# DEVELOPMENT OF AGE-RELATED MACULOPATHY:

# A HISTOCHEMICAL AND MOLECULAR APPROACH

Antoinette Carolien Lambooij

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Development of age-related maculopathy: a histochemical and molecular approach
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Thesis Rotterdam

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# DEVELOPMENT OF AGE-RELATED MACULOPATHY:
# A HISTOCHEMICAL AND MOLECULAR APPROACH

## ONTWIKKELING VAN
## OUDERDOMS-GEBONDEN MACULOPATHIE:
## EEN HISTOCHEMISCHE EN MOLECULAIRE BENADERING

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*Voor mijn ouders*
*Voor Erik*

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# PUBLICATIONS AND MANUSCRIPTS

## BASED ON THE STUDIES DESCRIBED IN THIS THESIS

CHAPTER 4 Lambooij AC, Kliffen M, Kuijpers RW, Houtsmuller AB, Broerse JJ, Mooy CM (2000) Apoptosis is present in the primate macula at all ages. *Graefes Arch Clin Exp Ophthalmol* 238:508-514

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CHAPTER 9 Lambooij AC, Kuijpers RWAM, Mooy CM, Kliffen M (2001) Radiotherapy of exudative age-related macular degeneration: a clinical and pathologic study. *Graefes Arch Clin Exp Ophthalmol*, 239:539-543

# ABBREVIATIONS

| | |
|---|---|
| **AMD** | age-related macular degeneration |
| **ARM** | age-related maculopathy |
| **BLD** | basal laminar deposit / basal linear deposit |
| **bp** | base pair |
| **CNV** | choroidal neovascularization |
| **CNVM** | choroidal neovascular membrane |
| **PDR** | proliferative diabetic retinopathy |
| **FAS-L** | fas-ligand |
| **FGF** | fibroblast growth factor |
| **GCL** | ganglion cell layer |
| **GH** | growth hormone |
| **HPRT** | hypoxanthine-guanine phosphoribosyl transferase |
| **IGFBP** | insulin-like growth factor binding protein |
| **IGF-I** | insulin-like growth factor-I |
| **IGF-IR** | insulin-like growth factor receptor type I |
| **IL** | interleukine |
| **INL** | inner nuclear layer |
| **MMP** | matrix metalloproteinase |
| **ONL** | outer nuclear layer |
| **rd-mice** | retinal degeneration-mice |
| **RPE** | retinal pigment epithelium |
| **RT-PCR** | reverse transcriptase-polymerase chain reaction |
| **SSD** | source skin distance |
| **SST** | somatostatin receptor |
| **TGF-β** | transforming growth factor-β |
| **TNF-α** | tumor necrosis factor-α |
| **TUNEL** | terminal deoxynucleotidyl transferase mediated deoxyuridine biotin nick end labeling |
| **VA** | visual acuity |
| **VEGF** | vascular endothelial growth factor |

# PART I

# INTRODUCTION

# CHAPTER 1

## GENERAL INTRODUCTION: AGE-RELATED MACULOPATHY

Age-related maculopathy (ARM) is the major cause of blindness in people over 65 years of age in the Western world. ARM involves the central part of the retina, called the macula or yellow spot, where visual acuity is highest.
Clinically, early stages of ARM show drusen and pigment alterations, associated with minimal or mild vision loss. Histopathologically, the first signs are deposits between the retinal pigment epithelium (RPE) and Bruch's membrane, drusen and RPE alterations. Late stages of ARM, also called age-related macular degeneration (AMD), include geographic atrophy and exudative macular degeneration. They are associated with severe vision loss. The exudative form is characterized by choroidal neovascularization (CNV). In CNV, newly formed vessels sprout from the underlying choroid and grow through breaks in Bruch's membrane beneath the retinal pigment epithelium (RPE) and the retina.[1] Clinically, visual acuity decreases rapidly because of hemorrhages or serous detachments.
Geographic atrophy is characterized by areas of degenerated RPE and neural retina in the absence of breaks in Bruch's membrane and subretinal new vessels, and has been suggested to be the natural endstage of ARM.[2]
In the Netherlands, the prevalence of late stages of ARM is 1.7% in people over 55 years of age and up to 11% in people over the age of 85.[3] Sixty-five % of patients with AMD have the exudative form, while 35% show geographic atrophy.[3] Exudative AMD is responsible for 80% of the cases of severe vision loss.[4,5] Numbers will increase because of the population's increasing age. Both aging,[6] genetic factors,[7-9] and environmental factors such as cigarette smoking[6,10,11] and antioxidant status[12] are acknowledged risk factors in the aetiology of ARM. Only a limited percentage of AMD patients is amenable to treatment.[13]

This thesis focusses on the pathogenesis of photoreceptor atrophy and on the pathogenesis of exudative AMD.

# CHAPTER 2

# PATHOGENESIS AND HISTOLOGY OF AGE-RELATED MACULOPATHY

## 2.1 ANATOMY OF THE NORMAL RETINA

The human retina (Figure 2.1A) comprises the neuroretina and the RPE, which is a monolayer of pigmented cells in close contact to the photoreceptors and is situated on top of Bruch's membrane. Bruch's membrane consists of three layers: a middle layer of elastic tissue and two outer layers of collagen. Beneath Bruch's membrane is the choriocapillaris, which is part of the choroidal vascular network and responsible for the nutrition and oxygenation of the RPE and outer neuroretina. The inner retinal layers on the other hand are supplied with blood from the central retinal artery.

The optical center of the human retina is called the macula lutea (Figure 2.1B). This is an area of about 5 mm with an indentation in the middle, the foveola. The yellow coloration of the macula is derived from the presence of macular pigment, chemically defined as xanthophyll carotenoids.[14,15] Surrounding the foveola the annular regions towards the margin of the macula are called fovea, parafovea and perifovea, respectively.

The neuroretina consists of different layers, which are described in figure 2.1A. In the macula, the ganglion cell layer consists of a minimum of two cells thick. Furthermore, this region has the highest concentration of photoreceptors, permitting high-resolution visual acuity. The photoreceptors are organized in a mosaic of rods and cones. The mosaic in the fovea is composed entirely of cones. In the periphery of the fovea more rods are present. Outside the macula, cones are scarce.

## 2.2 AGING OF THE RETINA

### 2.2.1. Theories on Cellular Aging

Aging or senescence, can be defined as a series of time-related processes occurring in the adult individual that ultimately bring life to a close.[16] These processes involve the

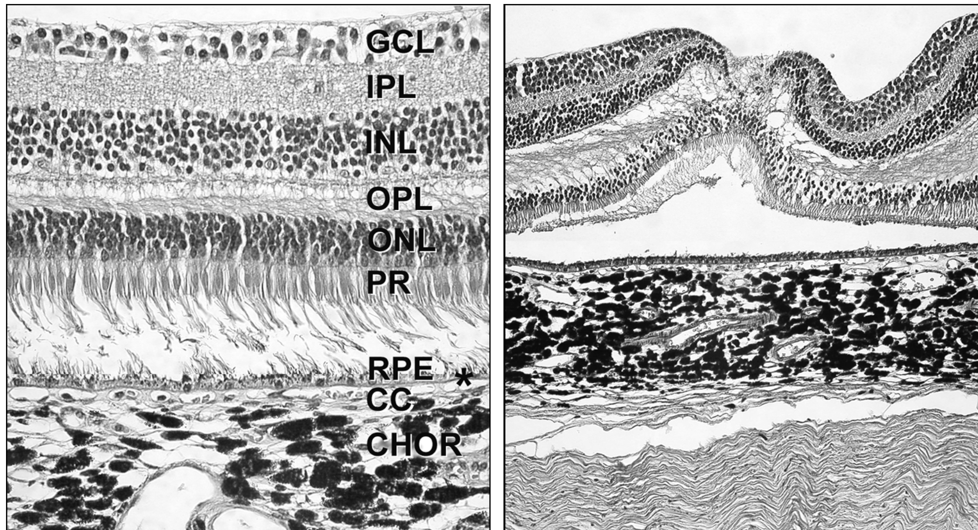


***Figure 2.1 Anatomy of macula (A)*** *Macular region of non-human primate, indicating the retinal layers. Bruch's membrane (*) is located in between the RPE and CC.* ***(B)*** *Foveal indentation at visual centre. (GCL = ganglion cell layer; IPL = inner plexiform layer; INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer; PR = photoreceptor layer; RPE = retinal pigment epithelium; CC = choriocapillaris). Mallory staining; original magnification x200 (A) and x100 (B)*

cumulative effects of extrinsic influences and an intrinsic molecular program of cellular aging.[17,18]

Theories concerning **extrinsic influences** imply that exogenous damaging factors exceed the cells' regenerative capability, thus causing senescence. The *free radical theory of aging* centers on the long-term deleterious oxidative effects of the physiologically generated free radicals.[19,20] Because oxidative damage is generally thought to play a major role in aging of the retina, this will be discussed in paragraph 2.2.2. Closely related to the *free radical theory of aging* is the *mitochondrial theory of aging*. The fact that mitochondria posses their own genetic material and that they only have a limited arsenal of DNA repair processes makes them especially vulnerable to oxidative damage.[21] This theory assumes that oxidative damage to mitochondrial DNA in postmitotic cells leads to mutations and blocks to replication, and consequently to mitochondrial dysfunction and physiological cellular decline.

**Intrinsic cellular aging** theories assume that genetic factors predispose to progressive cellular changes, leading to senescence.

The *somatic mutation hypothesis* proposes that an accumulation of DNA mutations leads to nonfunctional proteins and enzymes, and thus is responsible for senescence.[16] A second theory on intrinsic aging, the *programmed aging hypothesis*, assumes a predetermined, genetically programmed, sequence of events ultimately leading to senescence. The *telomere hypothesis of cellular aging* explains that proliferation stops after a defined number of cell divisions, because of telomere shortening.[22,23] At a critical telomere length, the cell irreversibly exits the cell cycle and enters a stage called

senescence. The senescent cells are metabolically active but cannot proliferate, and can be considered as replicative or telomeric aged.
How these concepts on intrinsic aging apply to post-mitotic cells is still unclear. Probably, in age-related disease, a combination of both intrinsic and extrinsic aging plays a role. With aging and accumulation of genetic damages, functional cellular capacity decreases until the disease threshold is achieved. Earlier onset of disease could occur because of genetic differences and by further loss of function due to environmental agents.[24,25]

The cells of the retina are post-mitotic and differentiated, and under normal circumstances they are unable to regenerate new cells after the loss of old or damaged ones. Therefore, the thickness of the human retina decreases with advancing age, due to loss of photoreceptors and ganglion cells.[26,27] Retinal cell loss probably occurs via apoptotic cell death. The apoptotic phenomenon is discussed in paragraph 2.2.3.

### 2.2.2. Oxidative Stress

Oxidative stress refers to cellular damage caused by oxidative processes and has been implicated in many disease processes, specially age-related disorders. The retina is particularly susceptible to oxidative stress, firstly because of its high consumption of oxygen, secondly because the membranes of photoreceptor outer segments contain a high concentration of polyunsaturated fatty acids that are highly susceptible to oxidation,[28] and thirdly because of its exposure to visible light.[29] The free radical theory of aging proposes that aging and age-related disorders are the result of cumulative damage arising from reactions involving oxidative processes.[20,29]
Many of the oxygen radicals are produced as byproducts of normal physiology. For instance, lipofuscin is an autofluorescent material which accumulates within the RPE throughout life. Lipofuscin is formed by the undegradable endproducts resulting from the phagocytosis of photoreceptor outer segments.[30] Lipofuscin is capable of light-induced generation of reactive oxygen species,[31,32] supporting the relationship between light, age-induced changes in the retina and retinal degeneration.[33] Although the RPE is rich in antioxidants, these may be insufficient to detoxify all the radicals and there may be an accumulation of oxidative damage throughout life that only manifests itself in older people.[30]

### 2.2.3 Apoptosis

Apoptosis is a cell suicide program in which the cell triggers a process of events that results in its own death.[34-37] Apoptosis plays a crucial role in many physiological processes such as embryonic development and homeostatic maintenance of several adult tissues. Also many disease processes are associated with apoptosis or a lack thereoff, for instance degenerative diseases and malignant tumours, respectively.

Apoptosis is an active process, that is usually dependent on protein synthesis[38] and on the expression of certain genes. The process of apoptosis can be initiated by a variety of stimuli such as irradiation, growth factor withdrawal, hormones, cytokines, natural killer cells, and a variety of chemical, viral, and physical agents.
After the initial stimulus has activated the process of apoptosis, a cascade of biochemical events is switched on, leading to the irreversible execution of the cell death. Cystein proteases known as caspases are key-players in the catabolic cascade, leading to DNA fragmentation and cellular degradation. The nuclear DNA is cleaved into internucleosomal fragments of multiples of 180 base pairs (bp) by endonucleases. Analysis by gel electrophoresis shows a characteristic ladder formation. DNA-nick ends of individual apoptotic cells can be visualized *in-situ* by the TUNEL method (terminal transferase-mediated dUTP nick end labeling).[39] Morphologically, apoptosis characteristically affects single cells in stead of groups of adjoining cells. Apoptotic cells show nuclear and cytoplasmic condensation. Cells shrink and are fragmented into apoptotic bodies, which are removed by macrophages or neighboring cells,[40] without any inflammation in contrast to cell death by necrosis.

