on color photography (D) in the peripheral retina. Optical coherence tomography (oblique section) of patient A-II:2 showed small dome-shaped elevations of the retinal pigment epithelium (H).
Figure 3.4. Retinal phenotypes of patients carrying the CFH p.Lys204fsX frameshift mutation. Fundus photography of the right eyes showed extensive drusen in the posterior pole extending to the peripheral retina of patients B-II:1 (A), B-II:4 (B), and B-II:6 (C). The green line indicates the optical coherence tomography section. Fluorescein angiography of patient B-II:4 showed similar but more numerous lesions (D) compared with color photography (B). Optical coherence tomography (oblique section) showed small dome-shaped elevations of the retinal pigment epithelium (E).

Figure 3.5. Retinal phenotype patient C-II:4, carrier of the CFH c.1697-17_-8del splice-acceptor site mutation. Fundus photography of the right eye showed, besides the extensive drusen in the posterior pole, a subretinal hemorrhage (A), which is clearly visualized with fluorescein angiography at age 55 years (B). At age 58 years, fundus photography showed large, soft, confluent macular drusen surrounded by many hard drusen in the right eye (C). Fluorescein angiography at age 58 years showed densely packed hyperfluorescent drusen in the posterior pole of the right eye (D). Optical coherence tomography (oblique section) showed the density of the drusen by the dome-shaped elevations of the retinal pigment epithelium (E).
Together with a previous report on CD caused by \textit{CFH} gene mutations,\textsuperscript{25} our findings suggest that only patients having specific gene mutations will develop this clinical phenotype of CD or have a greater genetic predisposition to develop CD. This is in contrast to typical AMD, which is a multifactorial disorder caused by accumulating genetic and environmental risk.\textsuperscript{3,8,11,34} This also might be a plausible explanation for the earlier onset of CD compared with typical AMD. In our study, the 10 affected individuals with CD who carried mutations in the \textit{CFH} gene showed a heterogeneous clinical presentation. A robust genotype-phenotype correlation of the severity of the disease is therefore not possible because only the identified \textit{CFH} mutations were taken into consideration.

Besides CD, specific mutations in the \textit{CFH} gene can also cause MPGN type II (dense deposit disease).\textsuperscript{35} However, the mutations we describe in this study are novel and, to our knowledge, have never been identified in patients with MPGN type II. To date, only 9 patients with MPGN type II have been reported to carry \textit{CFH} mutations, and nearly all of them were homozygous or compound heterozygous for missense mutations in \textit{CFH}.\textsuperscript{36,37} Only 1 patient was reported to carry a single heterozygous missense mutation and to develop late-onset MPGN type II and CD.\textsuperscript{38} Because of the relatively late onset of MPGN type II in the 2 patients (B-II:1 and C-II:4) of our families, we reason that single heterozygous mutations in \textit{CFH} may cause late-onset MPGN type II. Given that patient B-II:1 had early-onset CD at the initial examination before renal disease was diagnosed, we recommend that patients with extensive early-onset CD undergo screening for renal dysfunction. Despite urea and creatinine clearance within reference limits, MPGN and future renal dysfunction might develop because MPGN may be at a subclinical stage.\textsuperscript{39}

Fundus changes in patients with MPGN type II vary from pigmentary changes and CD to larger soft drusen and CNV, finally leading to visual loss.\textsuperscript{27,28,40,41} The 2 cases reported in our study are the second and third reported in the literature who developed a triad of MPGN type II, CD, and CNV caused by a specific mutation in \textit{CFH}.\textsuperscript{38} In both cases, the CNV was effectively treated with intravitreal injections of bevacizumab.

With upcoming treatment modalities to target specific components of the complement system, early identification of the CD subgroup of patients with AMD becomes relevant. The strong association of this group of patients with complement abnormalities may translate into a better response to complement-blocking therapy than among patients with AMD in general. Treatment with a humanized monoclonal antibody that blocks complement activity was shown to be successful in a patient with atypical hemolytic uremic syndrome.\textsuperscript{42-46} In 30% of all patients with atypical hemolytic uremic syndrome, \textit{CFH} gene mutations are the cause of the disease. This manifests as a loss of function of \textit{CFH}, resulting in increased activity of the complement system’s alternative pathway.\textsuperscript{47,49} Humanized monoclonal antibodies can inhibit the overactivated complement system. For this reason, humanized
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monoclonal antibody seems to be a rational candidate treatment for patients with CD caused by mutations in the \textit{CFH} gene.

In summary, our findings confirm the important role of heterozygous mutations in the \textit{CFH} gene in the development of CD. The genotype-phenotype correlation is not straightforward, and other genetic and possibly environmental factors may contribute to the development or severity of the disease. We recommend monitoring the renal function in patients with extensive CD because some of these patients may develop MPGN type II. Conversely, ophthalmic screening for CD in patients with MPGN type II is recommended because of the risk of developing CNV and/or geographic atrophy. The association of heterozygous \textit{CFH} mutations and presumed ensuing complement dysfunction in patients with AMD who also have CD provides us with a promising target for future treatments.
References


CHAPTER THREE


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Chapter Four
A functional variant in the CFI gene confers a high risk of age-related macular degeneration

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Up to half of the heritability of age-related macular degeneration (AMD) is explained by common variants. Here, we report the identification of a rare, highly penetrant missense mutation in CFI encoding a p.Gly119Arg substitution that confers a high risk of AMD ($P = 3.79 \times 10^{-6}$, odds ratio = 22.20; 95% confidence interval 2.98-164.49). Plasma/serum of cases carrying the p.Gly119Arg substitution mediated the degradation of C3b both in the fluid phase and on the cell surface at a lower level compared to controls. Recombinant protein studies revealed that the p.Gly119Arg mutant protein is both expressed and secreted at lower levels than wildtype protein. Consistent with these findings, 119Arg-encoding human mRNA had reduced activity compared to 119Gly in regulating vessel thickness and branching in the zebrafish retina. Taken together, these findings demonstrate that rare, highly penetrant mutations contribute to the genetic variance of AMD, with implications for predictive testing and personalized treatment.
Age-related macular degeneration (AMD) is the most common cause of blindness among the elderly in the industrialized world.\textsuperscript{1,2} Genome-wide association studies (GWAS) revealed that common variants in or near genes within the complement cascade, high-density lipoprotein cholesterol pathway, extracellular collagen matrix, and vascular endothelial growth factor are associated with AMD.\textsuperscript{3-6} Combined, these variants explain 50\% of the heritability of AMD.\textsuperscript{6-10} A fraction of the “missing heritability” in AMD may be explained by rare, highly penetrant variants.\textsuperscript{11-14}

To explore the role of rare, highly penetrant variants in AMD, and to provide direct causal evidence for particular genes and their protein products, we focused on \textit{CFI}, a gene that encodes Factor I (FI), a serine protease that circulates in its inactive form and can inactivate all complement pathways by cleaving the α’ chain of activated complement factors C3b and C4b.\textsuperscript{15,16} The importance of this FI-mediated regulatory mechanism is highlighted by the fact that rare \textit{CFI} variants predispose to diseases such as atypical hemolytic uremic syndrome (aHUS)\textsuperscript{17} and systemic lupus erythematosus.\textsuperscript{18} The hypothesis that altered FI function increases AMD risk has been raised,\textsuperscript{19} and an association between AMD and a haplotype block spanning the last two exons of \textit{CFI} and the adjacent \textit{PLAG12A} transcript was reported.\textsuperscript{20} However, in several GWAS, the most significant associations at this locus were reported for SNPs that are located downstream of \textit{CFI} and in the adjacent \textit{PLAG12A} and \textit{CCDC109B} genes.\textsuperscript{3,5,6} Therefore, these studies cannot distinguish between the involvement of \textit{PLAG12A} or \textit{CCDC109B} — rather than \textit{CFI} — as the source of the association signal.

\textbf{Methods}

\textit{Ethics Statement}

This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the participating centers. All subjects provided signed informed consent for participation in the study and the use of their blood and DNA for AMD research.

\textit{Study sample descriptions}

\textit{Case definitions.} Each subject was either clinically evaluated by dilated slit-lamp biomicroscopy and non-stereoscopic color fundus photography or assessed by reviewing the full ophthalmologic medical records. Cases in the EUGENDA, Baltimore and Rotterdam collections had either early AMD (stage 2; soft indistinct drusen (≥ 125 µm) only, reticular drusen only or soft indistinct drusen (≥ 63 µm) with pigmentary irregularities or stage 3; soft indistinct drusen (≥ 125 µm) or reticular drusen with pigmentary irregularities) or advanced AMD (stage 4; central chorioretinal atrophy or choroidal neovascularization) according the Rotterdam classification (Table 4.1).\textsuperscript{21} All subjects were classified based on the eye with the more advanced diagnosis. Control subjects did not have AMD (stage 0; no or only small hard drusen) in either eye and exhibited no other macular pathology. Individuals with AMD stage 1 (soft
Table 4.1 Age-related macular degeneration (AMD) staging results from six case-control datasets

<table>
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<td>3</td>
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<tr>
<td>1. EUGENDA-discovery</td>
<td>8 (9.5%)</td>
<td>27 (32.1%)</td>
</tr>
<tr>
<td>2. EUGENDA-replication</td>
<td>151 (14.9%)</td>
<td>50 (4.9%)</td>
</tr>
<tr>
<td>3. Baltimore-replication</td>
<td>65 (9.1%)</td>
<td>143 (20.0%)</td>
</tr>
<tr>
<td>4. Rotterdam Study I-replication</td>
<td>799 (61.0%)</td>
<td>298 (22.8%)</td>
</tr>
<tr>
<td>5. Rotterdam Study II-replication</td>
<td>219 (77.4%)</td>
<td>32 (11.3%)</td>
</tr>
<tr>
<td>6. Rotterdam Study III-replication</td>
<td>129 (80.1%)</td>
<td>23 (14.3%)</td>
</tr>
</tbody>
</table>

indistinct drusen (≥ 63 µm) only or pigmentary irregularities only) were excluded to avoid ambiguous diagnoses.

**EUGENDA.** Subjects were recruited through the ongoing EUGENDA study protocol.22,23 All subjects in the current study were unrelated, self-described Caucasian individuals from the Nijmegen (the Netherlands) or Cologne (Germany) area. All EUGENDA cases were ≥ 50 years of age, and the control subjects were ≥ 65 years of age.

**Rotterdam Study I, II and III.** Collections were derived from the three population-based Rotterdam Studies.2,24 In the current study, only cases ≥ 50 years of age and control subjects ≥ 65 years old were included. Ninety-eight percent of individuals in the Rotterdam Studies were self-reported Caucasians.

**Baltimore.** Unrelated subjects were recruited at Johns Hopkins Hospital in Baltimore, Maryland, USA, as previously described.3,25,26 The subjects were self-described Caucasian individuals of European descent from the Baltimore replication collection.

**Genotyping**

The genotyping in the present study was performed in primary and replication stages. In the primary stage, a panel of 84 cases (from the “EUGENDA-discovery” data set) was screened for CFI gene variants by polymerase chain reaction (PCR) amplification of all 13 coding exons and splice junctions. The primers and PCR conditions used to screen CFI are listed in Table 4.2.

Direct sequencing of the PCR amplicons was performed using an automated sequencer (BigDye Terminator, version 3 on a 3730 DNA analyzer; Applied Biosystems). The sequences were assembled using ContigExpress (Vector NTI Advance, version 11.0). Each novel variant was validated by and independent PCR. The p.Gly188Ala variant was screened in three affected family members of the proband, 809 unrelated cases and 254 control individuals by restriction enzyme
analysis using MnlI. In addition, we sequenced all coding exons of the \textit{CFH} gene in the EUGENDA-discovery cohort (Table 4.3).

In the second (replication) stage, the frequency of the \textit{CFI} p.Gly119Arg variant was measured by genotyping five replication cohorts (EUGENDA, Rotterdam Study I, Rotterdam Study II, Rotterdam Study III and Baltimore) using a custom-made TaqMan
CHAPTER FOUR

assay (Applied Biosystems). This assay consisted of two primers and two fluorescently labeled probes (Table 4.4). Genotyping was performed in a volume of 5 µl containing 10 ng of genomic DNA according to the manufacturer’s recommendations. 2.5 µl of 2x Taqman Mastermix (Applied Biosystems), 0.0625 µl of the Taqman assay (40x) and 1.44 µl of water. Genotyping was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: pre-denaturing at 94°C for 12 minutes, followed by 40 cycles of a denaturation step at 94°C for 15 seconds and an annealing/extension step at 60°C for 60 seconds. Genotypes were scored using the algorithm and software supplied by the manufacturer (Applied Biosystems). Eight wildtype controls and 24 positive p.Gly119Arg samples were included in each real-time PCR run and were 100% concordant, confirming the reproducibility of the Taqman assay. Samples identified as carriers by this assay were confirmed by independent PCR and sequencing.

Haplotype analysis was performed in carriers of the p.Gly119Arg and p.Gly188Ala variants using selected SNPs spanning the CFI, PLAG12A, and CCDC109B genes. SNPs were genotyped by PCR amplification and direct sequencing. Haplotypes were constructed using first-degree relatives of the cases.

Proteins

C4BP and factor H (FH) were purified as described previously, and FH was purified further by affinity chromatography using a MRCOX24 (monoclonal anti-FH) column. Factor B, factor D, C3, C3b and C4b were purchased from Complement Technology. C3b and C4b were labeled with 125I (PerkinElmer) using iodination beads (Pierce).

Factor I (FI) plasma concentration measurements

The concentration of FI in the plasma samples was measured by ELISA. Maxisorp Nunc Immunoplates (Nunc) were coated with rabbit anti-human FI Ig (generated in-house) in 50 mM sodium carbonate (pH 9.6) at 4°C overnight. Recombinant wt FI was used as a standard, and FI was detected using goat anti-human FI (Quidel) followed by rabbit anti-goat Ig conjugated with horseradish peroxidase (Dako); 1,2-phenylenediamine dihydrochloride (Dako) was used as the substrate. The experiment was performed three times.

<table>
<thead>
<tr>
<th>Table 4.4. Primers and probes used for the CFI p.Gly119Arg Taqman-assay</th>
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<tbody>
<tr>
<td><strong>Primers</strong></td>
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<td></td>
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<tr>
<td><strong>Probes</strong></td>
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CFI cDNA cloning and mutagenesis

cDNA encoding the histidine-tagged full-length human FI was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen). The mutations identified in the AMD patients were introduced using the QuikChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) with the following primers for p.Gly119Arg: 5’- GTTTCCCTTGAGCATAGAAATACAGATTCAG -3’ and G188A: 5’- GTGCAATTGCCGAGCATTAGAGACCAG -3’ and complementary reverse primers. The variants are underlined in the primer sequences. The variants were confirmed by automated DNA sequencing using the Big Dye terminator kit v3.1 cycle sequencing kit (Applied Biosystems).

Expression of wt and mutant factor I (FI)

HEK293 cells (ATCC number 1573-CRL) were transiently transfected with wt FI, one of the two mutant constructs or empty vector (pcDNA3) using Lipofectamine 2000, according to the manufacturer’s instructions (Invitrogen). Six hours after transfection, the transfection medium was replaced with Dulbecco’s modified Eagle’s medium with high glucose (HyClone) and 10% fetal bovine serum (Invitrogen). Two days after transfection, the medium was replaced with serum-free Opti-MEM Glutamax (Invitrogen), and the cells were cultured for an additional three days. After collecting the conditioned medium, the cells were washed in ice-cold Dulbecco’s phosphate-buffered saline (HyClone) and lysed in ice-cold solubilization buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% aprotinin and 2 mM phenylmethanesulfonyl fluoride. The supernatants and cell lysates were analyzed by ELISA. The complete experiment was performed three times.

Purification of recombinant factor I (FI)

Wildtype and p.Gly119Arg FI were expressed in HEK293 cells in a stable manner and purified by affinity chromatography using nickel-nitrilotriacetic acid Superflow resin (Qiagen) as described previously. All proteins were stored at -80°C. Protein concentrations were determined by measuring the absorbance at 280 nm and subtracting the absorbance at 320 nm. The concentrations were then verified by SDS-PAGE followed by Coomassie staining of the gel.

Degradation of C3b and C4b in the fluid phase and C3b degradation on the cell surface

To study how the CFI variants would affect the protein’s ability to degrade C3b in the fluid phase, plasma samples were obtained from 97 healthy controls, 15 cases with a heterozygous p.Gly119Arg variant and four cases with a heterozygous p.Gly188Ala variant and diluted 30x in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0 and mixed with trace amounts of 125I-labeled C3b. A mixture of recombinant FI (20 μg/ml), 20 μg/ml plasma purified FH, 150 μg/ml C3b and 125I-labeled C3b was used as a positive control, and 125I-labeled C3b was used as a negative control. For recombinant wt
and mutant Fl 1, 2.5 or 5 μg/ml was mixed with 20 μg/ml FH, 150 μg/ml C3b and 125I-labeled C3b or 100 μg/ml C4BP, 50 μg/ml C4b and 125I-labeled C4b. A mixture of 20 μg/ml Fl, 100 μg/ml C4BP, 50 μg/ml C4b and 125I-labeled C4b was used as a positive control for C4b degradation, and 125I-labeled C4b was used as a negative control. The samples were incubated at 37°C for 90 minutes, and the reaction was terminated by adding reducing sample buffer and boiling for three minutes. The proteins were separated on a 10-15% gradient SDS-PAGE gel and visualized using a Phosphoimager (GE Healthcare). The intensities of C3b and C4b and the 43-kDa and C4d cleavage products were analyzed using the ImageGauge software (Fujifilm). Each experiment was performed three times. The degradation of C3b in the plasma samples was calculated as the ratio between the 43-kDa product and the α’ chain; this ratio was normalized to the ratio of normal human serum to compare the results across different gels.

The ability of the CFI mutants to degrade C3b, deposited on the surface of erythrocytes, was also tested, essentially as described previously. Serum samples were obtained from 10 patients with the heterozygous G119R mutation, two with the heterozygous G188A mutation and 17 AMD patients without any CFI mutation. Sheep erythrocytes were coated with C3b, followed by incubation with 0.5% serum, diluted in TBS supplemented with 10 mM EDTA, for 1 h incubation at 37°C. To control the amount of deposited C3b on the erythrocytes as well as generated iC3b, the cells were incubated with specific monoclonal antibodies directed against either C3d (Quidel), which recognizes C3b or the degradation product iC3b (Quidel) and a ratio between these two was calculated.

Modeling of factor I (Fl) and structural analysis of the mutations
The ternary complex was modeled using the human Fl crystal structure (PDB entry 2XRC) according to the procedure described by Roversi et al. All figures were generated using the YASARA program.

Zebrafish analyses
A morpholino (MO) against zebrafish Fl (12ng) was obtained from Gene Tools. eGFP:Fli1 transgenic zebrafish embryos were injected at the one- to two-cell stage with 1nl of solution. Overexpression of both 119Gly and 119Arg as well as rescues were performed by either injecting the corresponding human wildtype mRNA (100pg) alone and coinjecting with MO. mRNA was transcribed in vitro using SP6 Message Machine Kit (Ambion). At 24hpf, zebrafish were grown in 1-Phenyl-2-Thiourea (PTU) to prevent pigmentation. Zebrafish were collected and observed at 5 days post fertilization. Vasculature was observed using the Nikon AZ100 Microscope with a using a 5.0-megapixel DS-Fi1 color digital camera head. Software to take measurements of the vessel diameter was NIS Elements AR 3.2.
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Assessing renal function
The creatinine, urea nitrogen, and albumin concentrations were measured in stored serum obtained from ten cases carrying the p.Gly119Arg variant and 20 cases without the p.Gly119Arg variant (EUGENDA) using the ARCHITECT c16000 system (Abbott Diagnostics). Variant carriers and non-carriers were matched with respect to gender, age, and AMD stage for each eye. We calculated the estimated glomerular filtration rate (eGFR) using the MDRD equation as described previously\textsuperscript{32} and compared the eGFR of p.Gly119Arg cases to matched cases using an independent-samples Mann-Whitney test.

Statistical analysis
Except where indicated otherwise, differences between the mutant and wildtype groups were analyzed using the one-tailed Fisher’s exact test. Differences with a P-value of < 0.05 were considered statistically significant. The data were analyzed using SPSS software, version 18.0 (SPSS, Chicago, IL).

Results
We sequenced all CFI coding exons from genomic DNA of 84 unrelated cases with AMD (“EUGENDA-discovery” data set), and found two heterozygous variants in four cases (Table 4.5). Three cases carry the p.Gly119Arg substitution; the fourth case carries the p.Gly188Ala substitution. Neither of these variants was identified in 192 ethnicity- and age-matched controls. The p.Gly188Ala substitution was also detected in three affected family members of the proband, although it was not found in 809 unrelated AMD cases. No co-existing mutations in the complement factor H (CFH) gene were identified in cases carrying the CFI p.Gly119Arg or p.Gly188Ala substitutions (Table 4.3). Haplotype analysis revealed that the p.Gly119Arg and p.Gly188Ala variants reside on different haplotypes (Figure 4.1).