In the eye, apoptosis occurs under both physiological and pathological conditions. During normal retinal development, many more retinal neuronal cells are produced than will ultimately survive in the adult retinal system; the redundant cells die by apoptosis. The survival of developing neurons depends on the correct connection to both their efferents and afferents, on the interaction with neighboring glial cells, and on the availability of neurotrophic proteins and neurotransmitters.[41] The removal of redundant cells in the developing mouse retina occurs by apoptosis in the various retinal layers in time waves.[42]
Under pathological circumstances, retinal cell loss via apoptosis is considered as a final common pathway resulting from a variety of primary defects. Recent studies indicate that apoptosis is a mechanism of cell death in several ocular diseases including glaucoma, retinitis pigmentosa, cataract formation, retinoblastoma, retinal ischemia, and diabetic retinopathy.[43-53] Apoptotic cells have also been identified in late stages of AMD, both in the neovascular form as well as in the geographic form.[54-56] It is suggested that apoptosis is one of the pathways of photoreceptor degeneration in AMD.[55]

Many inhibitory and stimulatory genes regulating apoptosis have been identified (Figure 2.2). In the retina some of these have been studied under experimental conditions.
The **Bcl-2** family consists of many proteins, with an important role in both the induction and protection from apoptosis, depending on specific ratios of pro- or anti-

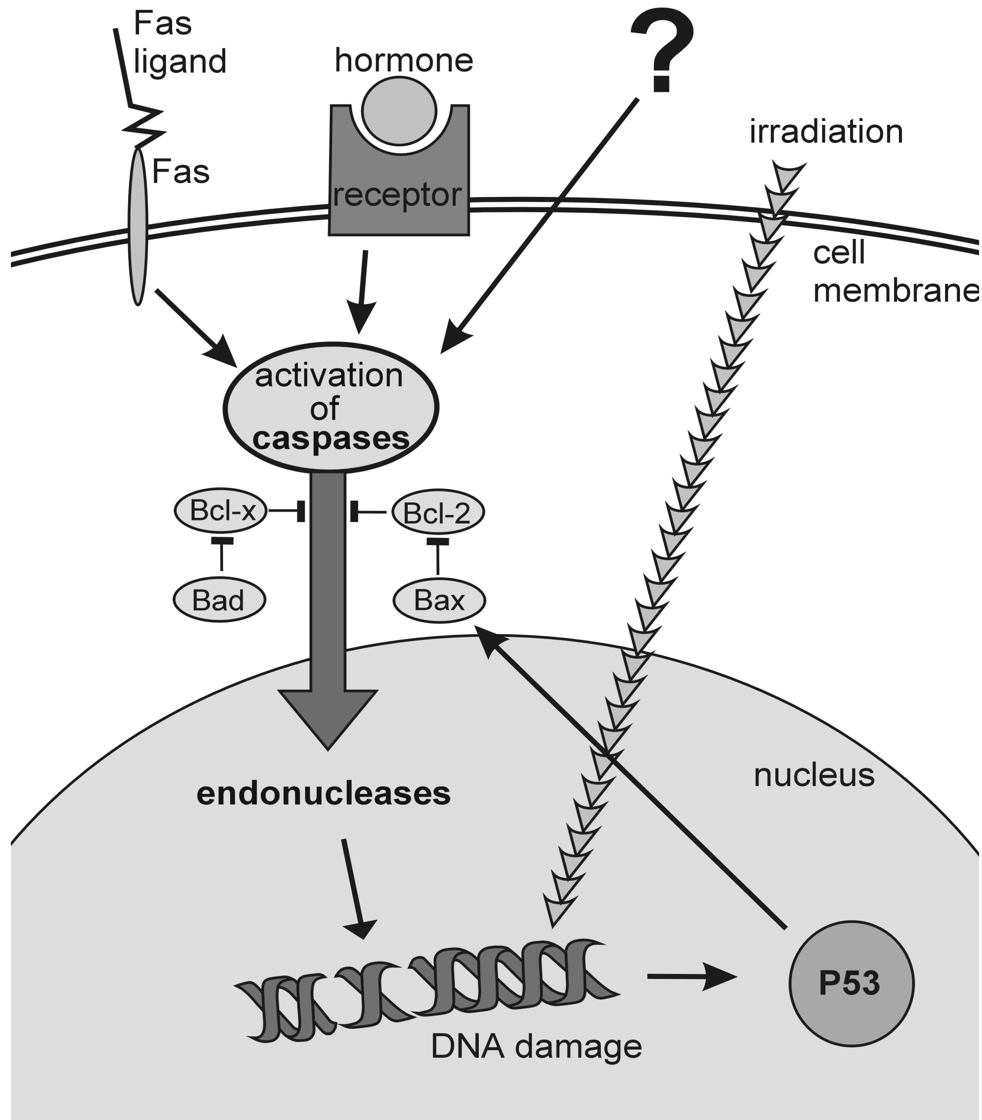


***Figure 2.2 Cellular processes involved in apoptosis.*** *Simplified scheme of cellular processes involved in apoptosis. Activation of caspases by Fas-ligand binding to Fas, hormone-receptor activation and many other factors, several still unidentified, induce a cascade of events. This ultimately leads to cleavage of DNA by endonucleases. The process can be inhibited by Bcl-2 or Bcl-x, while p53 has a pro-apoptotic effect when DNA damage is irreparable.*

apoptotic members of the family.[57] The Bcl-2 family of proteins are located mostly in the mitochondrial membrane. The apoptosis-protective protein Bcl-2 is expressed widely in the developing neuronal system but downregulated in the adult neuronal system.[58] Bcl-x is another apoptosis protective protein and is predominantly present in postnatal neural tissues[57] and adult rat retina.[59] Overexpression of Bcl-2 or Bcl-x delayes photoreceptor cell death in a mouse model of retinal degeneration,[60] although conflicting reports exist.[61]

**Fas** (CD95) receptor mediates apoptosis when triggered by its ligand, Fas-ligand (FasL) or by agonistic antibodies.[62] In the eye, FasL expression helps to maintain immune privilege by inducing apoptotic cell death of invading lymphoid cells that enter in response to infection.[63] Thus, infiltrating inflammatory cells are killed before

they can damage the eye, thereby helping to preserve vision. Furthermore, FasL expressed on RPE cells is suggested to control growth and development of new subretinal vessels[64] by inducing apoptosis of endothelial cells.

The **p53** tumor suppressor protein is involved in the control of the cell cycle, and is associ-ated with apoptosis in various cell types, especially following DNA damage.[65] DNA dama-ge induces p53 to stop the cell cycle allowing the DNA damage to be repaired. If the da-mage is beyond repair, p53 activates the apoptotic program. The p53 product is thought to play a dynamic role in the process of apoptosis due to retinal ischemia.[66,67] In a mouse model of retinal degeneration, absence of p53 delays photoreceptor cell loss.[68] However, also p53-independent apoptosis has been described in studies on retinal degeneration.[69]

The proteins **Jun** and **Fos** are proto-oncogenes.[70] There is evidence that both Fos and Jun are involved in apoptosis of various cell types, including neurons[71] and other retinal cell types.[41] In both differentiated and undifferentiated retinal cells, expression of c-Jun is correlated with apoptosis, preceding the morphological and biochemical characteristics of apoptosis.[41] c-Fos deficient mice are protected against light-induced photoreceptor apoptosis.[71]

## 2.3 AGE-RELATED MACULOPATHY

### 2.3.1 Pathogenesis of Age-related Maculopathy

The first histologic signs of ARM are deposits (basal laminar and linear deposits, and drusen) between the RPE and Bruch's membrane. The deposits are accompanied by attenuation of the RPE and thickening of Bruch's membrane. The depositions probably form a barrier for oxygen and nutrition transport from the choriocapillaris to the RPE and outer neuroretina, culminating in further degeneration and ultimately death of RPE cells and secondary degeneration of rods and cones.[1] Furthermore the extended deposits in and along Bruch's membrane provide a cleavage plane for ingrowing choroidal neovascularization.

The site of the primary lesion in the pathogenesis of ARM is still unclear. Several theories have been hypothesized, in which the primary defect is allocated either to the RPE, to Bruch's membrane, to the choriocapillaris or to the photoreceptors.

Several authors assume that ARM is caused by gradual failure of the metabolic integrity of the RPE,[1,72,73] giving rise to other signs of deterioration, such as deposits in Bruch's membrane and RPE and associated photoreceptor cell death.[1] The RPE dysfunction may be due to imperfections in the cell’s digestive mechanisms,[1] or to

oxidative stress due to free radical chain reactions.[29] Abnormal molecules such as lipofuscin, gradually accumulate within the RPE and normal metabolism is disrupted, leading to aberrant deposition of debris in and along Bruch's membrane. In addition, there is evidence that RPE produces endothelial cell growth inhibitors and trophic factors which maintain the normal function of the choriocapillaris.[74] This biochemical communication between the RPE and choriocapillaris may be disturbed by a thick layer of basal deposits, which interferes with the diffusion of those factors.[74]
Other authors assume choroidal circulatory abnormalities to be the primairy event in the pathogenesis of ARM.[75] Vascular insufficiency could lead to insufficient removal of waste products from the outer retina and to a disrupted supply of oxygen and nutrients.[76] Friedman proposes that AMD is the result of atherosclerotic changes in choroidal vasculature and deposition of lipids in Bruch's membrane.[77] Some authors argue that because in the macular area a great number of photoreceptor cells are located, characterized by a high energy turnover, even a minor compromise of blood flow and oxygen supply causes cellular hypoxia, leading to degeneration.[78,79] Studies in support of this theory demonstrated choriocapillary atrophy,[80] reduced choroidal arteries and reduced choroidal blood flow in ARM patients.[79,81-85] In contrast, other authors assume that the blood flow through the choriocapillaris is in excess of the amount required to nourish the retina,[86] and changes in choroidal blood flow may be secondary to changes in the RPE-Bruch's membrane complex.
Closely related to hypotheses on vascular abnormalities are theories considering Bruch's membrane as the primary lesion. They propose that depositions of neutral lipids in Bruch's membrane may cause hydrophobicity and predispose to detachment of the RPE and cause functional loss.[83,87] Also other depositions in Bruch's membrane such as cholesterol,[88,89] probably derived from the choriocapillaris, may impair the nutrient exchange along Bruch's membrane.
Yet another line of theory proposes that macular rod dysfunction (for instance a defected rim protein like ABCR) is the primary factor in AMD. This rod dysfunction in turn induces RPE dysfunction and ultimately cone photoreceptor death.[90] In the case of ABCR-mediated retinal degeneration, the defected rod rim protein causes RPE dysfunction because of excessive lipofuscin accumulation.[91,92] This theory is strengthened by the detection of mutations in the ABCR gene in some patients with AMD,[9] although the detected mutations may simply reflect polymorphisms found in normal healthy people.[93,94]

### 2.3.2 Early stages of Age-related Maculopathy

Morphologic changes of early stages of ARM include drusen, basal deposits, retinal pigment alterations and deterioration of Bruch's membrane. These changes have extensively been described in the theses of van der Schaft[95] and Kliffen.[96] They will be described here in short.

*Drusen*

Drusen are extracellular deposits situated between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane. It must be noted that the term 'drusen' raises confusion, because it is used in clinical and in histopathological setting, to describe a variety of deposits which differ morphologically, biochemically as well as ophthalmoscopically.[97] The morphology and biogenesis of drusen have recently been reviewed by Hageman.[98] It is beyond the scope of this thesis to discuss all types of drusen, however two types will be distinguished here.

**Hard drusen** usually appear fundoscopically as small yellow-white deposits with well-demarcated boundaries. Histologically, hard drusen are accumulations of homogeneous hyaline material along Bruch's membrane with attenuation of the overlying RPE (Figure 2.3A). Multiple hard drusen are recently acknowledged to be predictive of ARM progression[99] and even for development of AMD [van Leeuwen, personal communication].

**Soft drusen** are funduscopically seen as amorphous deposits with indistict borders, usually larger than 63 µm in size. Histologically, soft drusen appear as large drusen with sloping edges, containing less homogeneous membranous or fibrillar material[100] (Figure 2.3B). The overlying RPE is often attenuated and atrophic. In contrast with hard drusen, soft drusen are significant risk factors for developing late stage AMD.[101] Multiple drusen (5 or more), large drusen (sized larger than 63 µm), and confluence of drusen are associated with increased risk of progression to exudative AMD.[1,83,102] The origin of the drusenoid material is unclear. It may be derived from the RPE,[1,98,103,104] from the chorio-capillaris,[88,105,106] or both.[98] Recently a role for inflammation and immune-mediated processes in drusen biogenesis has been proposed.[98,107]

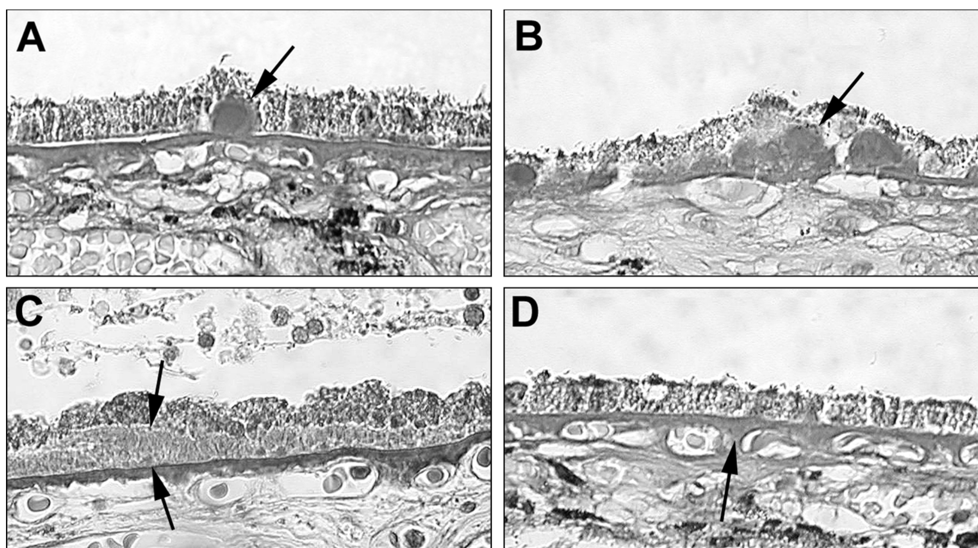


***Figure 2.3 Sub-RPE deposits.*** ***(A)*** *Hard drusen.* ***(B)*** *Soft drusen.* ***(C)*** *Basal laminar and/or linear deposits (in between arrows)* ***(D)*** *Thickening of Bruch's membrane with formation of intercapillary pillars (arrow). Mallory staining. Original magnification x400.*

*Basal deposits*

Two types of basal deposits are distinguished by electon microscopy: firstly basal laminar deposits which are localized between the basal cytoplasmic membrane of the RPE and its

basement membrane, composed of granular material with wide-spaced collagen.[108] The second type of deposits is called basal linear deposits. These deposits are located between the basement membrane of the RPE and the remainder of Bruch's membrane,[108] composed of granular and vesicular lipid-rich material. Basal linear deposits can appear similar to soft drusen, with the exception that they are not heaped up.[109] Both basal linear deposits and soft drusen provide a cleavage plane within Bruch's membrane which may facilitate the ingrow of choroidal neovascularization.[110] Basal laminar and linear deposits (BLD) are detectable by light microscopy (Figure 2.3C) but are clinically only detectable by secondary changes of RPE.[73] Basal deposits are positively associated with early ARM lesions and may be a significant indicator of progression to late AMD.[110,111] The origin of basal deposits is unclear. Several authors suggest that these deposits are released from the RPE via the basal plasma membrane.[110]

*Deterioration of Bruch's membrane*

With advancing age, the thickness of Bruch's membrane increases,[80,112,113] expanding between the choriocapillary vessels, the so-called intercapillary pillars (Figure 2.3D). Further changes include hyalinization, densification and calcification.[1] Thickening and hyalinization of Bruch's membrane appears to be caused by accumulation, predominantly in the outer collagenous zone, of coated membrane-bound bodies and of wide-spaced collagen.[114]

*Retinal Pigment Epithelium abnormalities*

Lipofuscin accumulates in the RPE with age as a byproduct of photoreceptor outer seg-ment phagocytosis.[1,115] Further RPE changes in ARM include attenuation of the RPE overlying drusen and BLD (Figure 2.3B), RPE atrophy, hypertrophy, hyperplasia and pigment clumping.[116] RPE hyper-pigmentation and hypo-pigmentation are significant inde-pendent risk factors for the development of exudative AMD.[117]

### 2.3.3 Geographic Age-related Macular Degeneration

Histopathologically, atrophic or geographic AMD involves choroidal atrophy, involution of the RPE, and involution of the adjacent photoreceptors and outer retinal layers in the macular region. At the edge of the area of atrophy pigment clumps accumulate.[2] In the absence of neovascularization, geographical atrophy probably is the natural end-result of ARM.[2]

It is currently unknown what factors determine the development of the disease towards either the geographic or to the neovascular form. In 42% of eyes histologically diagnosed with geographic atrophy, neovascularization was demonstrated,[2] although the fibrovascular invasion had not obscured the underlying choroid on clinical examination. The clinical picture is therefore determined by the extent of the neovascular response and different manifestations may occur in the two eyes.[2] In a histologic and morphometric study on geographic and neovascular AMD, no differences were detected in measured variables between eyes with the neovascular and geographic forms of AMD. This may indicate that the underlying pathophysiologic mechanisms are not different in these two AMD groups.[118]

### 2.3.4 Exudative Age-related Macular Degeneration

Exudative AMD is characterized by RPE detachment, choroidal neovascularization and disciform scarring. The presence of confluent soft drusen and BLD predisposes to a detachment of the RPE basement membrane from Bruch's membrane.[73] The RPE detachment often goes hand in hand with serous detachment of the neuroretina.

In neovascular AMD, vessels from the choroid invade Bruch's membrane and grow beneath the degenerating RPE or beneath the neural retina (Figure 2.4). Fibrovascular tissue proliferates, involving transdifferentiated RPE cells and inflammatory cells such as macrophages and (myo-)fibroblasts.[119] In later stages this proliferation leads to the formation of a fibrocellular disciform scar (Figure 2.5). Photoreceptor cells disappear rapidly in this stage. The formation of the disciform lesion is regarded as the end result of CNV and also as normal wound repair.[1,74,119,120] The newly formed vessels have a tendency to leak and bleed. Additionally, the normal blood-retinal barrier from the outer retinal blood supply, which is situated in the tight junctions of the RPE monolayer, is broken and thus the new vessels may give rise to serous detachments or hemorrhages.[86] CNV also occurs in other ocular diseases such as the presumed ocular histoplasmosis syndrome, posterior uveitis, multifocal choroiditis, ocular toxoplasmosis, birdshot chorioretinopathy, ocular sarcoidosis, rubella retinopathy, Vogt Koyonagi Harada syndrome, Behçet's disease and chronic uveitis.[78]

Neovascular AMD has a chronic inflammatory component.[78,121-124] CNV contains chronic inflammatory cells such as macrophages[121-123] and multinucleated giant cells which participate in the breakdown of Bruch's membrane and may provide an angiogenic stimulus for CNV.[78,124-127] It is possible that inflammatory changes are a result, rather than a cause, of the degenerative changes that subsequently lead to CNV. However, the occurrence of CNV in diseases in which the chorioretinal inflammation clearly precedes the degenerative changes, as it does in posterior uveitis, supports the hypothesis that the development of chorioretinal inflammation is a critical, late step in the pathogenesis of CNV in both ARM and posterior uveitis.[78]

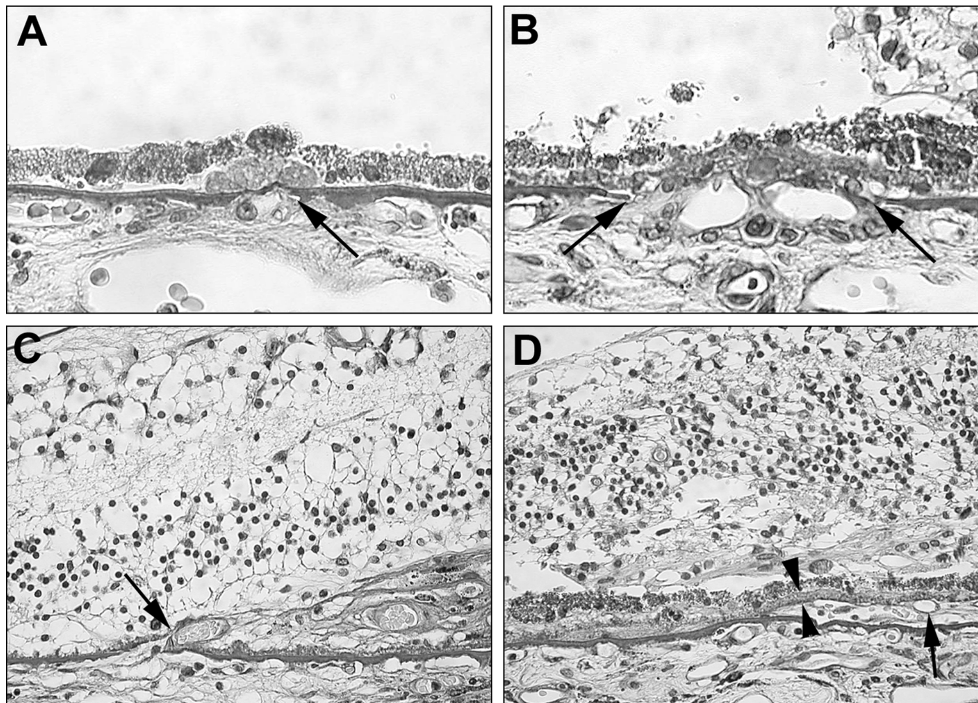


***Figure 2.4 Early choroidal neovascularization (A)*** *Thinning of Bruch's membrane below soft drusen, with a possible break through by a capillary.* ***(B)*** *Capillaries growing through a defect in Bruch's membrane (in between arrows) towards the RPE.* ***(C)*** *Capillary growing through a break in Bruch's membrane into a choroidal neovascularization.* ***(D)*** *Capillary (arrow) in a sub-RPE choroidal neovascularization. A layer of BLD is indicated by arrowheads. Mallory or PAS staining. Original magnification x400 (A-C) and x200 (D).*

**Classification of Choroidal Neovascularization**

Clinically, two types of CNV are distinguished based on the pattern at fluorescein angiography: classic CNV and occult CNV. [128] Classic CNV is characterized by an area of hyperfluorescence with well-demarcated boundaries on the early phase of fluorescein angiography. In occult CNV, the borders are usually poorly demarcated, and there is late leakage of undetermined source. Also mixtures of classic and occult CNV occur. Occult CNV covers up to 87% of all CNV associated with ARM.[129,130] Occult CNV can be visualized with indocyanine green videoangiography.[131,132]

Histologically, two different types of CNV can be distinguished[120,133] (Figure 2.6). Type 1 CNV is located beneath the RPE and is usually associated with ARM (Figures 2.5A and 2.6A). Type 2 CNV is present between the neuroretina and RPE (Figures 2.5B and 2.6B). This type is associated with focally destructive lesions affecting Bruch's membrane and the RPE, such as focal chorioretinal scars in ocular histoplasmosis syndrome.[120] In ARM, with decreased coherence of the RPE, Bruch's membrane and choriocapillaris, the CNV is more likely to develop between the RPE and Bruch's membrane (type 1). This in contrast to younger patients with an intact RPE-Bruch's membrane-choriocapillaris complex, who are more prone to develop a type 2 membrane. When proliferation of CNV associated with ARM continues, the subRPE

type 1 membrane may grow through the RPE into the subretinal space, resulting in a mixed pattern[120] (Figure 2.6C).

The correlation of the clinical and histological classifications is still not clear. Although with fluorescent angiography, the well-defined, classic type of CNV is more frequent in a subretinal type 2 membrane, this is not a reliable sign in differentiating the two types of membranes.[120]

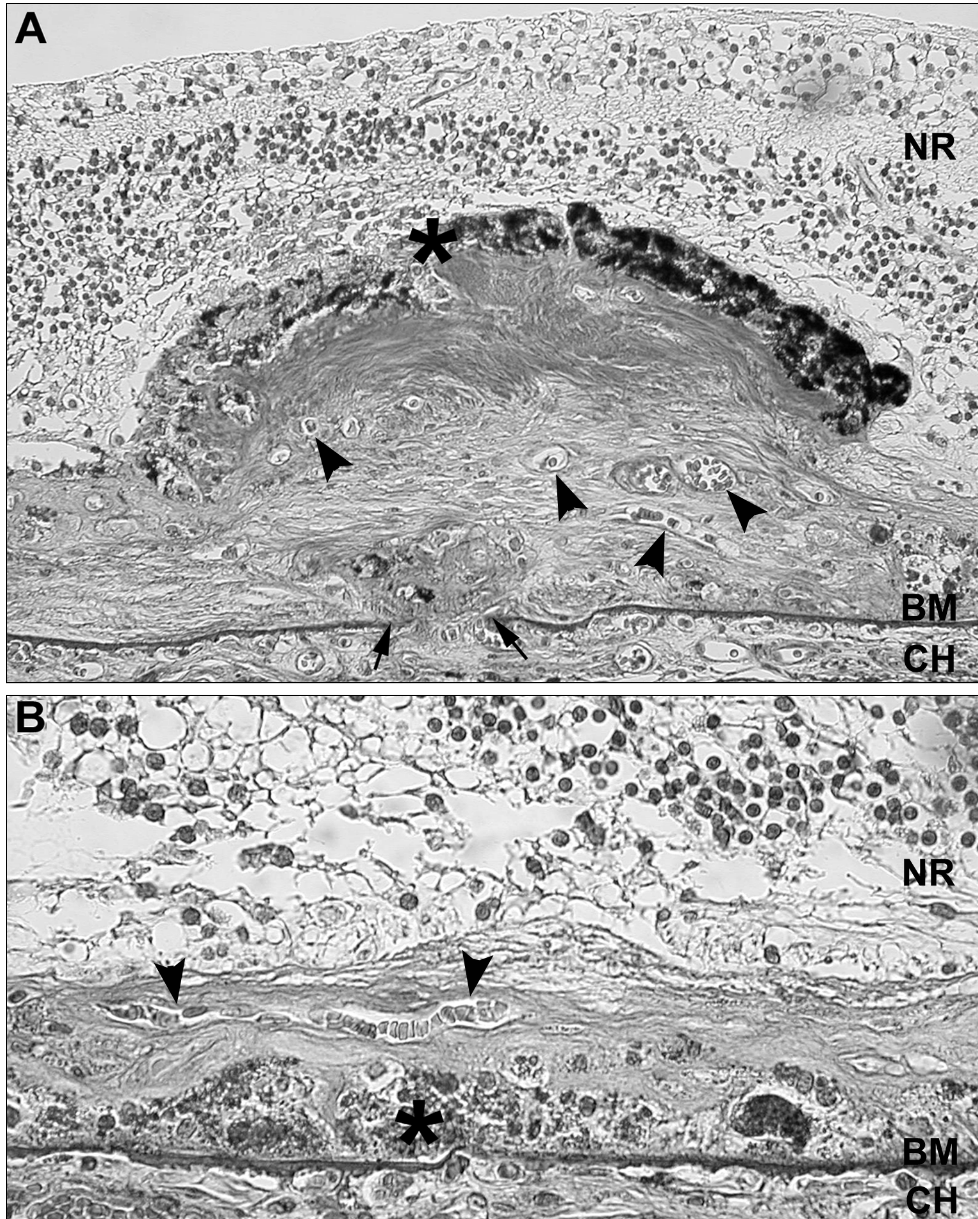


***Figure 2.5 Fully developed choroidal neovascularizations**. **(A)** Sub-RPE choroidal neovascularization. A large defect in Bruch's membrane may serve as the original site of the neovascularization. In the outer portion of the membrane several vital vessels (arrowheads) are seen, while the inner portion (darker area just below the RPE) the membrane has turned into a fibrocellular scar. The overlying neuroretina is disorganized, all photoreceptors have disappeared.**(B)** Subretinal choroidal neovascularization, with several capillaries containing erythrocytes (arrowheads). The overlying neuroretina is disorganized. RPE is indicated by an asterix. NR = neuroretina; BM = Bruch's membrane; CH = choroid. Mallory-staining. Original magnification x 200*

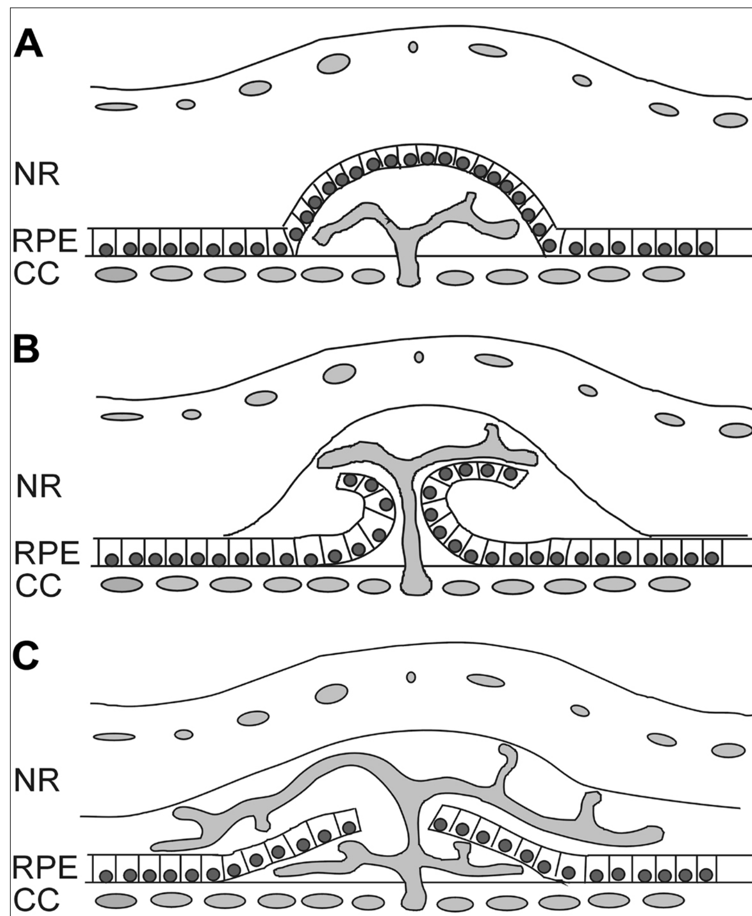


***Figure 2.6 Schematic histologic classification of CNV. (A)*** *Type 1 membrane, which is localized sub-RPE.* ***(B)*** *Type 2 membrane, with subretinal localization. The adherence of the RPE to Bruch's membrane is largely undisturbed.* ***(C)*** *Combined (mixed) membrane, partly localized sub-RPE with outgrow in the subretinal space.*
*NR = neuroretina; RPE = retinal pigment epithelium; CC = choriocapillaris. Adapted from: Grossniklaus H.E. and Gass, J.D.M., Clinicopathologic correlations of surgically excised type 1 and type 2 submacular choroidal neovascular membranes. Am J Ophthalmol 1998;126:59-69.*

**Growth Factors involved in Pathogenesis of Choroidal Neovascularization**
In the complex process of angiogenesis, vascular endothelial cells are activated to migrate and proliferate by angiogenic factors. The surrounding vascular basement membrane and the extracellular matrix are degradated by proteolytic enzymes (called matrix metalloproteinases), enabling proliferating vascular endothelial cells to migrate towards the stimulus and form sprouts. Sprouts then connect to form vascular loops, which are canalized to establish blood flow. During the last stage pericytes and smooth muscle cells are recruited to stabilize the new vessels, and the extracellular matrix is remodelled.[134]
Under physiological circumstances, the quiescent vascular homeostasis of the retina is regulated by a balance between naturally occurring pro-angiogenic factors and angiogenesis inhibitors.[134-136] Several factors, among which hypoxia,[137,138] oxidative stress,[139-

[141] and many still unidentified other factors, are capable of regulating growth factor expression. These factors may be capable of disturbing the natural homeostasis, thus allowing neovascularization. The definite pro-angiogenic action of growth factors is further dependent on extracellular matrix composition and the expression of receptors on target cells.[142,143] The provoking factors of angiogenesis in AMD are still unidentified. Hypoxia as a stimulus has been suggested by several authors.[74,144,145] A thick layer of BLD may serve as a barrier for oxygen diffusion and cause relative hypoxia in the outer retinal layers, inducing angiogenic factor release. Upregulation of several growth factors by reactive oxygen intermediates in the RPE and macrophages[139-141] may highlight oxidative stress as a provoking factor.