<table>
<thead>
<tr>
<th>Variant</th>
<th>rs-number</th>
<th>Exon</th>
<th>Patient number</th>
<th>Population allele frequency*</th>
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<tbody>
<tr>
<td>c.355G&gt;A;p.G119R</td>
<td>rs141853578</td>
<td>3</td>
<td>#8, #47, #69</td>
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</tr>
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<td>-</td>
<td>3</td>
<td>#3</td>
<td>-</td>
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<tr>
<td>c.563G&gt;C;p.G188A</td>
<td>-</td>
<td>4</td>
<td>#73</td>
<td>-</td>
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<tr>
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<td>rs114013791</td>
<td>12</td>
<td>#21, #29</td>
<td>0.015</td>
</tr>
</tbody>
</table>

*Exome variant server database, European samples
The p.Gly119Arg variant was studied in 1,014 AMD cases and 711 controls (“EUGENDA-replication” data set). The variant was identified in eleven additional cases, but was absent from controls, demonstrating a strong association between AMD and p.Gly119Arg ($P = 2.16 \times 10^{-4}$, Table 4.6). In addition, we genotyped 2,469 cases (1,754 European and 715 US) and 3,035 controls (2,883 European and 152 US) from four independent studies (Table 4.6). In this replication cohort, the p.Gly119Arg variant was identified in six cases and one control ($P = 0.035$). Notably, this 73-year-old control individual had numerous hard drusen in all four quadrants of the peripheral retina but with a normal macula. In total, we identified the p.Gly119Arg variant in 20 of 3,567 cases versus only one of 3,937 controls, consistent with p.Gly119Arg conferring a high risk for developing AMD ($P = 3.79 \times 10^{-6}$, odds ratio = 22.20; 95%

![Figure 4.1. Haplotypes of SNPs surrounding the CFI gene in one case carrying the p.Gly188Ala variant (#73) and three unrelated cases carrying the p.Gly119Arg variant (#8, #47, #69). Haplotypes were constructed using first-degree relatives. The p.Gly119Arg and p.Gly188Ala variants reside on different haplotypes. A 140-kb shared haplotype spanning the CFI gene (extending from SNP rs13117504 to SNP rs7675460) was observed in three cases carrying the p.Gly119Arg variant.](image)

| Table 4.6 CFI genotyping results of the p.Gly119Arg variant in individuals of six case-control data sets |
|-----------------------------------------------|----------------|----------------|-----------------|----------------|
| G119R heterozygotes | G119 homozygotes | Fisher’s Exact |
| **AMD** | **Unaffected** | **AMD** | **Unaffected** | **One-tailed P value** |
| 1. EUGENDA-discovery | 3 | 0 | 81 | 192 | 2.16x10^{-4} |
| 2. EUGENDA-replication | 11 | 0 | 1,003 | 711 | 2.16x10^{-4} |
| 3. Baltimore-replication | 2 | 0 | 713 | 152 | 2.16x10^{-4} |
| 4. Rotterdam Study I-replication | 3 | 0 | 1,307 | 1,767 | 0.035 |
| 5. Rotterdam Study II-replication | 1 | 1 | 282 | 617 | 2.16x10^{-4} |
| 6. Rotterdam Study III-replication | 0 | 0 | 161 | 498 | 2.16x10^{-4} |
| Meta-analysis (1+2+3+4+5+6) | - | - | - | - | 3.79x10^{-6} |
confidence interval 2.98-164.49). Of note, the frequency of the p.Gly119Arg variant differed among the independent cohorts (Table 4.6). An explanation may lie in the difference in distribution of AMD stages in the EUGENDA and Baltimore cohorts (mainly stage 4) compared to the Rotterdam Studies (mainly stage 2). Since most carriers of the p.Gly119Arg variant have AMD stage 4 (Table 4.1), this may explain the lower frequency observed in the Rotterdam Studies.

The p.Gly119Arg and p.Gly188Ala variants affect highly conserved glycine residues in the CD5 domain of FI and are predicted to alter the packing and stability of this domain (Figure 4.2). To investigate the consequences of the AMD-associated variants on protein expression and function, we measured the concentration of FI and the degradation of C3b in plasma and serum samples. Plasma FI levels were measured in 15 cases carrying p.Gly119Arg, four cases carrying p.Gly188Ala, 97 age-matched controls and 100 cases without a CFI mutation. The cases carrying the p.Gly119Arg variant had lower plasma FI levels compared to both controls and cases without a CFI variant (P = 2.08 x 10^-8 and P = 1.69 x 10^-3, respectively, two-tailed Kruskal-Wallis with Dunn’s post-hoc test; Figure 4.3A). We observed similar differences for the p.Gly188Ala allele (P = 4.74 x 10^-3 and P = 4.74 x 10^-3, respectively; Figure 4.3A). Plasma samples of cases carrying the p.Gly119Arg or p.Gly188Ala variants had a lower capacity to degrade 125I-labeled C3b in the fluid phase compared to controls (P = 8.04 x 10^-4 and P = 0.029, respectively, two-tailed Kruskal-Wallis with Dunn’s post-hoc test; Figure 4.3B,C). Serum samples of cases carrying the p.Gly119Arg variant had a lower ability to degrade C3b on the cell surface, compared to cases without a CFI mutation (P = 5.43x10^-4, two-tailed Kruskal-Wallis with Dunn’s post-hoc test; Figure 4.3D).

To interrogate further the consequences of the p.Gly119Arg and p.Gly188Ala variants on the functional activity of FI, we expressed wt and mutant FI proteins in HEK293 cells. Both p.Gly119Arg and p.Gly188Ala were expressed and secreted at lower levels than wt FI (P = 8.45 x 10^-4 and P = 2.81 x 10^-4, respectively, two-tailed one-way ANOVA with Dunnett’s post-hoc test; Figure 4.4A). The small amounts of expressed p.Gly119Arg FI were purified and tested for the ability to cleave C3b and C4b (expression of the p.Gly188Ala variant was too low for the protein to be purified). At 5 µg/ml, the purified p.Gly119Arg FI degraded both C3b and C4b at modest, yet significantly higher efficiency than wt FI (Figure 4.4B-E, P = 1.19 x 10^-2 and P = 3.13 x 10^-3, respectively, two-tailed two-way ANOVA with Bonferroni post-hoc test). However, the severely impaired expression and secretion of the mutant protein led to a net effect (-17%) of significantly decreased C3b degradation (Figure 4.3).

Taken together, our in vitro data, as well as measurements from plasma samples and serum samples, pointed to a model in which the 119Arg-variant confers a lower FI activity compared to 119Gly. Next, we sought to test this model in vivo. It has been shown previously that genetic manipulation of known AMD loci in zebrafish embryos results in vascular or angiogenic perturbations; these can be monitored with the fli1-EGFP transgenic reporter, in which EGFP is expressed specifically in the vascular
Figure 4.2. Schematic and structural view of factor I (FI).

(A,B) The CFI gene contains 13 exons and the exons correspond well to the domain structure. (C) FI is composed of the following domains: FI membrane attack complex domain (FIMAC), a CD5-like domain, two low-density lipoprotein receptor 1 and 2 (LDLR1 and 2), and the serine protease (SP) domain. (D) Model of the ternary complex containing C3b, factor H (FH), and FI. A surface representation of C3b is shown in gray; the surface representation of the four FH domains (FH1-4) is shown in red. FI is shown as a ribbon style cartoon. The CD5 domain is shown in green, the FIMAC and LDLR domains are shown in blue, and the catalytic SP domain is shown in orange. (E) The Gly119 residue is located at the surface of the CD5 domain and is not involved in any inter-domain contacts. However, the surface loop containing Gly119 links the preceding FIMAC to the CD5 domain and therefore packs against the CD5 domain. The introduction of a much larger and charged arginine residue in the p.Gly119Arg variant is predicted to disturb this packing and thus destabilize the CD5 domain. (F) The Gly188 residue is located at the interface of the CD5 domain and the LDLR domains. Glycine-188 is important for the required structure of the loop, which mediates many inter-domain contacts in the overall FI structure and does not tolerate substitution by any other amino acids. Introduction of any other side chain at this position (such as an alanine or valine introduced by the p.Gly188Ala and p.Gly188Val variants) will perturb the orientation of the loop and disrupt inter-domain packing within the heavy chain.
A functional variant in the CFI gene associated with AMD

Figure 4.3. Factor I (FI) concentrations and degradation of C3b in plasma/serum of cases carrying the p.Gly119Arg or p.Gly188Ala variant, cases without a CFI variant and ethnicity- and age-matched controls. (A) FI concentration was measured by ELISA in plasma samples of 15 cases carrying p.Gly119Arg, four cases carrying p.Gly188Ala, 97 ethnicity- and age-matched control individuals and 100 cases without a CFI variant. The median FI concentration, shown as a horizontal line, is significantly lower in cases carrying the p.Gly119Arg variant and the p.Gly188Ala variant compared to cases without a CFI variant or controls. (B) Serum samples of 10 cases carrying p.Gly119Arg, two cases carrying p.Gly188Ala and 17 cases without a CFI variant were tested for their ability to degrade C3b deposited on the surface of sheep erythrocytes. The experiment was performed three times and the ratio between the generated iC3b and the deposited C3d (i.e. C3b) was calculated (the median is shown as a horizontal line). (C) Plasma samples of 15 cases carrying p.Gly119Arg, four cases carrying p.Gly188Ala and 97 controls (Ctrl) were incubated with trace amounts of 125I-labeled C3b for 90 minutes at 37°C and then separated on a 10-15% gradient SDS-PAGE gel. 125I-labeled C3b was loaded as negative control (-), and a mixture of 20 μg/ml recombinant FI, 20 μg/ml plasma purified factor H, 150 μg/ml C3b and 125I-labeled C3b was used as a positive control (+). (D) The ratio between the 43-kDa degradation product and the α’ band is plotted for cases carrying the p.Gly119Arg or p.Gly188Ala variant, and controls. The ratio was normalized to pooled normal human serum (NHS) to facilitate comparison of the individual gels. The experiment was performed three times, and the median is shown as a horizontal line.
Figure 4.4. Expression of recombinant factor I (FI) in HEK293 cells, and degradation of C3b and C4b by recombinant wt and mutant p.Gly119Arg FI. (A) HEK293 cells were transiently transfected with empty vector (pcDNA3) or a plasmid expressing wildtype (wt), p.Gly119Arg or p.Gly188Ala FI. The FI concentrations in the supernatants and cell lysates were measured by ELISA and the ratios between the concentrations of FI in the supernatants and cell lysates are plotted. The experiment was performed three times, and the results are presented as mean ± standard deviation (SD). (B, C) Recombinant wt or p.Gly119Arg FI (1, 2.5 or 5 μg/ml) was incubated with C3b and factor H (FH) or (D, E) C4b and C4BP with trace amounts of $^{125}$I-labeled C3b/C4b for 90 minutes at 37°C and then separated on a 10-15% gradient SDS-PAGE gel. $^{125}$I-labeled C3b was loaded as negative control (-), and a mixture of 20 μg/ml recombinant FI, FH/C4BP, C3b/C4b and $^{125}$I-labeled C3b/C4b was used as a positive control (+). The ratio between the 43-kDa and C4d products and the $\alpha'$ bands are plotted in (C) and (D). The experiment was performed three times, and mean ± SD is plotted. ns; non significant.
endothelium. For example, suppression of vascular endothelial growth factor A (VEGF-A) causes severe loss of vasculature throughout the organism, recapitulating the mouse phenotype. Similarly, suppression of the AMD candidate PLD1 results in loss of intersegmental vessels of the truncal vasculature. Given these observations, we asked whether expression of 119Gly- or 119Arg-encoding human CFI mRNA might have a differential effect on vascular architecture. Injection of 100 pg of 119Gly-encoding, capped human mRNA into 2-4 cell stage fli-EGFP embryos followed by scoring at five days revealed defects in the morphology of the hyaloid vessels in the developing retina; objective scoring (masked to injection cocktail) of the diameter of these vessels at three different branch positions in a field anchored in the central retina (n = 40-60 embryos per injection, optic nerve positioned always at bottom center of the field) showed reduction of tube thickness compared to dye-injected embryos (P < 0.0001, two-tailed student t-test; Figure 4.5). By contrast, embryos injected with 100 pg of human mRNA encoding the 119Arg allele were overall indistinguishable from controls; although occasional embryos showed structural defects of the hyaloid vessels, the average thickness was indistinguishable from sham (n = 40-60 embryos; Figure 4.5). Further, suppression of CFI with a morpholino (MO) against the acceptor site of exon 3 of the sole CFI zebrafish locus induced similar retinal pathology, albeit less severe compared to mRNA overexpression (P < 0.0001, two-tailed student t-test), which could be ameliorated by 100 pg of wt mRNA. Notably, MO suppression or overexpression of human CFI mRNAs did not induce abnormal phenotypes in the truncal vasculature (Figure 4.5) or any appreciable gross dysmorphology, intimating a specific effect in the retina and supporting the specificity of the observed phenotypes. Taken together, these data are consistent with the data derived from both patient sera and in vitro studies that the p.Gly119Arg variant reduces the activity of FI and also suggest that CFI might be dosage-sensitive in either direction (too much or too little).