In the retina, many growth factors have been identified that are involved in the pathogenesis of neovascular retinal disease. Some of the most important factors will be discussed.

***Vascular endothelial growth factor (VEGF)***

VEGF is a endothelial-specific mitogen, which is capable of stimulating all major functions of endothelial cells in the process of angiogenesis: increased permeability, migration, proliferation, and tube formation.[119,142] Under physiological circumstances, VEGF has a low constitutive expression in the eye.[146] In the RPE, this probably functions as a trophic factor in the maintenance of the endothelial cells of the choriocapillaris.[119,146,147] VEGF is upregulated by multiple factors, including hypoxia,[137,148-152] several growth factors such as fibroblast growth factors (FGFs),[153,154] transforming growth factor-β (TGF-β)[155] and insulin-like growth factor-I (IGF-I),[156] prostaglandins,[157] alterations in the extracellular matrix,[158] and also by oxidative stress.[139]

VEGF appears to play a central role in neovascular AMD. VEGF protein and mRNA have been identified in histopathologic specimens of early and neovascular ARM.[144,145,159,160] Animal models in which VEGF is overexpressed in the RPE show choroidal[161-163] or intrachoroidal neovascularization.[164] Blocking of VEGF receptor kinases causes dramatic inhibition of CNV under experimental circumstances,[165,166] indicating that VEGF may be required for development of CNV. However, additional factors are probably needed.

***Insulin-like growth factor-I (IGF-I)***

IGF-I is a growth promoting polypeptide that has mitogenic and differentiating effects on many cell types. The diverse activities of IGF-I are mediated through binding and activation of the type I IGF receptor (IGF-IR). In the circulation and extracellular space, IGF-I is usually bound to one of the IGF-binding proteins (IGF-BP) [reviewed by Baxter[167]]. Six major IGF-BPs are discerned currently. The circulating IGF-I/IGF-

BP complex limits access of IGF-I to specific tissues and to the IGF receptor. IGFBP-3 is the most abundant in serum, and binds more than 95% of the IGF. Intravascularly, the IGF-I/IGFBP3 dimer forms a complex with the acid-labile subunit, resulting in a prolonged half-life of several hours. When released from this complex, IGF-I can enter target tissues with help of other binding proteins.[168] Furthermore most IGF-BPs have actions that are independent of IGF-I binding, including inhibition or enhancement of cell growth and induction of apoptosis. IGF-I and the IGFBPs are mainly produced by the liver, however they are also synthesized locally by most tissues, where they act in an autocrine or paracrine manner.[168]
In many situations on pathological growth, multiple components of the IGF system may be dysregulated.[169] IGF-I participates in each step of ocular neovascularization.[170] It is involved in the degradation of basement membranes and extracellular matrix proteolysis, and in vascular endothelial cell migration and proliferation.[171] IGF-I also increases RPE cell migration and proliferation in vitro.[172] In the eye, IGF-I can act as a direct angiogenic factor on vascular endothelial cells of the retina[173] and choriocapillaris,[174] or indirectly through increased VEGF gene expression of cultured RPE cells.[156] Intravitreous injection of IGF-I in animals produces preretinal neovascularization in rabbits[175] or microangiopathy resembling diabetic microangiopathy in pigs.[176] In mice, inhibition of IGF-I can decrease ischemia-induced retinal neovascularization.[177] Inhibition of IGF-I can be achieved by somatostatin analogues or by transgenic downregulation of growth hormone (GH). In addition, antagonists of IGF-IR suppress retinal neovascularization and reduces the retinal endothelial cell response to VEGF. This may suggest that IGF-I has a permissive role in VEGF-induced neovascularization.[178] The effect of IGF-I on choroidal neovascularization has not been studied so far.

***Somatostatin***

Somatostatin is a neuropeptide with a wide variation of activities in various tissues [reviewed by Patel[179]]. In the retina somatostatin functions as a neurotransmitter. Under pathologic conditions, somatostatin and its analogues inhibit ocular angiogenesis, indirectly by downregulation of growth hormone and IGF-I,[177] by inactivating the IGF-I mediated activation of IGF-IR,[180] and by inhibiting VEGF expression in RPE cells.[180] In addition, somatostatin and its analogues can inhibit angiogenesis directly,[181] possibly by activation of somatostatin receptors located on capillary endothelial cells.[173] In the treatment of diabetic retinopathy patients, the somatostatin analogue octreotide may retard progression of advanced diabetic retinopathy and may delay the time to laser surgery.[182] A pilot study in which patients with neovascular AMD were treated with octreotide, showed stabilization or minor deterioration of visual acuity in the majority of patients after 2 years.[183]

***Angiopoietin (Ang)-Tie2 system***

Tie2 is an endothelial cell-specific receptor which is thought to stabilize vascular integrity.[184] Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are ligands for the Tie2 receptor. Ang2 is a natural antagonist for Tie2.[185] The system is thought to play a role in pathologic angiogenesis in which VEGF is involved. Both hypoxia and VEGF selectively enhance Ang2 expression in retinal vascular endothelial cells while the expression of Ang1 and Tie2 remains stable. Ang2 is up-regulated in hypoxic retinas and neovascular vessels in vivo.[186]. In CNV, Ang2 and VEGF are both upregulated, and Tie2 is expressed in a variety of cell types, supporting a role of the interaction between VEGF and Ang2 in the pathogenesis of CNV formation.[187]

***Fibroblast growth factor (FGF)***

FGFs are a family of heparin binding proteins, with mitogenic, neurotrophic and angiogenic properties.[119,188-190] In the macula, FGFs have a constitutive expression.[119] Some FGFs are non-secreted factors, which probably have autocrine functions in the retina. FGF5 is a secreted protein with probable paracrine functions as well.[119] FGF has synergistic angiogenic activity with VEGF.[191-193] In neovascular AMD, several FGFs have been identified[145,189,190] and FGF is capable of inducing subretinal neovascularization in rabbits.[194]

***Platelet-derived growth factor (PDGF)***

PDGF stimulates formation of granulation tissue and is involved in wound repair.[119] This could explain increased PDGF expression in RPE underlying retinal detachment,[195] and in eyes with epiretinal membranes.[195,196] In eyes with neovascular AMD, expression is upregulated in the outer nuclear layer of the retina.[144] Since PDGF is highly growth promoting and chemotactic to RPE cells,[195,197,198] this upregulation could be attributed to its participation in RPE migration towards the inner retina, often seen in neovascular AMD.[144]

***Transforming growth factor-beta (TGF-β)***

Activated TGF-β inhibits endothelial cell proliferation. It is secreted by pericytes in a latent form, which is then activated by the vascular endothelial cell, emphasizing the important role of pericytes in maintaining vascular quiescence.[134] The role of TGF-β in the process of angiogenesis is still controversial. TGF-β has been postulated to be an inhibitor of ocular angiogenesis,[199,200] however also pro-angiogenic functions have been attributed to this cytokine.[135,143] The angiogenic actions of TGF-β are indirect by modulating expression of other angiogenic factors such as VEGF,[155,197,201] or by recruitment of inflammatory cells, which in turn produce positive regulators such as VEGF.[143,202] In early ARM[144] and in neovascular AMD,[189,203] TGF-β expression is upregulated.

***Pigment Epithelium-Derived Factor (PEDF)***

PEDF is a neurotrophic factor[204] and one of the most potent inhibitors of ocular angiogenesis,[135] produced in the RPE.[204,205] PEDF is probably responsible for the physiological avascularity of the cornea and vitreous.[135] The amount of PEDF produced by retinal cells is positively correlated with oxygen concentration, suggesting that loss of PEDF plays a permissive role in ischemia-driven retinal neovascularization.[135] PEDF inhibits aberrant blood vessel growth in mouse models of ischemia-induced retinopathy,[206,207] and in experimental CNV.[207] This angiogenesis inhibiting effect is thought to be caused by induction of apoptosis of activated endothelial cells.[206]

***Angiostatin***

Angiostatin is a potent inhibitor of angiogenesis, selectively inhibiting endothelial cell proliferation.[208] It is composed of an internal fragment of plasminogen.[208] Angiostatin reduces neovascularization size in experimental rat CNV,[209] and prevents retinal neovascularization in a mouse model of retinopathy of prematurity without affecting physiological angiogenesis.[210] It is suggested that local release of angiostatin is one of the mechanisms that mediates the therapeutic effect of retinal photocoagulation in proliferative diabetic retinopathy.[211]

## 2.4 THERAPEUTIC MODALITIES

Therapies for AMD are mainly focussed on patients with neovascular AMD. Only studies on antioxidant vitamins and cofactors for antioxidant enzymes such as zinc also address early ARM. A recent report demonstrated a modest effect for antioxidant vitamins E, C and A in combination with zinc in preventing progression from early ARM to advanced AMD, particularly to neovascular AMD.[12]

Two treatment modalities with acknowledged beneficial effect on neovascular AMD are laser treatment[128,212] and photodynamic therapy.[213,214] Laser treatment reduces the risk of visual acuity loss, however, only a small group consisting of patients with classic CNV are eligible for laser therapy. Patients with subfoveal CNV experience an immediate central scotoma due to irreversible retinal and choroidal damage. Recurrences of CNV frequently occur, often within 1 year of laser treatment.[215] It is unclear in these cases whether the laser treatment is inadequate or whether the recurrent CNV consists of a new neovascularization.[108]

Photodynamic therapy (PDT) is a promising newly developed treatment modality, combining laser with light-sensitive drugs, intended to achieve isolated vessel occlusion. PDT also is most effective on patients with classic CNV, and multiple consecutive treatments are required.[213,214]
Radiotherapy is one of many experimental treatments for neovascular AMD. Varying results have been published with a variety of techniques and dosage-schemes, but a recently performed pooled analysis of different studies indicated that radiotherapy with higher dosages may only act to slow or delay the progress of the disease.[216]
Surgical treatments including surgical excision of the CNV and retinal rotation are still in an experimental stage. In general, surgical treatment does not improve vision in patients with AMD, but may be effective in patients with other causes of CNV.[120,217]
Currently a variety of trials on anti-angiogenic drugs to attack neovascular AMD is underway. Angiogenesis inhibitors that could be valuable against CNV are given in Table 2.1. Because of the multifactorial origin of vascular growth in CNV, inhibition of more than one growth factor is probably essential for a definite effect.

***TABLE 2.1 ANGIOGENESIS INHIBITORS POSSIBLY USEFUL IN NEOVASCULAR AMD***

| Angiogenesis inhibitor | Trials | Comments |
|---|---|---|
| angiostatin | | using gene-therapy |
| matrix metalloproteinase-inhibitors | | prevent enzymatic degradation of extracellular matrix |
| interferon-2α | | not effective in neovascular AMD |
| thalidomide | multicentre trial | no results yet |
| monoclonal antibodies | | against endothelial cell markers [218] or integrins |
| antisense-oligonucleotides against VEGF | phase II multicentre trial | prevents translation of mRNA into proteins [219,220] |
| steroids | uncontrolled pilot study of intravitreal triamcinolone | probable beneficial effect in neovascular AMD |
| somatostatin-analogues | randomized, double blind trial using octreotide | pilot study was promising in stabilizing visual acuity |

# CHAPTER 3

## AIM OF THE THESIS

Although the morphology of ARM has been described in detail, the pathogenesis is still poorly understood. Several important questions remain to be answered in order to develop new prevention and treatment strategies.
Although the most striking associated factor for ARM is age, it is still not clear whether ARM is an exaggeration of the normal aging process (in other words an advanced stage of a deteriorative process that takes place in all eyes[1]), or a fundamentally different disease-entity. All major signs of ARM increase with advancing age, but only in some individuals they progress to the stage of functional loss or cell death.[1] A well-known mechanism of cell death and subsequent atrophy is apoptosis. To address this topic, we studied the presence of apoptosis in the aging retina in the second part of this thesis. The RPE appears to play a vital role in the development of ARM. The expression of the apoptosis–regulating protein Fas-ligand on RPE is hypothesized to have an inhibitory effect on human neovascularization[64] by inducing apoptosis of active vascular endothelial cells. Therefore, we investigated whether Fas-ligand expression on RPE cells is associated with the stage of ARM and with age.
Focussing on neovascular AMD the question remains what factors trigger neovascular capillaries to develop from the choroid. It is acknowledged that growth factors are important in initiation and development of CNV. VEGF seems to play a central role in neovascular AMD,[144,145,159-166] however other growth factors are probably required in addition. Thus, in the third part of this thesis, we focus on the Insulin-like Growth Factor pathway in neovascular AMD. IGF-I is associated with ocular angiogenesis in animal models,[177,178] it has direct angiogenic effects,[170,173] and modulates the expression and effects of VEGF[156,178] Finally, in order to assess the effects of treatments, we describe the histopathological findings of radiotherapy on neovascular AMD.

# PART II

# MOLECULAR ASPECTS OF THE AGING RETINA

# CHAPTER 4

# APOPTOSIS IS PRESENT IN THE PRIMATE MACULA AT ALL AGES


## ABSTRACT

**Purpose:** It has become increasingly clear that apoptosis is a main event in photoreceptor cell death in a variety of retinal degenerations. We investigated the role of apoptosis in the physiologically aging primate macula.

**Methods***:* Twenty maculae of rhesus monkeys, aged 6 to 34 years, were investigated. Apoptosis was determined in formalin-fixed, paraffin-embedded eyes using the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method and quantitatively analyzed. Morphology of TUNEL positive cells was studied by confocal laser microscopy and transmission electron microscopy. The thickness of the outer nuclear layer (ONL) was determined by image analysis. Furthermore, expression of apoptosis-regulating proteins Bcl-x, Fas and Fas Ligand was studied by immunohistochemistry.

**Results:** TUNEL positive nuclei showed apoptotic features on confocal laser microscopy. They were scattered and sparsely found in the macula, most frequently in the ONL. The thickness of the ONL decreased with increasing age. Apoptosis was found equally distributed at all ages, although in the two oldest maculae up to 13 times more apoptosis was found. Expression of Bcl-x, Fas and Fas Ligand was equal at all ages.

**Conclusion:** Our findings indicate that apoptosis in the primate macula occurs at all ages at similar rate, possibly increasing in the oldest age group, and may account for the decreasing thickness of the primate macula with age.

## INTRODUCTION

The thickness of the human retina decreases with advanced age, due to loss of photoreceptors and ganglion cells.[26] Photoreceptor loss occurs in atrophic age-related macular degeneration (AMD) as well as in the more severe neovascular form.[90] Apoptosis was demonstrated in 4 out of 16 cases of AMD, neovascular as well as atrophic.[55] Therefore, it was hypothesized that apoptosis is involved in photoreceptor degeneration in AMD.[55] In animal models of retinal degeneration, photoreceptor cell death occurs by apoptosis.[43-45,221-223]

It has become increasingly apparent that apoptosis, a cell suicide program, plays a crucial role in many physiologic processes, such as embryonic development and homeostatic maintenance of tissues, and in many disease processes, for instance malignant tumors. The process of apoptosis is under genetic control and can be initiated by an internal clock (programmed cell death), or by extracellular agents such as hormones, cytokines, natural killer cells, and a variety of chemical, viral, and physical agents. During apoptosis, individual cells show nuclear and cytoplasmic condensation and biochemical analysis reveals internucleosomal DNA fragmentation.[34,35,37]

Apoptosis-regulating genes in the retina have been studied extensively in experimental conditions. The Bcl-2 family consists of many proteins, both inhibitors and stimulators of apoptosis. The apoptosis-protective protein Bcl-2 is expressed widely in the developing neuronal system but downregulated in the adult neuronal system.[58] Bcl-x is the predominant Bcl-2 family member in postnatal neural tissues[57] and adult rat retina.[59] Overexpression of Bcl-2 or Bcl-x is protective for photoreceptor cell death in rd mice.[60] Fas (CD95) receptor mediates apoptosis when triggered by agonistic antibodies or its ligand, Fas Ligand (FasL).[62] Fas and FasL are expressed in normal human retina.[54,64,224,225]

The ocular fundus of humans and rhesus monkeys is almost identical.[226,227] Aged maculae of rhesus monkeys have clinically and histologically detectable pathology associated with age related maculopathy (ARM). Clinically, macular drusen (6 to 74%) identical to human drusen, and alterations of the RPE (18 to 45%) have been demonstrated.[228-232] The maximum life span of the rhesus monkey is one third of humans implying that relative aging occurs three times more rapidly.[227]

The aim of this study was to ascertain whether apoptosis plays a role in the physiologically aging primate macula.

## MATERIALS AND METHODS

The animals were procured, maintained and used in accordance with the Dutch law and regulations, the Animal Welfare Act and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources-National Research Council (USA).

*Experimental animals*

Twenty rhesus monkeys (*Macaca mulatta*) studied were obtained from the Department of Clinical Oncology, University of Leiden, from the project 'Late effects of total body irradiation in rhesus monkeys'.[233,234] The animals used in this study belonged to the non-irradiated control group and were aged 6 to 34 years.

*Tissue preparation and histopathology*

The eyes of the monkeys were enucleated immediately post mortem and were processed as described before.[235] In short, a portion of the retina of approximately 1 $cm^2$ containing the macula was sectioned and horizontally divided in two parts. One part was fixed by immersion in formaline for 24 hours. After embedding in paraffin, sections through the centre of the macula were cut at 5 µm thickness and were mounted on silanized glass slides. For light microscopy, sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff and Mallory. The maculae were histologically examined by light microscopy for signs of aging and early macular degeneration, i.e. thickening of Bruch's membrane, basal laminar deposits (BLD), drusen and RPE abnormalities.[112,236]

*TUNEL staining*

To identify apoptosis, we used the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine (dUTP)-biotin nick end labeling (TUNEL) method, which labels the fragmented DNA ends with biotinylated poly(dU). Paraffin sections, within 1 mm of the foveola, were used for TUNEL staining, according to the method of Gavrieli et al.[39] with the following modifications. After deparaffinization and rehydration, the slides were pretreated with 20 µg/ml DNase-free proteinase K (Gibco Life Technologies, Breda, Netherlands) during 10 minutes at 37ºC. As a positive control one slide was treated by DNase I (Promega, Madison, WI, USA) dissolved in a DNase buffer (30 mM Trizma-base, 140 mM cacodylate, 4 mM $MgCl_2$, 0.1 mM dithiotreïtol, pH 7.2) during 1 hour at 37ºC or overnight at 4ºC to induce chromosomal breaks. Sections were washed in TdT buffer (0.5 M cacodylate, 1 mM $CoCl_2$, 0.5 mM DTT, 0.15 M NaCl, 0.05% bovine serum albumin, pH 6.8) and incubated for 3 hours in a mix containing 25 µl TdT buffer, 5 U TdT (Promega) and

0.5 nmol biotin-16-dUTP (Boehringer Mannheim, Germany). As a negative control, TdT enzyme was omitted. Labeling was done using biotinylated multilink antibodies followed by streptavidin-labeled alkaline phosphatase (Biogenex, San Ramon, CA, USA). New Fuchsin was used as chromogen, and the slides were counterstained with Mayer's hematoxylin. The number of TUNEL-stained nuclei was quantified in four random slides per macula by image analysis.

*Confocal laser microscopy*
To examine morphology, TUNEL-positive nuclei and apoptotic bodies were localized exactly in TUNEL-stained maculae by computer. Thereafter the slides were unmounted and stained with 5 ng/ml propidium iodide (Sigma, Steinheim, Germany) in Vectashield (Vector, Burlingame CA, USA). The TUNEL-positive nuclei were relocalized and examined using a confocal laser scanning microscope with a He/Ne-laser at 543 nm as an excitation light source. A long pass filter (> 570 nm) was used for the detection of propidium iodide emission light.

*Electron microscopy*
Another part of the macula was embedded for electron microscopy. The tissue was immersed in 4% paraformaldehyde and, after dehydration, embedded in Lowicryl (Aurion, Wageningen, Netherlands). The blocks were polymerized at -35ºC under ultraviolet light. Ultrathin sections were cut and stained with uranylacetate 6% and lead citrate. The sections were mounted on grids and examined for features of apoptosis using a transmission electron microscope.

*Image analysis*
Digital microscopic images consisting of 512×512 pixels (0.43 μm/pixel) of each section were recorded with a 40× objective using a Zeiss Axioplan Microscope (Zeiss, Oberkochen, Germany) equipped with a Sony DXC-930P 3-chip color CCD videocamera (with a 0.45× lens). The measurements and estimations were performed with a semi-automatic digital image analysis procedure (software: KS400, Kontron, Germany).
To determine the percentage of TUNEL-positive nuclei, the total numbers of nuclei present in the ONL and INL were estimated in the following way: 1) the mean surface area of individual nuclei that could be identified automatically by the image analysis software was measured ($A_{mean\ nucleus}$); 2) the total nuclear surface area of all nuclei, including the nuclei that were not segmented by the software ($A_{total}$), was measured, and 3) the total number of nuclei ($N_{total}$) was estimated as: $N_{total}=A_{total}/A_{mean\ nucleus}$. The number of TUNEL-stained nuclei per 1000 nuclei in the ONL and INL was recorded.
To relate thickness of the macula to age, we chose a section in the perifoveal region of the slides. Because the INL width has a large variance along the foveolar -

parafoveolar - perifoveolar region [237], we restricted this part of the study to the ONL width. We standardized according to the number of ganglion cells, to exclude any possible regional anatomic variation. We ensured that the plane of section was truly axial along the length of the rod inner segments. Therefore, five maculae had to be excluded. The ONL width was measured from the outer limiting membrane to the innermost cell of the ONL.

*Immunohistochemistry*

Expression of Bcl-x, Fas and FasL was determined as follows. Paraffin slides were deparaffinized and rehydrated. For the Fas antibody, antigen retrieval was performed (pronase treatment for 10 minutes at 37ºC). Incubation was performed with polyclonal rabbit anti-human antibodies against Bcl-x and FasL (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and monoclonal mouse anti-human antibodies against CD95 (Fas) (Immunotech, Marseille, France) for 1 hour at room temperature. Labeling was done using biotinylated multilink antibodies followed by streptavidin-labeled alkaline phosphatase (Biogenex, San Ramon, CA, USA). New Fuchsin was used as chromogen. The slides were counterstained with Mayer's hematoxylin, and examined by light-microscopy. Negative controls for immunohistochemistry included (1) omission of the primary antibody, (2) use of irrelevant antibodies of the same isotype, and (3) preabsorption of the Fas and FasL antibodies with a tenfold concentration of the immunizing peptide for 4 hours.

*Statistical analysis*

To analyse the data, Spearman's correlation was used. A value of $P<0,05$ was considered significant.

## RESULTS

*Histopathology*

BLD and soft drusen were not found in any of the maculae. Hard drusen were noted in 8 maculae. The correlation ($r_S=0.57$) between number of drusen and increasing age was significant ($P=0.01$). Diffuse thickening of Bruch's membrane with intercapillary pillars was noted in one macula from a monkey 21 years of age.

*TUNEL staining*

All maculae were included except for two that after processing did not contain a retina (n=18). TUNEL-stained nuclei, indicating apoptosis, were observed sparsely in the

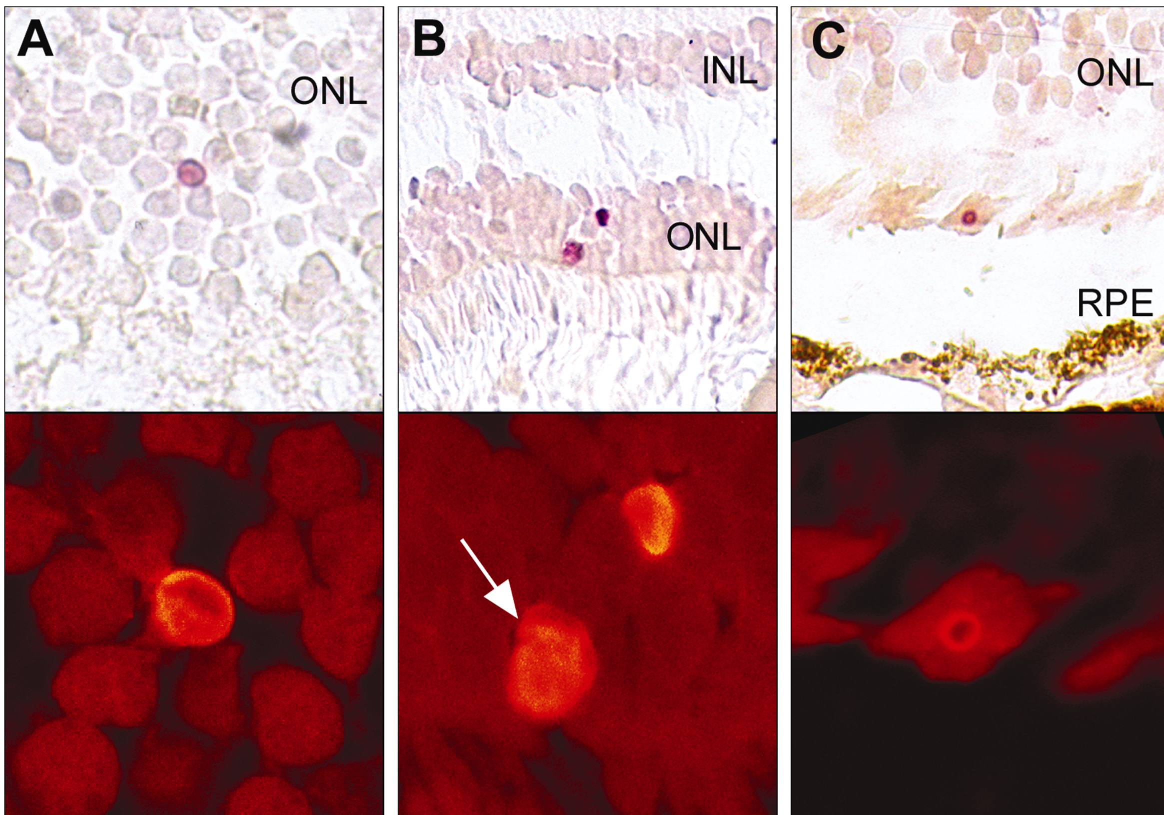


***Figure 4.1*** ***Apoptosis in rhesus monkey macula****. In-situ 3'end labeling of apoptotic DNA by alkaline phosphatase detection (upper row) and confocal laser microscopy images of same cells (lower row). (****A****) TUNEL-positive nucleus in ONL of 8-year-old monkey. Nucleus appears shrunken in relation to surrounding cells, and nuclear material appears condensed in periphery of nucleus. (****B****) TUNEL-positive nuclei in ONL of 34-year-old monkey. One nucleus shows budding (arrow). (****C****) Apoptotic body in the photoreceptor layer of 18 year old monkey. ONL = outer nuclear layer; INL = inner nuclear layer, RPE = retinal pigment epithelium. Original magnification upper row, 400×; estimated magnification confocal images, 1300× – 1800×.*

nuclear layers of the retina but were most numerous in the ONL (Figure 4.1A, B). TUNEL-stained nuclei were also found in the photoreceptor layer, as well as small labeled particles, consistent with apoptotic bodies[238] (Figure 4.1C). The red-colored apoptotic bodies were clearly distinguishable from RPE pigment. In four maculae TUNEL-stained nuclei were sporadically found in the RPE. The negative controls did not stain, and positive controls showed adequate labeling of DNA fragments.

*Confocal laser microscopy*

Confocal microscopy of TUNEL-stained nuclei revealed the presence of condensed nuclei with morphologic features of apoptosis: condensation of nuclear chromatin, cell body shrinkage and cell budding (Figure 4.1).

*Electron microscopy*

In the ultrathin sections of the macula of the oldest monkey we found focally in the ONL a cell, showing phagocytosis of condensed material, consistent with nuclear chromatin (Figure 4.2).