The p.Gly119Arg variant was described previously in several individuals with aHUS, hinting at a direct link between AMD and aHUS in a fashion similar to recent reports for CFH. We assessed renal function in p.Gly119Arg AMD cases; we measured the estimated glomerular filtration rate (eGFR; normal > 90 ml/min/1.73m²) using serum creatinine, urea nitrogen, and albumin measurements of ten cases. None of these cases had clinically significant renal dysfunction (defined as eGFR < 30 ml/min/1.73m²); however, a mild subclinical decrease in renal function was noted (median eGFR of 81 ml/min/1.73m²). Because AMD patients generally have subclinical renal dysfunction, we compared the renal function of these p.Gly119Arg cases to that of 20 cases who were matched for age, gender and disease severity but did not carry the p.Gly119Arg variant. No significant difference was observed (P = 0.860; two-tailed Mann-Whitney test). In addition, none of the cases carrying the p.Gly119Arg variant had malignant hypertension, pulmonary edema, thrombocytopenia or anemia, features that were observed previously in aHUS patients carrying the p.Gly119Arg variant. This suggests that the p.Gly119Arg variant is not sufficient and that additional factors — such as a second variant or external trigger — are required to develop aHUS.
**Discussion**

Here we report that rare, highly penetrant **CFI** variants that reduce the expression and secretion of F1 can cause AMD. These data represent the first direct evidence that F1 deficiency confers a high risk of developing AMD and increase support for the hypothesis that the previously observed association between AMD and SNPs near the **CFI** gene are driven by **CFI** rather than the downstream genes **PLAG12A** or **CCDC109B**. However, further research is needed to explain the functional effect of the associated SNPs or an unidentified variant tagged by these SNPs. In the context of our findings, it is plausible that these SNPs might be associated with altered F1 expression levels, since our in vitro data indicate a loss of function mechanism, while our zebrafish studies, with the caveat that caution must be exercised in interpreting data from a developmental system for an adult-onset disorder, suggest a “goldilocks effect” in which dosage perturbations at either end of the spectrum can be deleterious to retinal vasculature. A functional link between F1 dysfunction and AMD pathogenesis was proposed recently. F1 is constitutively expressed by the retinal pigment epithelium (RPE), the site of drusen formation in AMD. Amyloid-β, a main
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constituent of drusen, binds to FI, which inhibits the ability of FI to cleave C3b to inactivated iC3b. In addition, exposure of RPE cells to amyloid-β decreases secretion of FI. This supports a central role of impaired FI function in deregulated complement activation in AMD.

Our findings have implications for both predictive testing and for the development of new AMD treatments. The first prognostic AMD tests, which are based on a small number of common variants, are currently being marketed. However, these tests are not reliable for individuals carrying rare variants that confer a high risk of developing AMD. It is therefore essential to understand the role of these rare variants in AMD before reliable genetic tests can be developed. These tests should not only be based on a small number of common variants, but will also need to address rare, highly penetrant variants, particularly in the case of densely affected families.43

Finally, new treatments are currently being developed to selectively inhibit complement activation in AMD. However, recent phase 2 clinical trials using eculizumab, an inhibitor of complement component C5, appeared not to effectively restrict geographic atrophy or drusen area in AMD patients.44,45 Complement inhibitors are likely to be more effective for individuals carrying rare variants that severely affect complement activation. Future research is warranted to unravel pathogenic mechanisms in various subgroups of AMD, and to develop personalized treatments tailored to the patient’s individual genetic makeup.
References

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Chapter Five
Short-term changes of cuticular drusen on spectral-domain optical coherence tomography

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Objectives: To determine if small hard drusen in patients with cuticular drusen (CD) show short-term changes.

Methods: Ten subjects with CD were longitudinally followed during a period of 4 months by spectral-domain optical coherence tomography. Drusen that showed a spontaneous change in volume were further analyzed according to 5 morphologic parameters: shape, reflectivity, homogeneity, and concurring photoreceptor layer/retinal pigment epithelium damage. Odds ratios (OR) and risk for regression and progression of drusen volumes were calculated.

Results: One hundred and five small hard drusen in 19 eyes showed a spontaneous change in volume over the period of follow-up. Drusen with a “pointed” shape were significantly associated ($P = 0.031$; OR $4.89$; 95% confidence interval [CI] 1.16-20.67) with spontaneous progression in drusen volume, with a chance of 0.80 (95% CI 0.55-0.93) to progress. Drusen that showed a decreased reflectivity of overlying photoreceptor layer ($P = 0.041$; OR $7.67$; 95% CI 1.09-54.24) or retinal pigment epithelium ($P = 0.022$; OR $12.38$; 95% CI 1.44-106.57), showed a significant association with spontaneous regression in drusen volume, with chances of regression of 0.86 (95% CI 0.41-0.98) and 0.89 (95% CI 0.49-0.99), respectively.

Conclusions: Small hard drusen in patients with the CD phenotype are subject to a process of short-term remodeling. The dynamic nature of this disease points to high biochemical activity that may be sensitive to future pharmacologic treatment strategies. In addition, these short-term changes of drusen may be a source of misclassification in disease staging.
Introduction

Macular drusen are the hallmark lesions of age-related macular degeneration (AMD).\textsuperscript{1,2} They are identified on ophthalmoscopy as focal yellow-white subretinal deposits, which are pathologic extracellular deposits between the basal lamina of the retinal pigment epithelium (RPE) and the inner collagenous layer of Bruch’s membrane.\textsuperscript{3-5} Drusen contain a wide variety of compounds that appear to reflect the complex pathogenesis of AMD. Important constituents of drusen are neutral lipids,\textsuperscript{6,7} carbohydrates,\textsuperscript{8} zinc,\textsuperscript{9} and a wide variety of proteins. Many proteins found in drusen are associated with inflammation and/or immune-associated processes, including a broad spectrum of complement components.\textsuperscript{10,11} In addition, associations between AMD and genetic variants in complement genes have been reported, which supports the role of low-grade inflammation and an abnormal regulation of the complement system in drusen pathogenesis.\textsuperscript{12-20} Drusen are an important quantifier of the severity of AMD. Therefore, the size and number of drusen are used for AMD staging and for the prediction of the likelihood of disease progression and vision loss.\textsuperscript{1,2,21}

Different clinical subtypes of drusen have been described in AMD, but all drusen seem to be indistinguishable in location, composition, and substructure.\textsuperscript{5} “Cuticular drusen (CD),” also termed “basal laminar drusen,” refers to an early-onset drusen phenotype with innumerable small (25 to 75 µm) hard drusen.\textsuperscript{22,23} This subtype of AMD is more easily visualized angiographically than biomicroscopically, with a typical “stars-in-the-sky” appearance during the early arteriovenous phase of fluorescein angiography (Figure 5.1).\textsuperscript{24} In later stages, the number of drusen often increases, with clustered groups of drusen scattered throughout the retina.\textsuperscript{22}

Figure 5.1. Fluorescein angiographic photograph of the left eye of a 45-year-old patient with cuticular drusen.
In general, color fundus photographs are used to evaluate the morphology of drusen over time. However, color images do not provide detailed information about the changing morphology of small drusen. The introduction of spectral-domain optical coherence tomography (SD-OCT) has enabled an improved in vivo visualization of drusen morphology. SD-OCT is able to acquire 3-dimensional images of the retina with high speed and high resolution. Subsequently, studies of the fine details of small drusen and adjacent retinal structures become possible.

After we observed occasional changes of drusen morphology in routinely followed eyes with CD, we decided to longitudinally investigate the appearance of small hard drusen in eyes with this phenotype. The focus of our investigation was to determine whether morphologic parameters may be predictive for processes of progression or regression of small hard drusen in CD affected eyes.

Methods

Patients
A total of 10 subjects who met the diagnostic criteria of CD were retrieved from the European Genetic Database (EUGENDA, www.eugenda.org), a large multicenter database for clinical and molecular analysis of AMD and different early-onset drusen phenotypes.

For inclusion in the study, subjects had CD of the posterior pole and ocular media allowing adequate SD-OCT scanning, defined by a maximum score of NO3/NC2/C1/P1 according the Lens Opacities Classification System III. Study participants had to be able to fixate for at least 1 minute per eye to allow adequate SD-OCT scanning. The CD phenotype was defined as a symmetrically distributed pattern between both eyes of at least 50 scattered, uniformly sized, small (25 µm to 75 µm), hyperfluorescent drusen on fluorescein angiography in each eye, of which at least 20 drusen are located outside the Wisconsin age-related maculopathy grading template. Eyes with choroidal neovascularization (CNV), a large area of central geographic atrophy (> 1500 µm), and retinal abnormalities other than AMD-related were excluded from the study. In order to exclude possible effects of antioxidant agent use on changing drusen morphology, the use of antioxidant agents was prohibited 1 month prior to study entry and during the follow-up period.

Image acquisition
All study participants were examined 3 times with a 2-month interval during a follow-up period of 4 months. During each visit, visual acuity was recorded with Early Treatment Diabetic Retinopathy Study (ETDRS) charts. Multimodal imaging was performed by a combined confocal scanning laser ophthalmoscope (cSLO)/SD-OCT device (SPECTRALIS; Heidelberg Engineering, Heidelberg, Germany).
after pupil dilation with 1 drop of 2.5% phenylephrine hydrochloride and 1 drop of 1% tropicamide. A standardized imaging protocol was performed in each study eye, which included near-infrared reflectance imaging in high-speed mode (λ = 815 nm; scan angle, 30 degrees; image resolution, 768 x 768 pixels) and simultaneous SD-OCT scanning (λ = 870 nm; 40 000 A-scans per second; number of B-scans, 38; distance between B-scans, 122 µm) using a second, independent pair of scanning mirrors. Because of the independent pairs of scanning mirrors, eye movements were registered and corrected automatically ("eye tracking") by the cSLO/SD-OCT device. The eye tracker enabled the identification of the same scanning location during follow-up visits. By this means, a very high repeatability and reproducibility of longitudinal measurements with an exceedingly small measurable change (1.5 µm) of retinal structure has been shown for this device. Therefore, a highly reliable comparison of drusen as small as 25 to 75 µm, like in cuticular drusen, is possible on follow-up OCT scans.

To increase image quality, the Automatic Real-Time (ART) function was used. With ART activated, 35 SD-OCT B-scans of the same scanning location were performed during the scanning process and images were averaged for noise reduction.