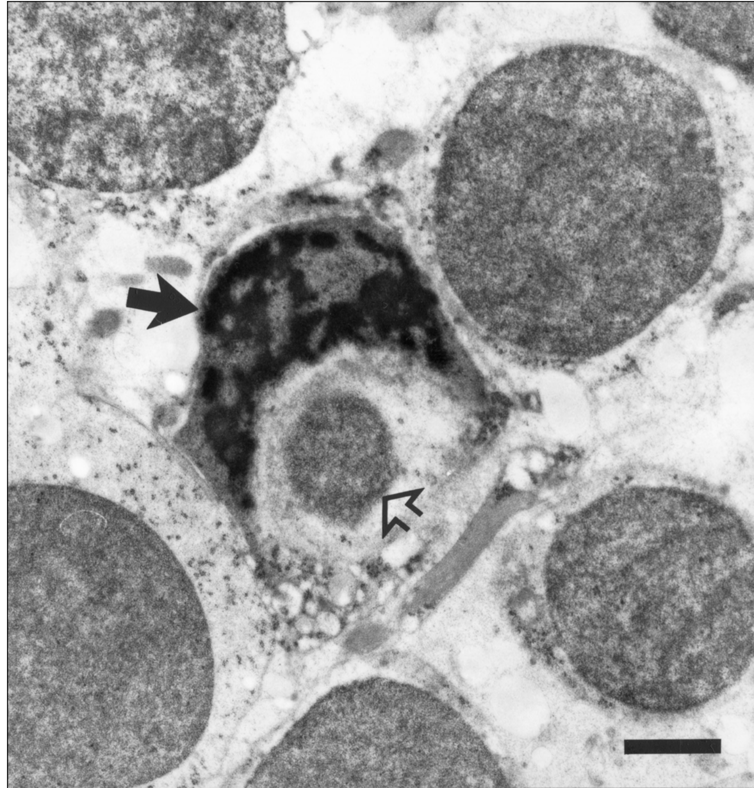

***Figure 4.2*** ***Transmission electron microscopy*** *of nuclei in the ONL of the oldest monkey (34 years of age) with phagocytosis of condensed nuclear material (filled arrow) by a neighboring cell. The open arrow indicates the original nucleus of the cell. Scale bar represents 1,59 μm.*

*Image analysis*

TUNEL-positive nuclei in the ONL were found at a rate of 0 to 0.53‰ of the total number of ONL nuclei in each section. TUNEL staining was found equally distributed at all ages, although in the two oldest maculae (32 and 34 years of age) 6 and 13 times more positive nuclei were found in the ONL, respectively (corrected for the amount of nuclei in the ONL) (Figure 4.1B). We found a non-significant correlation of $r_S$ = 0.14 for the ONL and $r_S$ = 0.10 for the INL between TUNEL staining and increasing age (ONL: $P$ = 0.59, INL: $P$ = 0.69; Spearman's correlation coefficient) (Figure 4.3). The thickness of the ONL decreased significantly with increasing age ($r_S$ = -0.56; $P$ = 0.029) (Figure 4.4).

*Immunohistochemistry*

Expression of Bcl-x was observed in the RPE, in the outer limiting membrane, and in nuclear and plexiform layers, particularly in the outer plexiform layer (Figure 4.5A). Strong expression of Fas was found in ganglion cells and INL, less strong expression in the RPE and variously in choriocapillaris and choroidal vessels (Figure 4.5B). FasL expression was found throughout the neuroretina, in the RPE, and variously in the choriocapillaris (Figure 4.5C). In negative controls, no staining or slightly aspecific

staining was seen (Figure 4.5D, E, F). Expression of Bcl-x, Fas, and FasL in the neuroretina was similar at all ages.

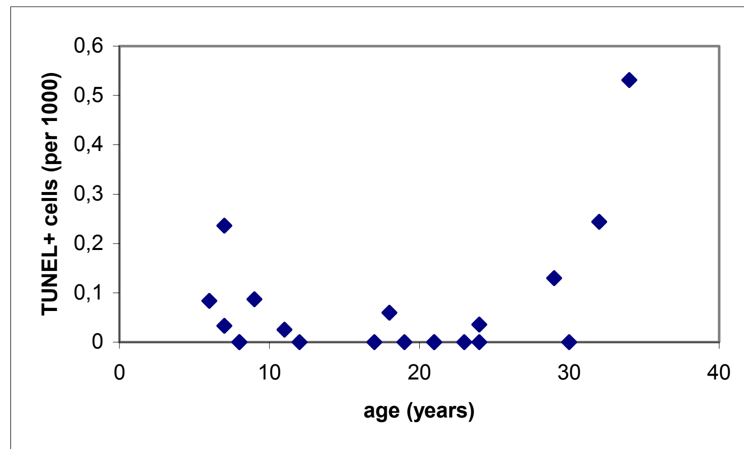


***Figure 4.3*** *Relationship between TUNEL-stained nuclei in ONL and aging, in years. Number of apoptotic nuclei is quantified per 1000 nuclei of ONL.*

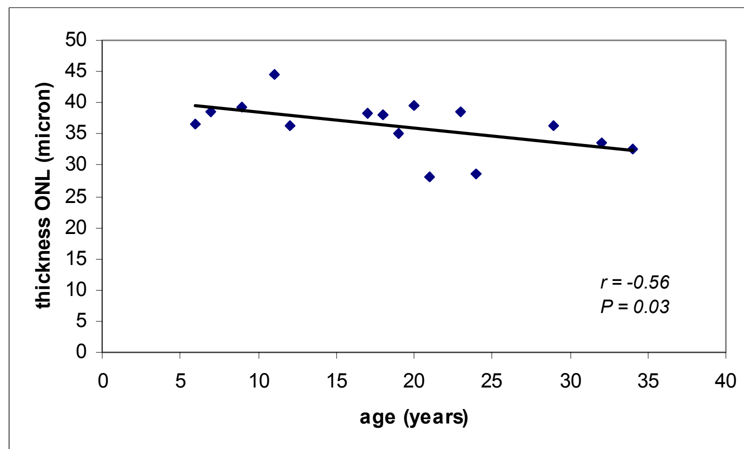


***Figure 4.4*** *Relationship between age and thickness of ONL (in micrometers) in rhesus monkey, indicating that thickness of the macula decreases with increasing age.* $r_S = -0.56$, $P = 0.03$ *(Spearman's correlation).*

## DISCUSSION

We found that apoptosis in the primate macula occurs at all ages at similar rate, even in the youngest age group. In the 2 oldest maculae (>32 years) we found approximately 6 and 13 times more apoptotic nuclei, respectively. However, a significant positive correlation of apoptosis with increasing age could not be demonstrated. This may be due to the relatively small number of aged maculae. Moreover, the occurrence of apoptosis in postmitotic tissues should be a rare event.

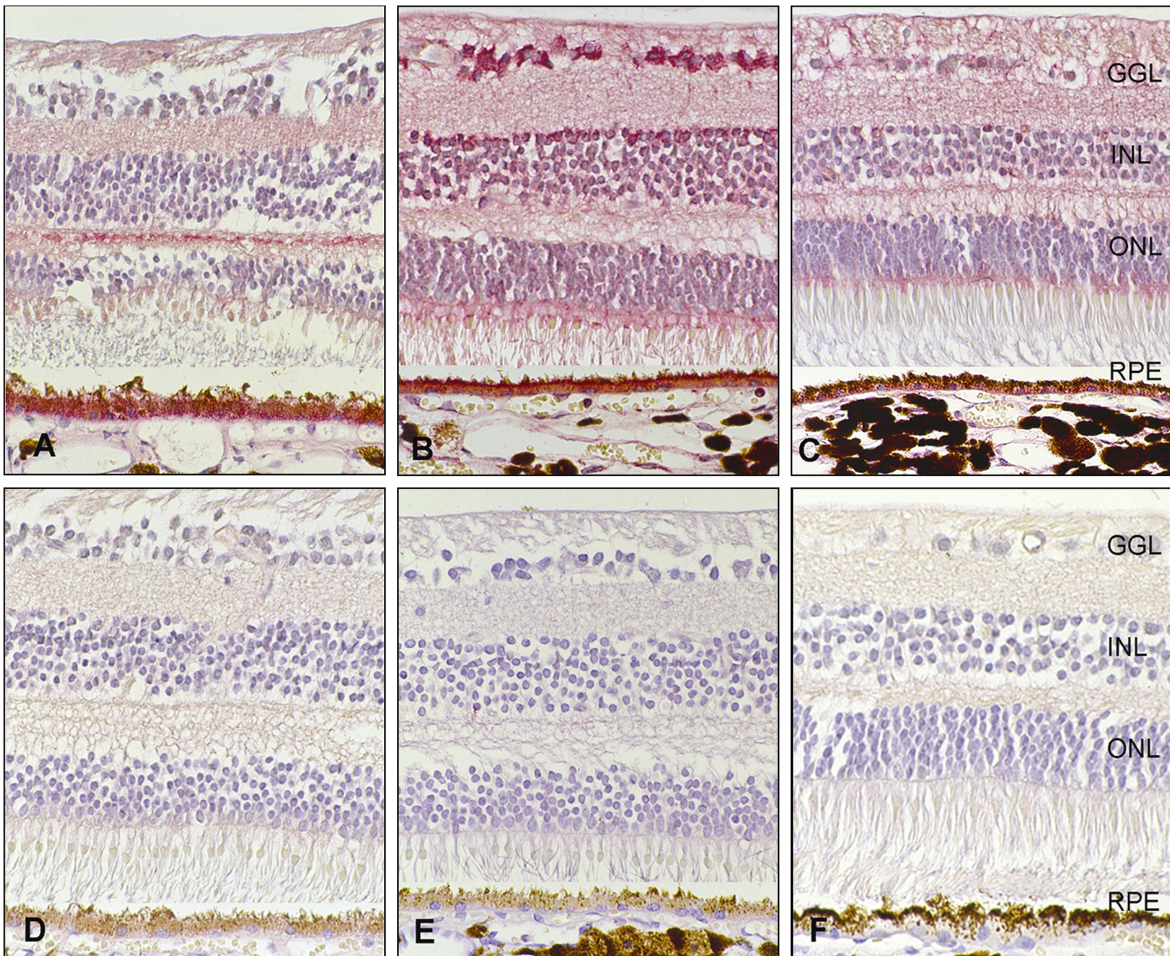


*Figure 4.5* ***Immunolocalization of apoptosis-regulating proteins*** *in rhesus monkey macula. Immunohistochemistry was performed on paraffin-embedded tissue and visualized with an alkaline phosphatase system using a red chromogen. Expression of Bcl-x* ***(A)****, Fas (CD95)* ***(B)****, and FasL* ***(C)****. Negative control staining using irrelevant polyclonal rabbit IgG antibodies* ***(D)****, irrelevant monoclonal mouse $IgG_1$ antibodies* ***(E)****, and peptide blocking of the anti-FasL antibodies* ***(F)****. GGL = ganglion cell layer, INL = inner nuclear layer, ONL = outer nuclear layer, RPE = retinal pigment epithelium. Original magnification ×400*

With confocal laser microscopy, we demonstrated features of apoptosis, such as nuclear chromatin condensation, cell shrinkage and cell budding in TUNEL stained nuclei. Therefore, we assume that TUNEL positive nuclei in our study represent apoptosis.

The apoptotic process is accomplished quickly[34] and the period through which dying cells can be revealed by the TUNEL method is also relatively short: it is estimated at about 10 hours in rat retinal ganglion cells.[51] Thus, because of the short duration of apoptosis a relatively low incidence of histologic signs of apoptosis can indicate a considerable rate of cell loss.[239] Our findings of constant levels of apoptosis in the ONL might explain the decrease of ONL thickness with increasing age. These findings are in concordance with decreasing of thickness of the human retina with age,[26,27] although a different method was applied in those studies.

Apoptotic cells break up in membrane-bound fragments, so-called apoptotic bodies, that are phagocytosed by neighboring cells and induce no inflammatory response.[34,35,37] On electron microscopy we found phagocytosis of condensed nuclear material by a neighboring cell. Furthermore, we found some apoptotic bodies in the photoreceptor layer by confocal laser microscopy. Therefore it is conceivable that some apoptotic bodies migrate from the ONL and are phagocytosed by the RPE. Apoptosis in RPE cells was a rare event at all ages. However, with a small number of RPE cells present per slide, this result can indicate RPE cell loss by apoptosis. This is in accordance with the observations of decreased RPE cell density in the macular area with age.[27,240] In vitro, cultured RPE cells can be triggered to undergo apoptosis by a variety of agents, such as oxidative stress[25] and lipofuscin components.[241]

Apoptosis in the younger age group may partly be explained by a continuation of the apoptotic process, responsible for the death of redundant cells during development of the retina.[42,242] Another explanation may be that apoptosis resulting from external stimuli is already present at young age and functions in order to remove damaged or dysfunctional cells. Our findings are in concordance with findings of apoptosis in control monkey retinas in other studies[52] and indicate that some apoptosis of the retina occurs as part of normal aging. The equal protein expression of the apoptosis-regulating genes *Bcl-x, Fas* and *FasL* at all ages is in accordance with the steady amount of apoptosis during the aging process.

The apoptotic rate increases in the oldest age group and in maculae with signs of ARM, possibly under influence of other stimuli, internal as well as external. Internal stimuli may be genetic predisposition, as is shown in colonies of rhesus monkeys with high rates of ARM.[229] Likewise, in humans there is evidence for genetic predisposition in AMD.[7,9] A number of external stimuli may be postulated. Hypoxia and ischemia of the outer retina are thought to contribute to the development of AMD.[144] In a recent study on retinal ischemia in rats, apoptosis appeared as late component of neuronal death.[67] Environmental factors such as light are assumed to play a role in retinal degeneration. In albino rats some of the damage inflicted by light may result in apoptosis of retinal photoreceptor cells. [243,244] Apoptosis is also induced by ultra violet light damage.[245] In our study, the investigated rhesus monkeys, being captive, were underexposed to ultraviolet light, and were not exposed to other known risk factors for ARM,[3] although atherosclerosis was present in the older monkeys. In rhesus monkeys, end stage AMD is rare.[231] This may reflect either a slower degenerative process, or may represent an environmentally selective phenomenon.[228] Furthermore, we did not find any BLD in the maculae of these monkeys, which is consistent with findings of other studies.[103,228,231,232] This might implicate a degenerative process in the rhesus monkey eye somewhat different from that in the human eye.

In summary, we found that apoptosis occurs at similar rates at all ages in the primate macula. The process of apoptosis may account for the decreasing thickness of the ONL with age.

## ACKNOWLEDGEMENTS

The authors thank Frieda van der Ham, Kees Vissers, Ton de Jong and Alex Nigg for technical assistance, Frank van der Panne for photography and Theo Stijnen for statistical advice.

# CHAPTER 5

# ROLE OF FAS-LIGAND IN AGE-RELATED MACULOPATHY NOT ESTABLISHED

## ABSTRACT

**Purpose:** Fas-ligand (FasL) expression on retinal pigment epithelium (RPE) is hypothesized to have an inhibitory effect on human ocular neovascularization.

**Methods:** We studied FasL expression in the aging RPE and in early and late stages of age-related maculopathy (ARM). Immunohistochemistry with antibodies against FasL was performed on paraffin-embedded sections of 23 human eye bank eyes (aged 45 to 96 years) and 12 eyes with neovascular AMD.

**Results:** FasL expression in RPE was not related to age or to the presence of early ARM. Furthermore, FasL expression in RPE was similar in subretinal and sub-RPE choroidal neovascular membranes (CNVM).

**Conclusions:** It appears to be unlikely that FasL expressed on RPE controls the extension of CNVM from sub-RPE to subretinal.

## INTRODUCTION

Age-related maculopathy (ARM) is the major cause of blindness in people over 65 years in the Western world.[3] Late stages of ARM, also called age-related macular degeneration (AMD), include geographic atrophy and neovascular macular degeneration. The neovascular form is characterized by choroidal neovascular membranes (CNVM). In CNVM new vessels grow beneath the retinal pigment epithelium and the retina from the underlying choroid. Many growth factors have been identified that might influence angiogenesis in CNVM, such as VEGF, bFGF and somatostatin.[145,159,160,189,190,246-248]
Recently, the role of Fas and its natural ligand, Fas-ligand (FasL) has been acknowledged in the process of angiogenesis.[249] Fas and FasL are important for apoptosis in T-lymphocytes but are also expressed on non-lymphoidal tissue. In the eye Fas-FasL interactions appear to be an important mechanism for the maintenance of immune privilege by inducing apoptosis of invading lymphocytes.[63] Kaplan and coworkers[64] studied the role of FasL in surgically excised CNVMs of patients with AMD. They demonstrated FasL-positive RPE cells in close proximity to and surrounding Fas-positive vascular endothelial cells in new vessels. They also found an increased incidence of neovascularization in Fas-deficient and FasL-defective mice compared with normal mice. Fas-FasL interaction on RPE induced apoptosis of cultured choroidal endothelial cells. They concluded that FasL expressed on RPE may control the growth and development of subretinal neovascularization. They hypothesized that with RPE senescence, subretinal neovascularization in AMD may result from a decreased inhibitory effect of FasL-positive RPE cells on angiogenesis.

The purpose of our study was to investigate FasL expression in the aging RPE and in early stages of ARM, and to study FasL expression on RPE in subretinal (clinically defined as classic) CNVMs, as well as subretinal RPE (clinically defined as occult) CNVMs.

## MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

*Patient materials*

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. For determination of FasL expression on RPE related to age and early ARM, 22 human eye bank eyes were used. The donors were 45 to 96 years of age (mean 75 years) with postmortem time from 2 to 11 hours. The donors had no history of eye disease, and the samples were macroscopically and microscopically checked for retinal diseases that might stimulate angiogenesis. The macular area (about 1 $cm^2$) was dissected from the ocular tissue, fixed in phosphate buffered formaldehyde and embedded in paraffin. Sections of 5 µm were made and classified for the presence of ARM as described before[144] (Table 5.1).

Furthermore, FasL expression on RPE in CNVM was determined on 12 eyes (6 enucleated eyes, 4 donor eyes and 2 surgically removed subretinal neovascularizations) of 11 patients with neovascular AMD, described before[248] (Table 5.2). All eyes were processed for routine diagnostic procedures by fixation in formaldehyde and embedded in paraffin. Five µm sections were prepared for immunohistochemistry.

***TABLE 5.1. CLASSIFICATION OF HUMAN MACULAE AND FASL EXPRESSION IN RPE***

| Case no. | Age | PM | Classification of macula | FasL expr RPE |
|---|---|---|---|---|
| **1** | 38 | 9.5 | no ARM | 1 |
| **2** | 45 | 8.5 | no ARM | 2 |
| **3** | 55 | 7 | no ARM | 2 |
| **4** | 64 | 10 | ARM | 2 |
| **5** | 65 | 8 | ARM | 0 |
| **6** | 67 | 11 | ARM | 3 |
| **7** | 74 | 8 | ARM | 1 |
| **8** | 74 | 8 | ARM | 2 |
| **9** | 76 | 7 | no ARM | 3 |
| **10** | 77 | 9 | no ARM | 0 |
| **11** | 77 | 9 | no ARM | 2 |
| **12** | 80 | 9.5 | no ARM | 2 |
| **13** | 81 | 5.5 | no ARM | n.c. |
| **14** | 81 | 7 | ARM | 0 |
| **15** | 81 | 7 | ARM | 2 |
| **16** | 85 | 4.5 | ARM | 3 |
| **17** | 86 | 5 | ARM | 2 |
| **18** | 86 | 10 | ARM | 3 |
| **19** | 87 | 5 | ARM | 0 |
| **20** | 88 | 8 | ARM | 0 |
| **21** | 91 | 4.5 | no ARM | 3 |
| **22** | 96 | 2 | ARM | 2 |

*Categories of FasL expression: 0 (0 – 25% positive cells), 1 (26 – 50% positive cells), 2 (51 – 75% positive cells) and 3 (76 – 100%). PM = postmortem time in hours; ARM = age-related maculopathy; n.c. = not classifiable.*

*Immunohistochemistry*

Polyclonal rabbit antibodies against Fas (C20) and FasL (N20) for immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The sections were deparaffinated and rehydrated. After blocking with normal goat serum (Dako, 1:10) for 15 minutes, the slides were incubated with the antibodies (Fas, 1:500; FasL 1:100) for 1 hour. The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both Biogenex, San Ramon, USA) for 30 minutes. The complex was visualized by incubating the sections with new fuchsin for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. We graded the expression in 4 categories of positive cells: 0 (0 – 25%), 1 (26 – 50%), 2 (51 – 75%) and 3 (76 – 100%). Negative controls for immunohistochemistry included 1) omission of the primary antibody, 2) incubation with an irrelevant polyclonal rabbit antibody and 3) preabsorbtion of the antibodies with a tenfold of the immunizing receptor peptide for 4 hours. The manufacturer has described the specificity of the antibodies.

## RESULTS

*FasL expression in aging human macula and early ARM*

FasL protein was found mostly in a membranous pattern at the basal side of the RPE (Figure 5.1A). Incidental cells stained in a more diffuse pattern. In early ARM, FasL staining was similar to non-ARM maculae (Figure 5.1B).

FasL expression in RPE cells was not related to age (the Spearman coefficient, $r = -0.14$ , $P = 0.95$ ), nor to presence of early ARM (logistic regression adjusted for age, FasL > 25% vs. FasL < 25%; odds ratio = 2.3; 95% CI: [0.2 to 28.2]) (Table 5.1). In negative controls, no staining was detected.

*FasL expression on RPE in CNVM*

In CNVMs, strong FasL and less intense Fas staining were found in RPE monolayers (Figure 5.1C-D, Table 5.2). Endothelial cells of newly formed vessels had both FasL and Fas expression in most cases. FasL staining in RPE was similar in sub-RPE (Figure 5.1C) and subretinal CNVMs (Figure 5.1D), as well as in fibrovascular and fibrocellular CNVMs. In negative controls with CNVMs, no staining was detected.

*TABLE 5.2. PATIENT DATA AND EXPRESSION OF FASL AND FAS IN EYES WITH CNV*

| No. | Age/sex | OD/OS | Clinical description | Histological classification CNV | | | Fas/FasL expression | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | subretinal/sub-RPE | FV/FC | other | Fas | | FasL | |
| | | | | | | | EC | RPE | EC | RPE |
| **1. CNV1** | 79/M | U | surgically excised CNV | mixed | FV and FC | hemorrhage | 1 | 1 | 2 | 0 |
| **2. CNV2** | 79/F | U | surgically excised CNV | subretinal | FV and FC | hemorrhage | 0 | 1 | 2 | 2 |
| **3. CNV6** | 72/M | OS | disciform MD | mixed | FV and FC | BLD, hemorrhage | 2 | 1 | 1 | 2 |
| **4. CNV7** | 86/M | OS | disciform MD, acute glaucoma | sub-RPE | FV and FC | BLD, hemorrhage, retinal detachment; posterior uveitis | 1 | 0 | 1 | 0 |
| **5. CNV8** | 91/M | OS | donor eye | mixed | FC | BLD | NC | NC | NC | NC |
| **6. CNV9** | 87/M | OS | donor eye | mixed | FV and FC | BLD | 2 | 0 | 0 | 0 |
| **7. CNV10** | 83/M | OD | painful eye, suspected uveal melanoma | mixed | FV and FC | BLD, hemorrhage, ischemic retinal disease | 1 | 1 | 2 | 1 |
| **8. CNV11** | 73/M | OS | disciform MD | subretinal | FC and FV | | 2 | 0 | 2 | 1 |
| **9. CNV12** | 73/M | OD | disciform MD, post irradiation | subretinal | FV | | 1 | 1 | 2 | 0 |
| **10. CNV13** | 82/M | OD | disciform MD | mixed | FC | confluent soft drusen | 1 | 1 | 0 | 2 |
| **11. CNV14** | 85/F | OS | post surgical endophthalmitis | sub-RPE | FV | endophthalmitis, uveitis | 0 | 1 | 1 | 2 |
| **12. CNV15** | 84/F | OD | expulsive hemorrhage, cataract | sub-RPE | FV | hemorrhage | 1 | 1 | 1 | 2 |

*Categories of Fas and FasL expression: 0 (0 – 25% positive cells), 1 (26 – 50% positive cells), 2 (51 – 75% positive cells) and 3 (76 – 100%). U = unknown; CNV = choroidal neovascular membrane; FV = fibrovascular; FC = fibrocellular; ARM = age-related maculopathy; NC = not classifiable.*

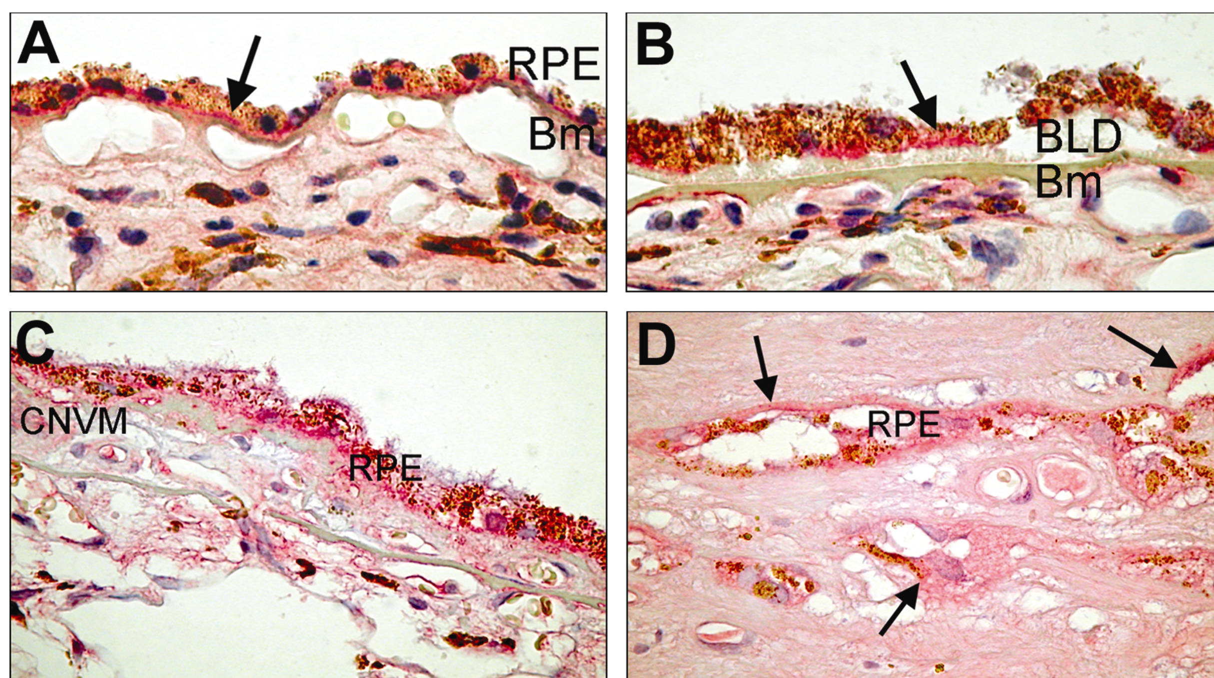


***Figure 5.1*** ***FasL expression in RPE (A)*** *Fas-ligand staining of human macular RPE in 80-year old donor eye. The FasL protein is found mostly in a membranous pattern at the basal side (arrow).* ***(B)*** *FasL staining of 86-year old donor eye with ARM.* ***(C)*** *Fas-ligand staining of subRPE choroidal neovascular membrane (CNVM) secondary to ARM.* ***(D)*** *FasL staining on RPE (arrows) of mixed subRPE and subretinal CNVM secondary to ARM. RPE = retinal pigment epithelium, Bm = Bruch's membrane, BLD = basal laminar deposits, CNVM = choroidal neovascular membrane (original magnification ×400, counterstaining Mayer's hematoxylin).*

## DISCUSSION

Our results show similar expression of FasL in the RPE in maculae of different age and ARM status. This might indicate that FasL expression in the RPE is not age-related or related to the presence of early ARM. This is not in line with Kaplan's hypothesis of FasL reduction with RPE senescence.[64]

In earlier studies, immunohistochemical Fas and FasL expression was found constitutively in the normal human retina and the choroid.[224] Both were also detected in cultured human RPE.[250]

Furthermore, we found similar FasL RPE expression in subretinal and in sub-RPE CNVM secondary to ARM. In subretinal CNV, the fibrovascular tissue grows through the RPE beneath the retina, while in sub-RPE CNV the neovascularization is restricted to the sub-RPE level. From experimental studies, and a study on excised human subretinal CNVMs it was postulated that decreasing FasL expression in sub-RPE CNVMs fails to inhibit subretinal extension of CNVM.[64] We did not find decreased expression in sub-RPE or mixed sub-RPE/subretinal CNVMs compared with subretinal CNVMs. Kaplan and coworkers used surgically excised CNV, which are mainly localized subretinal, while CNVMs in our study were localized mainly sub-RPE and mixed sub-RPE/subretinal.

This might partly explain the different expression pattern of Fas and FasL in our study compared to the results of Kaplan and coworkers. However, subretinal CNVMs are less common in ARM. [120] Therefore, it appears to be unlikely that FasL expression in RPE controls the extension of CNVM from sub-RPE to subretinal in human ARM. It may be possible that the Fas-FasL system is still important in this process, but through soluble- rather than membrane bound factors. Further investigation about the role of FasL in ARM is necessary.

# Part III

# Molecular Aspects of Neovascular Age-related Macular Degeneration

# CHAPTER 6

# INSULIN-LIKE GROWTH FACTOR-I AND ITS RECEPTOR IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION


## ABSTRACT

**Purpose:** The insulin-like growth factor-I (IGF-I) protein is a growth promoting polypeptide that can act as an angiogenic agent in the eye. The purpose of our study is to localize the expression of IGF-I and its receptor (IGF-IR) mRNA and IGF-IR protein *in situ* in the normal human eye and to examine the presence of expression in eyes with neovascular age-related macular degeneration (AMD).
**Methods**: Formalin-fixed, paraffin-embedded slides of 4 normal control eyes, 14 eyes with choroidal neovascularization (CNV) secondary to AMD were used. Three eyes with proliferative diabetic retinopathy were studied as positive control. IGF-I and IGF-IR mRNA was detected by *in-situ* hybridization with digoxigenin-labeled RNA probes. IGF-IR protein was studied by immunohistochemistry.
**Results**: In the normal retina, IGF-I and IGF-IR mRNA expression was found throughout the neuroretinal layers, in the retinal pigment epithelium (RPE) and in some choriocapillary and retinal capillary endothelial cells. In eyes with CNV we found IGF and IGF-IR mRNA in capillary endothelial cells, some transdifferentiated RPE, and fibroblast-like cells.
IGF-IR protein was found in normal eyes in all neuroretinal layers, in the RPE, and in the choroidal vessels. In eyes with CNV, we found IGF-IR protein in the RPE monolayer, in transdifferentiated RPE and in newly formed vessels.
**Conclusions**: The co-localization of protein and receptor indicates an autocrine function of IGF-I in the normal human retina. Since IGF-I participates in ocular neovascularization, synthesis of IGF-IR and IGF-I in endothelial cells, RPE cells and fibroblast-like cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD.