**Image analysis**

For the morphologic evaluation of the small hard drusen, baseline SD-OCT scans and SD-OCT scans after 4 months were studied side by side by 2 graders (J.v.d.V. and Y.L.). Only drusen that showed increasing volume over time or decreasing volume over time were analyzed for this study (Figures 5.2 and 5.3). The analysis focused on 5 morphologic parameters (Figure 5.4), partly adopted from Khanifar and associates, with the additional parameters for photoreceptor layer and RPE layer damage, scored as follows: the shape of the drusen was characterized as dome-shaped (basal diameter ≥ height), pointed (basal diameter < height), or saw-toothed (grouped pointed drusen edging each other at the level of Bruch’s membrane); the predominant internal reflectivity between RPE elevation and the Bruch’s membrane was characterized as low (isoreflective or hyporeflective compared to the photoreceptor outer segment layer), medium (hyperreflective compared to the photoreceptor outer segment layer but hyporeflective relative to the RPE), or high (isoreflective or hyperreflective in relation to the RPE); the homogeneity of internal drusen reflectivity was characterized as homogeneous (uniform internal reflectivity), nonhomogeneous with core (varying internal reflectivity with a distinct single focus of hyperreflectivity), or nonhomogeneous without core (varying internal reflectivity without a distinct focus of hyperreflectivity); photoreceptor layer damage was characterized as present (hyporeflective photoreceptor layer overlying a druse relative to the average photoreceptor layer reflectivity in the surrounding areas without drusen) or absent (isoreflective photoreceptor layer overlying a druse relative to the surrounding average photoreceptor layer reflectivity); and RPE damage was characterized as present (hyporeflective RPE overlying a druse relative to the surrounding average RPE reflectivity in retinal areas without drusen) or absent (isoreflective RPE overlying a druse relative to surrounding average RPE reflectivity).
Figure 5.2. Drusen volume at regression in a patient with cuticular drusen recorded by spectral-domain optical coherence Tomography (SD-OCT). (A) Baseline: Homogeneous, domeshaped hard drusen (white arrow heads) without damage of the overlying retinal pigment epithelium or photoreceptor layer. (B) After 2 months of follow-up. (C) After 4 months of follow-up; note the total regression of the hard drusen (black arrow heads) without detectable changes on SD-OCT at the former areas of homogeneous, dome-shaped hard drusen.

Statistics
Statistical analysis was performed by SPSS statistical software, version 18.0 (SPSS Inc., Chicago, Illinois, USA). The prevalence (number of eyes and number of drusen) of each basic morphologic pattern was calculated and analyzed with descriptive statistics. Drusen were measured by the Heidelberg Eye Explorer software, version 1.6.4.0 (Heidelberg Engineering GmbH, Heidelberg, Germany), and a ratio between height and basal diameter was calculated. For interindividual correction, a model for generalized estimating equations for binary outcome was used to analyze
differences in drusen characteristics between drusen that showed a progression in drusen volume (the “drusen progression” group) and drusen that showed an decreasing drusen volume (the “drusen regression” group). Strength of association of the different drusen characteristics between the “drusen regression” group and “drusen progression” group is shown as odds ratios (ORs) with a 95% confidence interval (95% CI). The chance of drusen morphology change was expressed as a value between 0 (0% chance) and 1.0 (100% chance). Reported $P$ values are 2-sided and a value of $< 0.05$ was considered statistically significant.
Results

SD-OCT was performed on 19 eyes of 10 patients. One eye was excluded from this study because of a large area of central geographic atrophy. The mean age of the patients was 64.6 ± 13.9 years, ranging from 45 to 86 years. Nine patients were female and 1 patient was male. The mean baseline best-corrected visual acuity was 78 letters (range, 20 to 95). In all eyes visual acuity remained stable ($P = 0.231$) during the period of follow-up, with a mean increase of 1 letter on the ETDRS visual acuity chart.

The morphologic results of small hard drusen with spontaneous volume regression and the morphologic results of small hard drusen with progression are depicted in the Table 5.1. The most common small hard drusen that showed short-term changes were homogeneous, dome-shaped drusen with medium internal reflectivity and without overlying RPE or photoreceptor layer damage. Dome-shaped small hard drusen ($n = 67$) showed an average base-to-height ratio of 1:0.58, while pointed small hard drusen ($n = 25$) showed an average base-to-height ratio of 1:1.43. Of the cases with drusen volume regression, 30.6% (15/49) completely regressed during follow-up, whereas 69.4% (34/49) showed a decreased drusen volume only. In cases of small hard drusen with increased drusen volume, 33.9% (19/56) showed development of new drusen, whereas 66.1% (37/56) of those small hard drusen showed an increased drusen volume.

Pointed drusen showed a significant association with a progression in volume ($P = 0.031$; OR 4.89; 95% CI 1.16-20.67), with a chance of 0.80 (95% CI 0.55-0.93) for volume progression. No significant longitudinal changes were observed for dome-shaped and saw-toothed drusen. Drusen with overlying photoreceptor layer or RPE damage showed a statistically significant association with a regression in volume.

Figure 5.4. Five of the combined morphologic drusen features seen in patients with cuticular drusen. (A) Dome-shaped, medium reflectivity, homogeneous, no retinal pigment epithelium (RPE) or photoreceptor layer damage. (B) Pointed, medium reflectivity, nonhomogeneous with core, no RPE or photoreceptor layer damage. (C) Saw-toothed, medium reflectivity, homogeneous, no RPE or photoreceptor layer damage. (D) Pointed, medium reflectivity, RPE and photoreceptor layer damage. (E) Dome-shaped, medium reflectivity, homogeneous, photoreceptor layer damage without RPE damage.
Table 5.1. Summary of drusen patterns seen in the drusen regression group and the drusen progression group, organized by basic morphologic parameters

<table>
<thead>
<tr>
<th></th>
<th>Drusen Regression Group</th>
<th>Drusen Progression Group</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Drusen (%)</td>
<td>No. Eyes (%)*</td>
<td>No. Drusen (%)</td>
</tr>
<tr>
<td>Shape</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dome</td>
<td>37 (75.5%)</td>
<td>16 (84.2%)</td>
<td>30 (53.6%)</td>
</tr>
<tr>
<td>Pointed</td>
<td>5 (10.2%)</td>
<td>3 (15.8%)</td>
<td>20 (35.7%)</td>
</tr>
<tr>
<td>Saw-toothed</td>
<td>7 (14.3%)</td>
<td>5 (26.3%)</td>
<td>6 (10.7%)</td>
</tr>
<tr>
<td>Reflectivity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (≤ photoreceptor layer)</td>
<td>2 (4.1%)</td>
<td>2 (10.5%)</td>
<td>1 (1.8%)</td>
</tr>
<tr>
<td>Medium (between photoreceptor layer and RPE)</td>
<td>47 (95.9%)</td>
<td>18 (94.7%)</td>
<td>55 (98.2%)</td>
</tr>
<tr>
<td>High (≥ RPE)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Homogeneity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogeneous</td>
<td>43 (87.8%)</td>
<td>19 (100.0%)</td>
<td>51 (91.1%)</td>
</tr>
<tr>
<td>Nonhomogeneous with core</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>2 (3.6%)</td>
</tr>
<tr>
<td>Nonhomogeneous without core</td>
<td>6 (12.2%)</td>
<td>5 (26.3%)</td>
<td>3 (5.4%)</td>
</tr>
<tr>
<td>Photoreceptor layer damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>6 (12.2%)</td>
<td>4 (21.1%)</td>
<td>1 (1.8%)</td>
</tr>
<tr>
<td>Absent</td>
<td>43 (87.8%)</td>
<td>18 (94.7%)</td>
<td>55 (98.2%)</td>
</tr>
<tr>
<td>RPE damaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>9 (18.2%)</td>
<td>6 (31.6%)</td>
<td>1 (1.8%)</td>
</tr>
<tr>
<td>Absent</td>
<td>40 (83.3%)</td>
<td>17 (89.5%)</td>
<td>55 (98.2%)</td>
</tr>
</tbody>
</table>

Abbreviations: n.s., not significant; RPE, retinal pigment epithelium.

*No. Eyes represents the total number of eyes out of 19, harboring drusen with specific morphologic parameter.

†P Value represents the difference in association between the drusen regression group and the drusen progression group.
(P = 0.041; OR 7.67; 95% CI 1.09-54.24 and P = 0.022; OR 12.38; 95% CI 1.44-106.57), with similar chances for drusen volume regression (0.86 [95% CI 0.41-0.98] and 0.89 [95% CI 0.49-0.99], respectively). Drusen reflectivity and homogeneity did not appear to have significant impact on drusen change.

Discussion

In this study, we were able to show that small hard drusen in patients with the CD phenotype are subject to a constant dynamic process of drusen remodeling. The initial drusen morphology seemed to predict the future course of drusen development. Small hard drusen with a decreased reflectivity of overlying RPE or photoreceptor layer were more likely to show a regression in drusen volume, whereas pointed small hard drusen were more likely to show volume progression.

Although the exact mechanism of drusen biogenesis in CD as well as in “typical” AMD is still unclear, an identical mechanism in the developmental courses may be expected because of the similar topographic, structural, and compositional features. In both drusen types, RPE cell pathology seems to play a major role in drusen development. Cellular remnants and debris derived from degenerated RPE cells become sequestered between the RPE basal lamina and the inner collagenous layer of Bruch’s membrane and provoke a chronic inflammatory response with complement activation. Simultaneous with this continuous process of accumulating extracellular debris, there is a process of drusen removal that may be related to at least 2 factors.

The first is the removal of these drusen constituents by macrophages. Different types of macrophages are present in the normal human choroid. In contrast to resident choroidal macrophages, Bruch’s membrane macrophages are only seen in eyes with drusen, making these macrophages a possible player in the process of drusen regression. A role for macrophages in the process of drusen removal is further supported by animal models that suggest that an impaired mobilization of macrophages may prevent the clearance of drusen-like lesions in mice.

A second mechanism that may explain the removal of drusen could be an altered function of protein expression in RPE cells. RPE cells produce and secrete their own complement inhibitors, such as complement factor H, complement factor I, membrane cofactor protein, vitronectin, and clusterin. The production of these complement inhibitors is upregulated in patients with AMD. Furthermore, vitronectin and membrane cofactor protein are upregulated in the RPE cells that flank or overlie drusen. This production of complement inhibitors by ocular tissues, like the RPE cell, plays an important role not only in protecting the eye against complement mediated damage but also in maintaining the immuneprivileged state of the eye.
Disturbance of the aforementioned factors that induce and sustain chronic local inflammation at the level of the RPE–Bruch’s membrane interface, and those that attenuate it, can explain the association of a decreased reflectivity of the overlying RPE and concomitant photoreceptor layer with drusen regression. A loss of RPE cells will result in a decreased generation of extracellular debris that makes up a druse, whereas macrophage recruitment and the upregulation of complement inhibitors by RPE cells flanking the druse will start a process of druse volume regression. It is this process of drusen remodeling that points to a high biochemical activity and suggests that future treatments targeting these biochemical processes in an early stage of the disease may have a significant role in prophylactic and therapeutic interventions in CD.

The finding that drusen progression and drusen regression occurred in all the study eyes within a very short period may have implications for clinical studies on patients with CD. Because number and size of drusen are important for disease staging, longitudinal changes in drusen morphology can be a potential source of misclassification and needs attention in epidemiologic studies investigating the natural history of CD as well in clinical trials evaluating the efficacy of possible therapies.

Our study has some limitations. First of all, the limited number of eyes restricts the general use of our data. However, because drusen remodeling was observed in all study eyes, those changes are very likely to occur commonly in eyes with CD. Secondly, slight variations of SD-OCT scan positions during follow-up visits cannot be excluded. However, eye movements were automatically registered and corrected for “eye tracking,” resulting in high repeatability and reproducibility of the SD-OCT scans; therefore, small shifts of only a few microns could have influenced the appearance of these very small drusen in basal laminar drusen. On the other hand, it is unlikely that random shifts may lead to nonrandom, continuous changes during the study period. We included only drusen with increasing or decreasing drusen volume at all time points (at baseline, after 2 months, and after 4 months) for progression analysis to further reduce the possibility for such a systematic measurement error.

In summary, we were able to demonstrate that small hard drusen in patients with CD show a constant remodeling process. This dynamic process may be a potential source of misclassification in disease staging at a single point of time. Changing the balance between the generation and the elimination of these drusen in an early stage of the disease may be a new target for therapeutic strategies.
References


Chapter Six
General discussion

Adapted from: Cuticular drusen: stars in the sky
Prog Ret Eye Res; under review
General discussion

This thesis focuses on the clinical and genetic characteristics of cuticular drusen, a subtype of age-related macular degeneration (AMD). We analyzed the genetic defects that might cause cuticular drusen, and we evaluated the association of possible genetic and/or environmental risk factors with this AMD subtype. In the previous chapters of this thesis, the merits and limitations of each study were discussed in detail. In this chapter, the primary findings of this thesis are reviewed and placed in a broader perspective, and particular emphasis is given to the role that genetic research has played in unraveling the etiology of cuticular drusen and the implications of our results with respect to clinical practice and future research.

Cuticular drusen and age-related macular degeneration

Cuticular drusen are typically first visible on ophthalmoscopy in early adulthood and are often an incidental finding during a routine ophthalmological examination. Cuticular drusen are usually small and, although they grow in size over time, they rarely exceed 125 µm in diameter. In the early stages—which are asymptomatic—cuticular drusen are distributed randomly throughout the fundus. In time, they tend to grow in number and become grouped in clusters with similar density in both eyes. These clusters can be arranged in a tightly knit pattern that gives the entire posterior pole an “orange-peel” appearance on ophthalmoscopy. In the later stages, cuticular drusen can reach confluence and may eventually damage/aff ect the adjacent RPE and neuroretina, causing localized or patchy chorioretinal atrophy. In the macula, these confluent drusen can also lead to serous “pseudovitelliform” RPE detachment, which can reduce visual acuity. An early report suggested a relatively benign course with spontaneous resolution of the RPE detachment in approximately 50% of cases. However, more recent studies found no cases of spontaneous resolution but instead reported enlarged areas of detached RPE that can become atrophic and/or result in the formation of choroidal neovascularization (CNV). In agreement with the relatively early onset of cuticular drusen, the onset of CNV in patients with cuticular drusen occurs nearly ten years earlier than in patients with other drusen phenotypes in AMD.

The lack of differences between cuticular drusen and other drusen phenotypes in AMD with respect to structure, composition and location—as well as the ability of cuticular drusen to progress into advanced AMD—suggest that cuticular drusen differ from other drusen phenotypes only by their early onset and number. However, this is an oversimplification: the characteristic early angiographic features of cuticular drusen also distinguish these drusen from other drusen, suggesting that there are indeed differences in composition. It has been hypothesized that this difference may be explained by differences in lipid composition, which could not be measured in previous immunohistochemical studies due to the use of xylene, which is necessary to extract the paraffin in which the cuticular drusen-affected eyes were embedded.
**CHAPTER SIX**

**Genetics of cuticular drusen**

The unique distribution and fluorographic characteristics of cuticular drusen allow clinicians to reliably differentiate between cuticular drusen and other drusen phenotypes. Nevertheless, the similarities between the various drusen phenotypes with respect to their location between the basal lamina of the RPE and the inner collagenous layer of Bruch’s membrane, substructure and composition suggest a common developmental pathway. It is therefore not surprising that we (see Chapter 2) and others have found that the cuticular drusen phenotype shares some of the same risk factors as AMD in general, although the degree of the association varies.\(^{16,17}\)

Whereas most of the previously identified demographic, environmental and genetic AMD risk factors confer a similar effect (i.e., odds ratio (OR)) in both groups, the effect of smoking and the frequency of the complement factor H (\(\text{CFH}\)) Y402H risk allele differed significantly between the groups. Specifically, smoking had a significantly lower association with the cuticular drusen phenotype (OR = 2.06) than with AMD in general (OR = 5.97). On the other hand, the \(\text{CFH}\) Y402H variant appeared to be more tightly correlated with the cuticular drusen phenotype—the allele frequency of the \(\text{CFH}\) Y402H variant was 64% in cuticular drusen versus 58% in AMD in general and 38% in normal controls.\(^{17}\)

Both of these findings suggest that one’s genetic constitution has a higher contribution to cuticular drusen than to AMD in general and highlight the importance of complement factor H in the development of cuticular drusen.

**Complement factor H**

Complement factor H is one of the principal regulators of the complement system. This protein’s primary function is to inhibit the alternative complement pathway by competing with factor B binding to C3b, thereby accelerating the decay of the alternative pathway C3 convertase and acting as a cofactor in the factor I-mediated proteolytic inactivation of C3b.\(^{18-20}\) RPE cells express factor H at a level that is comparable to the highest expression levels in the liver, where plasma factor H is synthesized.\(^{21}\)

*In vitro* studies have revealed that oxidative stress generally decreases factor H expression in the RPE, thereby increasing local complement activity.\(^{22,23}\) Individuals who are homozygous for the \(\text{CFH}\) Y402H polymorphism appear to have levels of \(\text{CFH}\) gene transcription and factor H protein levels in their RPE that are similar to the levels in individuals who are homozygous for the wildtype allele.\(^{24}\)

However, homozygous \(\text{CFH}\) Y402H carriers have decreased binding of factor H to sulfated glycosaminoglycans (which are found on host tissues) as well as decreased binding to other ligands, including C-reactive protein, necrotic cells and bacterial coat proteins.\(^{25}\) Among these effects, the change in the glycosaminoglycan-recognizing property of factor H is likely to be the most relevant to cuticular drusen and AMD in general.

The important role played by factor H in the development of cuticular drusen became even more evident following the identification of heterozygous loss-of-function mutations in the \(\text{CFH}\) gene among families with the cuticular drusen phenotype. In
a study published by Boon et al., a recessive disease model was suggested in which patients with cuticular drusen have a **CFH** mutation on one allele together with the **CFH** Y402H variant on the other allele.1 In Chapter 3 of this thesis, we confirmed that a heterozygous **CFH** gene mutation can cause cuticular drusen in a subgroup of patients, although a recessive disease model involving the Y402H allele was not apparent in these families. As we described, 5 out of 10 patients carried both the **CFH** mutation on one allele and the Y402H variant on the other allele. However, we cannot exclude the possibility that unidentified variants in other genes can contribute to the development of cuticular drusen, thereby adding to the burden of heterozygous **CFH** mutations. In addition to the loss-of-function mutations that were detected in our study, a recent study reported a rare heterozygous mutation (R1210C) in the **CFH** gene that confers a high risk of developing AMD.26 Because the patients in this study had progressed to late AMD, differentiating between “regular” AMD and cuticular drusen was not possible. Nevertheless, we speculate that these patients had cuticular drusen in the early stages of their disease. Indeed, our recent finding of the R1210C mutation in two unrelated patients with cuticular drusen (unpublished data) supports this hypothesis.

In addition to cuticular drusen, mutations in the **CFH** gene have also been associated with the renal diseases membranoproliferative glomerulonephritis (MPGN) type II and atypical hemolytic uremic syndrome (aHUS). Nearly 80% of MPGN type II patients have cuticular drusen and these are considered pathognomonic for this renal disorder.27 Histopathologically, the deposits in MPGN type II and AMD/cuticular drusen are similar in a number of aspects. In both diseases, the deposits have comparable saccharide compositions and contain cholesterol, amyloid P component, vitronectin and a wide variety of complement components.28-30 In view of the fundoscopical and histopathological similarities between MPGN type II and AMD-related cuticular drusen—as well as the association of both diseases with **CFH** gene mutations—one might expect a high rate of MPGN type II in patients affected by AMD. However, a high rate of co-morbidity has not yet been described in large epidemiological studies.31 Within the relatively small cohort of patients that we described in Chapter 3, two patients (patient B-II:1 and patient C-II:4) with cuticular drusen caused by a heterozygous mutation in their **CFH** gene also had MPGN type II.3 However, family members who had the same mutation showed no signs of renal failure based on their blood test results. This suggests that an additional factor (for example, a second mutation or an external trigger) is needed to develop MPGN type II. Indeed, several patients with MPGN type II have been reported to carry homozygous or compound heterozygous mutations in **CFH**.32-35 Interestingly, we recently saw a patient in our clinic with cuticular drusen who had developed MPGN type II following a streptococcal infection (unpublished data).

With respect to patients with aHUS, no drusen or other AMD-like changes have been described to date. This is remarkable, given that the genetic link between AMD/cuticular drusen and aHUS is even stronger than the link with MPGN type II, and both disorders have been associated with the heterozygote R1210C mutation in
the *CFH* gene.\textsuperscript{26,36-38} In aHUS, most R1210C mutation carriers are also heterozygous for mutations in other complement genes or tend to have more risk factors for developing aHUS.\textsuperscript{37} Therefore, the pathogenicity of a heterozygous *CFH* mutation alone is insufficient to develop aHUS, just as it is insufficient to develop MPGN type II. This additive model of multiple factors could explain why AMD/cuticular drusen patients who carry the R1210C mutation fail to develop aHUS. Patients with aHUS generally have a mutation on the other allele or in other complement genes. However, why no patients with the R1210C mutation and who have aHUS have been reported to have AMD/cuticular drusen remains a mystery.

**Complement factor I**

Complement factor I is a serine protease that circulates in an inactive form and can inactivate all complement pathways by cleaving the $\alpha'$-chain of the activated complement factors C3b and C4b.\textsuperscript{39,40} The presence of specific cofactors such as factor H, membrane co-factor protein, complement receptor 1 and C4b-binding protein is required for enhancing this cleavage.\textsuperscript{19,41-43} Like factor H, factor I is also required both to maintain complement homeostasis in plasma and to restrict complement activation. The importance of the regulatory role of factor I is highlighted by the fact that rare *CFI* mutations can predispose individuals to develop diseases such as aHUS and systemic lupus erythematosus.\textsuperscript{44,45}

In light of the functional similarities between factor I and factor H, it was no surprise that the *CFI* gene was recently identified as a second gene to harbor mutations in patients with cuticular drusen. As described in Chapter 4, two specific *CFI* gene mutations (G119R and G188A) confer a high risk of developing AMD. Most (n = 13) of the patients who carry a *CFI* mutation and for whom fundus photographs were available (n = 14) exhibited the cuticular drusen subtype of AMD (unpublished results). The G119R mutation has also been linked to aHUS,\textsuperscript{44,46} hinting again at a genetic association between cuticular drusen and aHUS. However, none of the patients in our cohort who had cuticular drusen and the G119R mutation had clinically significant renal dysfunction. As noted above, to develop aHUS, a single G119R mutation must be accompanied by a second mutation either on the other *CFI* allele or in another complement gene.\textsuperscript{44,46} Once again, a key question remains: why have patients with aHUS who carry the G119R mutation never been reported to develop cuticular drusen? This lack of patients with cuticular drusen may simply be due to a lack of observational reports; therefore, we strongly recommend that clinicians perform an ophthalmic examination in patients who have aHUS caused by a mutation in either their *CFH* or *CFI* gene.

**Identifying new mutations in cuticular drusen**

In Chapters 3 and 4, we demonstrated that in addition to polymorphisms (Chapter 2), *CFH* and *CFI* gene mutations can also contribute to the development of cuticular drusen. Indeed, theoretical modeling favors a scenario in which a significant portion of the genetic risk for developing common diseases such as AMD is due to mildly deleterious mutations that are maintained at a low frequency in the population by
weak purifying selection.\textsuperscript{47} Because several common variants in genes encoding components of the complement system are strongly associated with both AMD and the AMD subgroup cuticular drusen, selecting and prioritizing these genes in the search for additional disease-causing mutations seems to be the most logical next step. However, we cannot exclude the possibility that genes that have not yet been associated with AMD may contain mutations that can cause cuticular drusen. The use of new genetic techniques such as exome sequencing will definitely facilitate the identification of such mutations.

**Clinical implications and future perspectives**

At least two clinically relevant aspects can be drawn from the findings that are reported in this thesis. The first is genetic counseling, which became possible after the identification of the disease-causing genetic defect in a fraction of patients with cuticular drusen. By recognizing the genetic cause of the disease, the clinician can then inform the affected families more accurately regarding the prognosis, which can include an increased risk for developing cuticular drusen and cuticular drusen-related AMD. Furthermore, knowing the genetic defect may improve diagnostic accuracy and raise awareness of any disease-related co-morbidity that may develop in patients or their family members as a result of the same genetic defect. As shown in Chapter 3, patients with cuticular drusen who carry a mutation in the \textit{CFH} gene have a higher risk for developing MPGN type II. Screening these patients (in a preclinical stage) for MPGN type II will enable the clinician to initiate treatment in a much earlier stage, which may ultimately prevent end-stage renal failure.\textsuperscript{48}

As an integrative component of their genetic counseling, patients with cuticular drusen—and particularly their family members—should be made aware of the tests that are currently available for predicting AMD (e.g., ArcticDx, Sequenom). These tests are based on a small number of common variants and are therefore not reliable for individuals who carry a disease-causing mutation. Understanding the role of rare variants in AMD is therefore necessary before reliable genetic tests can be developed. Conversely, the relationship between genetic variants and AMD subtypes (for example, geographic atrophy and neovascular AMD, as well as cuticular drusen) must be understood better. To provide patients with timely and effective treatment options, reliable prognostic tests are needed to determine who is at high risk for developing AMD. These tests should be based on a small number of common variants but should also address rare, highly penetrant variants, particularly for the cuticular drusen phenotype, which is caused by rare mutations in a proportion (~8\%) of cases (unpublished data).

The second clinically relevant aspect of our findings is that patient care will improve with a better understanding of the etiology of cuticular drusen. This thesis supports the notion that considering the entire population of AMD patients as homogeneous is an oversimplification. Cuticular drusen is only one distinct AMD
subgroup and has an etiology that differs from other AMD subtypes, and this difference has consequences with respect to prevention and future treatments. Because complement abnormalities are more prevalent in patients with the cuticular drusen phenotype, therapies that target specific components of the complement system are—at least in theory—more promising for treating this AMD subgroup. It is important to differentiate this class of therapies from anti-VEGF-A agents (which are the current conventional treatment for end-stage neovascular disease), as complement-based therapeutics can intervene earlier in the disease process, perhaps preventing atrophic lesions and promoting recovery from cuticular drusen. The latter effect is supported by our findings in Chapter 5, which show that cuticular drusen can regress without anatomical damage. The therapeutic agents for treating AMD that are currently in either clinical trials or late-stage preclinical evaluation are listed in Table 6.1. To select the most suitable treatment, the drug's mechanism of action and its route/site of administration should be taken into consideration.

Based on their mechanism of action, the therapeutic agents that are currently under investigation can be divided into the following four classes: 1) monoclonal antibodies, 2) complement component inhibitors, 3) receptor antagonists, and 4) supplementation therapy. Monoclonal antibodies and complement component inhibitors suppress complement activation by binding to specific target cells and by preventing the formation of key elements within the proteolytic cascade, respectively. Each of these actions in turn prevents downstream complement activation and the formation of the membrane attack complex. It is important to note that most of these inhibitors target common complement pathway components and can therefore cause serious side-effects due to their concomitant inhibition of all three major complement pathways. This potential consequence can be avoided by selecting more specific agents such as receptor antagonists (e.g., the C5a receptor antagonist).49 Unlike C5a inhibitors, receptor antagonists competitively bind to the C5a receptor, thereby neutralizing its normal interactions. Receptor antagonists can therefore selectively suppress the inflammatory response without adversely affecting the entire complement-related immune system.50 The last class of therapeutics that is currently under investigation is the supplementation therapy group. Recombinant factor H is currently under preclinical development and may serve as a promising treatment option for patients with early stage cuticular drusen caused by a loss-of-function mutation in the CFH gene. In theory, this augmentation therapy should help restore homeostatic regulation of the alternative complement pathway.

To achieve the best results possible, clinicians must select the optimum route of administration for their drug of choice. The CFH Y402H variant that is associated with a high risk of developing AMD was recently found to protect against systemic inflammatory response syndrome in children.51 The R32Q variant of complement factor B,52 which decreases the risk of developing AMD, increases the susceptibility to develop cardiomyopathy in patients with Chagas disease.53 The above examples demonstrate that local delivery may be favored over systemic administration in order to avoid undesired systemic effects.
In conclusion, the findings reported in this thesis demonstrate that genetic factors play a larger role in patients with cuticular drusen AMD than in other AMD types. These findings may explain why, compared to AMD in general, cuticular drusen develop at a younger age and are more often clustered within families. Identifying the genetic defects that underlie the development of cuticular drusen will improve our understanding of this AMD subtype and will help clinicians customize the treatment to match the patient. To date, we have identified two genes that harbor rare, highly penetrant variants that can cause cuticular drusen. Determining which of the approximately 25,000 genes in the human genome harbor genetic defects that can contribute to the development of cuticular drusen will be a challenging task in the coming years.

![Table 6.1. Summary of anti-complement therapies for age-related macular degeneration](image)

<table>
<thead>
<tr>
<th>Drug (Trade name)</th>
<th>Class and mechanism of action</th>
<th>Current stage of development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soliris (Eculizumab)</td>
<td>1 - Monoclonal antibody targeting C5</td>
<td>Phase II (ongoing, no longer recruiting)</td>
</tr>
<tr>
<td>Anti-factor D (FCFD4514s)</td>
<td>1 - Monoclonal antibody that targets complement factor D</td>
<td>Phase II (ongoing, no longer recruiting)</td>
</tr>
<tr>
<td>TA106</td>
<td>1 - Monoclonal antibody that targets complement factor B</td>
<td>Preclinical</td>
</tr>
<tr>
<td>POT-4 (Compstatin)</td>
<td>2 - C3 inhibitor – prevents cleavage of C3 into C3a and C3b</td>
<td>Phase I (completed)</td>
</tr>
<tr>
<td>ARC1905</td>
<td>2 - C5 inhibitor – prevents cleavage of C5 into C5a and C5b</td>
<td>Phase I (ongoing, no longer recruiting)</td>
</tr>
<tr>
<td>C1-INH</td>
<td>2 - Protease inhibitor</td>
<td>Preclinical</td>
</tr>
<tr>
<td>JPE-1375/JSM-7717</td>
<td>3 - C5a receptor antagonist</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Recombinant CFH</td>
<td>4 - CFH supplementation</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>

Abbreviations: CFH, complement factor H
References


Chapter Seven
Summary / Samenvatting
Summary

Age-related macular degeneration (AMD) is a degenerative disease of the central retina and the most common cause of irreversible and progressive visual loss among the elderly in the Western world. AMD is a heterogeneous disorder, with several clinically recognized subtypes like the cuticular drusen phenotype. Despite intensive research on AMD in the last two decades, most of the AMD subtypes remain scarcely investigated. The aim of this thesis was to add to the insights in the clinical characteristics and genetic causes of cuticular drusen, and subsequently to contribute to a better understanding of this AMD subtype. This may enable better counseling of patients and their families, as well as tailor the choice of the therapeutic approach to this specific AMD subtype.

Chapter 1 provides a general introduction to the processes that lead to vision, the retinal anatomy, the imaging techniques used to evaluate retinal anatomy, molecular genetics, and describes the clinical and pathological aspects of AMD.

In Chapter 2, we investigated whether the association of cuticular drusen with several AMD risk factors is different from that of AMD as a whole. To perform this analysis, all major environmental and genetic risk factors previously found to be associated with AMD were evaluated in a cohort of 217 patients with cuticular drusen, a cohort of 540 patients with AMD who did not display cuticular drusen, and a cohort of 553 age-matched controls. We found that cuticular drusen was associated with most previously identified AMD risk factors. However, the association with the \textit{CFH} Y402H risk allele appeared to be stronger, whereas the association with smoking was less pronounced when compared to AMD as a whole. These findings suggest a more important role for genetic factors than environmental factors in the developmental course of cuticular drusen.

Chapter 3 describes a detailed clinical and molecular genetic analysis of 11 patients with cuticular drusen from 3 different families, who all carried a novel heterozygous mutation in the \textit{CFH} gene. A broad phenotypic variation was observed, ranging from limited macular drusen to extensive drusen in the posterior pole as well as the peripheral retina, even in association with a single \textit{CFH} mutation. Two patients developed end-stage kidney disease as a result of membranoproliferative glomerulonephritis type II, a second disease that can be caused by a mutation in the \textit{CFH} gene. These findings suggest that patients with extensive cuticular drusen caused by a \textit{CFH} gene mutation should undergo screening for renal function to preclude preclinical membranoproliferative glomerulonephritis type II.

Chapter 4 presents the identification of a rare, highly penetrant missense mutation, G119R, that confers a high risk of AMD in the \textit{CFI} gene, an AMD candidate by virtue of association of non-coding variants near the locus. We found that plasma of patients carrying the G119R mutation mediated the degradation of C3b at a significantly lower
level compared to patients without a mutation in \textit{CFI}. Recombinant protein studies revealed that the G119R mutant is both expressed and secreted at significantly lower levels than wild type protein. Consistent with these findings, 119R-encoding human mRNA had reduced activity compared to 119G in regulating vessel thickness and branching in the zebrafish retina. Taken together, these findings indicate an obligate role for \textit{CFI} in the pathogenesis of AMD and demonstrate that rare, highly penetrant mutations contribute to the genetic variance of AMD, with implications for predictive testing and personalized treatment.

In \textbf{Chapter 5}, we asked ourselves whether drusen in patients with cuticular drusen show short-term morphological changes. This question is interesting because the size and number of drusen are used for disease staging and for the prediction of the likelihood of disease progression and vision loss. In order to answer this question, ten subjects with cuticular drusen were longitudinally followed during a period of 4 months by spectral-domain OCT. Interestingly, all eyes showed drusen with a spontaneous change in volume. Subsequently, we investigated whether morphologic parameters may be predictive for processes of progression or regression of drusen. We found that pointed drusen showed a significant association with a progression in volume, while drusen with overlying photoreceptor layer or RPE damage showed a significant association with a regression in volume. These findings reveal a potential source of misclassification in disease staging. In addition, the dynamic nature of cuticular drusen points to high biochemical activity that may be sensitive to future pharmacologic treatment strategies.

In \textbf{Chapter 6}, the findings of the work described in this thesis are discussed and implications for scientists, ophthalmologists and patients are presented. After a brief introduction on the history of cuticular drusen, the role of rare mutations in the development of this AMD subtype is presented. Finally, the clinical implications of the genetic findings are discussed, like the possibility of genetic counseling and how a better understanding of the etiology of cuticular drusen will help us to tailor our choice of treatment.
Samenvatting

Leeftijdsgebonden maculadegeneratie (LMD) is een degeneratieve aandoening van de centrale retina en de meest voorkomende oorzaak van irreversibel en progressief visusverlies onder ouderen in de Westerse wereld. LMD is een heterogeen ziektebeeld met verschillende klinische subtypes, waaronder cuticular drusen. Ondanks intensief onderzoek naar LMD de afgelopen twintig jaar is er maar weinig bekend over de verschillende subtypes van LMD. Het doel van dit proefschrift was om meer inzicht te verwerven in de klinische kenmerken en de genetische oorzaken van cuticular drusen om zo bij te dragen tot meer kennis over dit subtype van LMD. Dit zou kunnen bijdragen aan een betere voorlichting voor patiënten en hun familieleden, alsook een meer toegespitste therapeutische aanpak van dit specifieke subtype van LMD.

Hoofdstuk 1 geeft een algemene introductie in het proces waardoor ons zicht tot stand komt, de anatomie van de retina, de technieken welke gebruikt worden om de retinale anatomie in beeld te brengen, de moleculaire genetica en beschrijft de klinische en pathologische aspecten van LMD.

In Hoofdstuk 2 hebben we onderzocht of de associatie tussen cuticular drusen en de verschillende risicofactoren van LMD anders is dan bij LMD in het algemeen. Om dit te onderzoeken hebben we alle belangrijke omgevings- en genetische risicofactoren die geassocieerd zijn met LMD geëvalueerd in een cohort van 217 patiënten met cuticular drusen, een cohort van 540 patiënten met LMD zonder het cuticular drusen subtype en in een cohort van leeftijdsgematchte controle personen. We vonden dat het cuticular drusen fenotype geassocieerd is met de meeste risicofactoren die ook geassocieerd zijn met LMD in het algemeen. Echter, de associatie met het CFHY402H risico allel bleek sterker, terwijl de associatie met roken minder uitgesproken bleek te zijn dan met LMD in het algemeen. Deze bevindingen suggereren dat genetische factoren een belangrijkere rol spelen in de ontwikkeling van cuticular drusen dan omgevingsfactoren.

Hoofdstuk 3 beschrijft een gedetailleerde klinische en genetische analyse van 11 patiënten met cuticular drusen uit 3 verschillende families die allen drager zijn van een nieuwe heterozygote mutatie in het CFH gen. Er werd een brede variatie gezien in de klinische presentatie, variërend van enkele drusen in de macula tot een grote hoeveelheid drusen verspreid over de hele retina. Deze variabele presentatie werd zelfs gezien in patiënten die dezelfde mutatie droegen. Twee patiënten ontwikkelden membranoproliferatieve glomerulonefritis type II, een aandoening die eveneens veroorzaakt kan worden door een mutatie in het CFH gen. Deze bevindingen suggereren dat een nierfunctiescreening bij patiënten met cuticular drusen nuttig kan zijn om membranoproliferatieve glomerulonefritis type II al in een preklinisch stadium te kunnen uitsluiten.
Hoofdstuk 4 beschrijft de ontdekking van een zeldzame, hoogpenetrante missense mutatie (G119R) in het CFI gen. In het plasma van patiënten met de G119R mutatie was een significant lagere afbraak van C3b in vergelijking met patiënten zonder mutatie te zien. Vervolgonderzoek liet zien dat het gametoomde G119R eiwit tot expressie komt en minder uitgescheiden wordt dan het wildtype eiwit. In lijn met deze bevindingen had het 119R coderende humane mRNA een verlaagde activiteit in vergelijking met 119G in de reguleringsfasen van vaatdikte en vaatvertakking in de retina van zebravissen. Concluderend laten deze bevindingen zien dat CFI een belangrijke rol speelt in de pathogenese van LMD en dat zeldzame hoogpenetrante missense mutaties bijdragen aan de genetische variatie van LMD. Bovendien heeft dit de nodige implicaties voor voorspellende testen voor LMD en behandeling op het niveau van de individuele patiënt.

In Hoofdstuk 5 vroegen we onszelf af of de drusen in patiënten met cuticular drusen morfologische veranderingen op korte termijn lieten zien. Deze vraag is interessant omdat de grootte en het aantal drusen wordt gebruikt om de ernst van het ziektebeeld te bepalen en de prognose van ziekteprogressie en visusverlies in te schatten. Om deze vraag te kunnen beantwoorden hebben we 10 patiënten met cuticular drusen gedurende een periode van 4 maanden gevolgd met spectrale-Domain OCT. Het opmerkelijke was dat alle ogen een spontane verandering in drusenvolume lieten zien. Vervolgens hebben we onderzocht welke morfologische parameters een voorspellende waarde hadden voor drusenprogressie of drusenregressie. Hierbij vonden we dat puntvormige drusen een significante associatie lieten zien met progressie van drusenvolume, terwijl drusen met overliggende fotoreceptorschade of RPE schade een significante associatie met regressie van drusenvolume lieten zien. Deze bevindingen laten een potentiële bron van misclassificatie van het ziektestadium zien. Tevens wijst de dynamiek van drusen op een hoge biochemische activiteit wat aangrijpingspunten kan bieden voor toekomstige farmacologische interventies.

In Hoofdstuk 6 worden de bevindingen uit dit proefschrift en de implicaties daarvan voor wetenschappers en klinici bediscussieerd. Na een korte inleiding over de geschiedenis van cuticular drusen, wordt de rol van zeldzame mutaties beschreven in de ontwikkeling van dit subtype van LMD. Tot sluit worden de klinische implicaties van de gevonden bevindingen besproken, zoals de mogelijkheid van genetische voorlichting en hoe een beter begrip van de etiologie van cuticular drusen kan helpen in de keuze voor therapie.
Chapter Eight
Curriculum Vitae
Curriculum Vitae

Johannes (John) Petronella Hubertus van de Ven was born on December 29th, 1981 in Tegelen, the Netherlands. After completing secondary school at “College Den Hulster” in Venlo he studied civil engineering for one year at the “Hogere Technische School” in ’s-Hertogenbosch. In 2002 he started his medical studies at the Catholic University Nijmegen (currently: Radboud University Nijmegen) where he graduated in August 2009. During his medical studies, he started as a student research assistant at the Department of Cardiology, investigating the inhibition of sympathetic activity in cardiovascular disease. In this period he got enthused in doing clinical research. His particular interest in ophthalmology arose in the third year of his medical studies, when he attended the course “sense organs and skin”, and was further reinforced during his internship in Ophthalmology.

Consequently, he started in September 2009 with his Ph.D. research on the clinical and genetic aspects of cuticular drusen, that resulted in this thesis, at the Department of Ophthalmology of the Radboud University Nijmegen Medical Centre in Nijmegen, headed by prof. dr. J.E.E. Keunen. In October 2012, he started a residency in ophthalmology at the same institute.
Chapter Nine
Dankwoord
Dankwoord

Bijna aangekomen bij het eind van mijn proefschrift, biedt er zich een mooie gelegenheid aan om even stil te staan en terug te blikken op een mooie en bijzondere periode uit mijn leven. Door de buitenstaander wordt promoveren vaak geassocieerd met het doen van onderzoek, het opschrijven van je resultaten in de vorm van een artikel en dit vervolgens te publiceren. Wie promoveren op deze manier definieert is waarschijnlijk zelf nooit gepromoveerd. Promoveren is namelijk veel meer! Het is tevens een ontdekkingsreis van jezelf, waarbij je prestaties leert neerzetten die je voorheen voor onmogelijk achtte, maar waarbij je ook zeker je beperkingen leert kennen. Er wordt een beroep gedaan op je creativiteit, flexibiliteit en je incasseringsvermogen wordt tot het uiterste getest. Gedurende dit proces wordt je bijgestaan door een heel team die je vanaf de zijlijn voorziet van goed advies. Promoveren doe je dan ook niet alleen, ondanks dat de enkele naam op de voorkant van dit proefschrift wellicht anders doet vermoeden. Ik realiseer me dat het onmogelijk is om eenieder te bedanken die een bijdrage heeft geleverd aan dit proefschrift. Toch wil ik een aantal mensen hier in het bijzonder noemen vanwege hun belangrijke bijdrage.

Mijn grootste dank ben ik verschuldigd aan alle patiënten en proefpersonen die deel hebben genomen aan mijn onderzoek. Zonder u was dit proefschrift simpelweg nooit tot stand gekomen. Met diepe respect heb ik mogen ervaren hoe u bereid was van heinde en verre naar Nijmegen af te reizen om de vaak lange onderzoekssessies te ondergaan. Ofschoon uw bijdrage formeel in anonimiteit plaatsvindt, vormt uw deelname een belangrijke bijdrage voor de toekomstige ontwikkelingen binnen de oogheelkunde. Dank daarvoor.

Prof. dr. C.B. Hoyng, beste Carel, ik heb je mogen ervaren als een man vol humor en relativeringsvermogen. Je gaf me veel vrijheid in het doen van onderzoek, maar wist me altijd weer een duw in de goede richting te geven als ik het grotere geheel uit het oog dreigde te verliezen. Jouw kracht schuilt in het herkennen van potentieel in alles en iedereen. Ik wil je dan ook graag bedanken dat je in mij geloofde en de kans die je me hebt geboden als onderzoeker.

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Mijn (studie)vrienden, Hilbert, Jacob, Guus, Bob, Bert-Jan, Stan, Pieter en Babak, dank voor jullie belangstelling, steun en broodnodige afleiding ondanks jullie eigen drukke agenda's. Vriendschap is geen illusie.
Ook mijn ouders Jo en Francien van de Ven wil ik graag apart noemen. Lieve ouders, jullie hebben mijn hele leven al achter me gestaan en met raad en daad ondersteund in alle beslissingen die ik nam. Ik begon aan mijn promotie in een moeilijke periode voor ons gezin. We dachten deze periode afgesloten te hebben, maar kregen vrij recent te horen dat pa ongeneeslijk ziek is. Ik houd onzettend veel van jullie en waardeer jullie enorm. Ik weet dat ik door alle drukte weleens vergeet te zeggen, daarom schrijf ik het hier nu voor eens en altijd op. En pa, dit proefschrift draag ik op aan jou.

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Chapter Ten
List of publications
List of publications

Evaluation of serum lipid concentrations and genetic variants at high-density lipoprotein metabolism loci and TIMP3 in age-related macular degeneration.

Central areolar choroidal dystrophy (CACD) and age-related macular degeneration (AMD): differentiating characteristics in multimodal imaging.

Clinical evaluation of 3 families with basal laminar drusen caused by novel mutations in the complement factor H gene.
Arch Ophthalmol 2012;130(8):1038-47

Short-term changes of basal laminar drusen on spectral-domain optical coherence tomography.

Genetic, behavioral and sociodemographic risk factors for second eye progression in age-related macular degeneration.
Invest Ophthalmol Vis Sci;53(9):5846-52

Cumulative effect of high risk alleles in CFH, ARMS2 and VEGF-A on response to Ranibizumab treatment in age-related macular degeneration.
Association analysis of genetic and environmental risk factors in the cuticular drusen subtype of age-related macular degeneration.
Mol Vis 2012;18:2271-78

Automatic drusen quantification and risk assessment of age-related macular degeneration on color fundus images.
Invest Ophthalmol Vis Sci 2013; epub ahead of print

A functional variant in the CFI gene confers a high risk of age-related macular degeneration.
Nat Genet 2013; in press

A rare nonsynonymous sequence variant in C3 confers a high risk of age-related macular degeneration.
Nat Genet; under review

Zinc supplementation inhibits complement activation in age-related macular degeneration.
Ophthalmology; under review

Cuticular drusen: stars in the sky.
Prog Retin Eye Res; under review