## INTRODUCTION

Neovascular age-related macular degeneration (AMD) is characterized by choroidal neovascularization (CNV), in which newly formed vessels from the underlying choroid grow beneath the retinal pigment epithelium (RPE) and the neuroretina. CNV may cause (sub) acute blindness because of bleeding or scar formation.[1] Although the morphology of angiogenesis in CNV secondary to AMD has been described in detail, the pathogenesis is still poorly understood.

Growth factors are acknowledged to play an important role in retinal neovascularizations. Vascular endothelial growth factor (VEGF), an endothelial specific mitogen, is regarded as one of the most important ocular angiogenic factors, especially under hypoxic circumstances.[166,251] Other angiogenic factors in ocular neovascularization include basic fibroblast growth factor, transforming growth factor-beta, platelet derived growth factor and insulin-like growth factor-I (IGF-I).[144,251]

In an earlier study we demonstrated that most CNV in AMD express somatostatin receptor type 2A that bind potential anti-angiogenic somatostatin analogues.[248] Somatostatin receptors are present on cultured human retinal endothelial cells. Proliferation of both retinal endothelial cells[173] as well as choroidal endothelial cells[174] can be inhibited by somatostatin analogues. In mice, inhibition of IGF-I by somatostatin analogues or by downregulation of growth hormone (GH), can decrease ischemia-induced retinal neovascularization.[177] IGF-I is a growth promoting polypeptide that has mitogenic and differentiating effects on many cell types, among which are ocular vascular endothelial cells[252] as well as RPE[172,253] and neuronal cells.[171] IGF-I can act as a direct angiogenic factor on retinal endothelial cells,[173,174] or indirectly through increased VEGF gene expression of cultured RPE cells.[156]

Most studies describe the role of IGF-I in *in vitro* models[173,174,254-257] or *in situ* in diabetic retinopathy.[258,259] So far IGF-I has not been studied in ARM.

The purpose of our study is to localize the expression of IGF-I and its receptor (IGF-IR) mRNA and IGF-IR protein *in situ* in normal human eyes and to examine the presence of expression in eyes with CNV, in order to elucidate its possible role in angiogenesis in AMD.

## MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

*Patients*

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. Four enucleated eyes without ischemic disease (enucleated for other reasons) and 3 enucleated eyes with proliferative diabetic retinopathy (PDR) were used as controls. Fourteen eyes (5 enucleated eyes, 4 donor eyes and 5 surgically removed subretinal neovascular membranes) of 13 patients with neovascular AMD were studied. The clinical and histological diagnosis of the eyes and the classification of CNV are described in Table 6.1. The eyes were processed for routine diagnostic procedures by fixation in 10% buffered formaldehyde and were embedded in paraffin. Five-μm sections were prepared for *in-situ* hybridization and immunohistochemistry.

*RNA probes*

The human IGF-I probe was a 258 bp fragment containing exon 2 and 3 of the IGF-I gene.[260] The human IGF-IR probe was generated using a cDNA clone with a unique insert of human IGF-IR (I.M.A.G.E. cDNA clone 150361, Research Genetics, Huntsville, AL, USA). The insert was reduced to 270 bp by restriction with BamHI and AvaI, and ligated in vectors pBluescript SK (antisense) and KS (sense) (Stratagene Europe, Amsterdam, The Netherlands). E. Coli Xl2Blue were transformed with these vectors and proper colonies were isolated and grown. Sequence analysis was performed to verify the inserts. Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) using T7 RNA polymerase.

*In-situ hybridization*

Sections were deparaffinated with xylene and rehydrated. The slides were incubated in the following solutions: 0.2 N HCL, 0.3% Triton X-100 in phosphate-buffered saline (PBS), RNase-free proteinase K (5 μl/ml for 20 minutes at 37ºC) and 4% formaline in PBS. Subsequently, acetylation was performed with acetic anhydride in 0.1 M triethanolamine. The slides were rinsed in 2x SSC (1xSSC = 150 mM NaCl and 15 mM sodium citrate) and preincubated in 50% formamide in 2xSSC at 37ºC. For hybridization, antisense and sense probes were diluted in hybridization solution (50% deionized formamide, 10% dextran sulphate, 4xSSC (IGF-I) or 2xSSC (IGF-IR), 1xDenhardt's solution, 1 μg/ml tRNA, 250 μg/ml herring sperm RNA) to a concentration of 400 ng/ml, and incubated at 68ºC for 30 minutes. The hybridization solution was then layered onto the sections and hybridized overnight at 55ºC in a humid chamber. Post hybridization washes were performed at 45ºC for 30 minutes in the following solutions: 50% formamide in 2xSSC, 50% formamide in 1xSSC, 0.1xSSC (IGF-IR) or 0.5xSSC (IGF-I). The slides were incubated with RNAse T1 (2 U/ml) in 2xSSC/1mM EDTA in 37ºC for 15 minutes and washed at 45ºC with

*TABLE 6.1 DIAGNOSIS OF STUDIED EYES AND HISTOLOGICAL CLASSIFICATION OF CNV*

| No | Age/ sex | OD/ OS | Clinical description | Histological classification CNV | | |
|---|---|---|---|---|---|---|
| | | | | subret/ sub-RPE | FV/FC | other characteristics |
| **Co1** | 83/M | OS | corneal ulcer | | | |
| **Co2** | 57/M | OS | recurrent conjunctival melanoma | | | |
| **Co3** | 42/M | OS | choroidal melanoma | | | |
| **Co4** | 69/F | OD | orbital metastasis, post irradiation | | | |
| **DM1** | 34/M | OS | PDR | | | |
| **DM2** | 63/F | OD | PDR | | | |
| **DM3** | 75/F | OD | PDR | | | |
| **CNV1** | 79/M | u | surgically excised CNV | mixed | FV + FC | hemorrhage |
| **CNV2** | 79/F | u | surgically excised CNV | subretinal | FV + FC | hemorrhage |
| **CNV3** | 76/F | OD | surgically excised CNV | subretinal | FV | BLD |
| **CNV4** | 79/M | u | surgically excised CNV | mixed | FV + FC | BLD, hemorrhage |
| **CNV5** | 78/M | u | surgically excised CNV | sub-RPE | FV + FC | BLD, confluent soft drusen |
| **CNV6** | 72/M | OS | disciform MD | mixed | FV + FC | BLD, hemorrhage |
| **CNV7** | 86/M | OS | disciform MD, acute glaucoma | sub-RPE | FV + FC | BLD, hemorrhage, retinal detachment; posterior uveitis |
| **CNV8** | 91/M | OS | disciform MD, donor eye | mixed | FC | disciform MD, BLD |
| **CNV9** | 87/M | OS | disciform MD, donor eye | mixed | FV + FC | disciform MD, BLD |
| **CNV10** | 83/M | OD | painful eye, suspected uveal melanoma | mixed | FV + FC | ischemic retinal disease; disciform MD, BLD, hemorrhage |
| **CNV11** | 73/M | OS | disciform MD | subretinal | FV + FC | |
| **CNV12** | 73/M | OD | disciform MD, post irradiation | subretinal | FV | |
| **CNV13** | 82/M | OD | disciform MD | mixed | FC | confluent soft drusen |
| **CNV16** | 80/F | OS | disciform MD, hemorrhage, secondary glaucoma | mixed | FV + FC | BLD, hemorrhage |

*CNV = choroidal neovascularization; OD = right eye; OS = left eye; FV = fibrovascular; FC = fibrocellular; PDR = proliferative diabetic retinopathy; u = unknown; MD = macular degeneration; BLD = basal laminar and linear deposits.*

1xSSC and at room temperature with 2xSSC. The digoxigenin-labeled hybrids were detected by antibody incubation performed according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) with the following modifications. A 1:1000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase was used for a 2.5 hour incubation at room temperature or overnight at

4ºC. Afterwards, an extra washing step of 0.025% Tween in Tris-buffered saline (pH 7.5) was introduced. For staining, sections were layered with detection buffer (0.1 M Tris-HC, 0.1 M NaCl, 0.05 M $MgCl_2$ pH 9.5) containing NBT (4-nitroblue tetrazolium chloride), BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (both from Vector, Burlingame, CA, USA) and 6% polyvinylalcolhol (m.w. 31.000-50.000, from Aldrich Chemical Milwaukee, WI, USA). The color reaction was performed in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Sections were washed in 10 mM Tris-HCl, 1 mM EDTA pH 8.0, counterstained with nuclear red solution, dehydrated with ethanol gradients and mounted.

*Immunohistochemistry*

Polyclonal rabbit antibodies against IGF-IR (1:750 dilution) were obtained from Research Diagnostics (Flanders, NJ, USA). Immunohistochemistry against IGF-I was not performed because of lack of adequate antibodies for paraffin-embedded material. Antibodies against pankeratine (monoclonal mouse antibodies; 1:100 dilution); factor VIII (monoclonal mouse antibodies; 1:50 dilution) and glial fibrillary acidic protein (GFAP; polyclonal rabbit antibodies; dilution 1:200) were obtained from DAKO (Glostrup, Denmark). Monoclonal mouse antibodies against smooth muscle actin (SMA; 1:150 dilution) were obtained from Biogenex (San Ramon, CA, USA). Sections were deparaffinated and rehydrated. Antigen retrieval was performed for the IGF-IR and pankeratin antibodies (microwave-heating for 10 minutes), and for the factor VIII antibodies (pronase treatment for 20 minutes at 37°C). After blocking with normal goat serum (Dako, 1:10) for 15 minutes, the slides were incubated with the primary antibodies for 1 hour at room temperature or overnight at 4°C (IGF-IR). The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both Biogenex) for 30 minutes. The complex was visualized by incubation with new fuchsin (as a red chromogen) for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. Negative controls for immunohistochemistry included 1) omission of the primary antibody, 2) incubation with an irrelevant polyclonal rabbit antibody and 3) preabsorbtion of the IGF-IR antibodies with a tenfold of the immunizing IGF-IR peptide (Research Diagnostics) for 4 hours.

*Grading of expression and statistics*

The slides were examined by light microscopy. Grading of mRNA and IGF-IR protein expression was performed in a masked fashion by two authors (AL and CM) twice or once, respectively. Slides were blinded and randomly graded per cell type in 3 categories (Table 6.2): 1: no staining; 2: staining in less than 50% of cells; 3: staining in more than 50% of cells.

Positive cell types in CNV were identified using pankeratine staining (RPE cells), factor VIII staining (vascular endothelial cells), GFAP staining (neuronal cells) and SMA (myofibroblasts) in consecutive slides, combined with cellular morphology at examination by light microscopy. Cells not meeting these criteria were classified as "other".
For inter- and intra-observer variability a kappa-value was calculated.

## RESULTS

In two eyes with neovascular AMD (Table 6.2: CNV 8 and CNV13) the normal retina, regarded as positive internal control, was negative with *in-situ* hybridization. All mRNA appeared to be lost in these eyes.

***TABLE 6.2 EXPRESSION OF IGF-I AND IGF RECEPTOR TYPE 1 IN CNV***

| | IGF-I mRNA | | | | IGF-IR mRNA | | | | IGF-IR protein | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **EC** | **RP** | **FB** | **O** | **EC** | **RP** | **FB** | **O** | **EC** | **RP** | **FB** | **O** |
| **1. CNV1** | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 |
| **2. CNV2** | 0 | 0 | 0 | 0 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 1 |
| **3. CNV3** | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 | 0 | 0 |
| **4. CNV4** | 1 | 2 | 1 | 1 | nc | nc | nc | nc | 1 | 2 | 0 | 0 |
| **5. CNV5** | 1 | 1 | 0 | 1 | 1 | 2 | 1 | 1 | 1 | 2 | nc | nc |
| **6. CNV6** | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 0 | 1 |
| **7. CNV7** | nc | 0 | nc | 1 | 1 | 2 | 1 | 1 | 0 | 1 | 0 | 0 |
| **8. CNV8** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| **9. CNV9** | 2 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | nc | nc | nc | nc |
| **10. CNV10** | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 0 |
| **11. CNV11** | 0 | 1 | 1 | nc | 2 | 2 | 2 | 2 | 1 | 1 | 0 | 1 |
| **12. CNV12** | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 1 | 1 |
| **13. CNV13** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| **14. CNV16** | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 |

*0 = no expression; 1 = 1 to 50% of cells; 2 = 51 – 100% of cells. EC = endothelial cells; RPE = retinal pigment epithelium; FB = fibroblasts and fibrocytes; O = other cell types.*

*In-situ hybridization*

### IGF-I

In the normal retina, IGF-I mRNA expression (Figure 6.1A,D) was found in the ganglion cell layer, inner nuclear layer, outer limiting membrane, RPE monolayer and in some cells in the choroid. Choriocapillary endothelial cells and retinal vessels were positive infrequently. Further expression was found in the lens epithelium, and in all

corneal layers (not shown). Hybridization with the sense probe was negative. In eyes with CNV we found IGF mRNA in the retina in the same pattern as in normal retina. Staining in preexistent RPE monolayer was similar to normal eyes. In 8 out of 14 eyes with CNV, expression was found in vascular endothelial cells, some RPE cells, and fibroblast-like cells (Table 6.2; Figure 6.2A,D,G,J). Eyes with PDR showed identical expression in retinal layers. In the preretinal membranes endothelial cells from newly formed capillaries and fibroblast-like cells stained positive (Figure 6.1G). Hybridization with the sense probe was negative.

**IGF-I Receptor**

In the normal eye mRNA of the IGF-IR (Figure 6.1B,F) was seen in the ganglion cell layer, inner and outer nuclear layer and outer limiting membrane. The RPE was strongly positive. Endothelial cells of the choriocapillaris, choroidal and intraretinal vessels were frequently positive. Further expression was found in the non-pigmented epithelium and to a lesser extent in the pigment epithelium of the ciliary body, the iris dilator muscle, the iris pigment epithelium and iris endothelial cells. The lens epithelium and all corneal layers were also positive. Hybridization with the sense probe was negative. In all eyes with CNV we found IGF-IR mRNA in endothelial cells of newly formed vessels, in RPE-cells and in the RPE monolayer, and in fibroblasts (Table 6.2; Figure 6.2B,E,H,K). Eyes with PDR showed expression in retinal layers and RPE similar to normal eyes. In the diabetic preretinal membranes (Figure 6.1H) endothelial cells from newly formed capillaries and fibroblast-like cells were positive. Hybridization with the sense probe was negative.

*Immunohistochemistry*

**IGF-I Receptor**

In the normal eyes, we found IGF-IR protein in the choroidal vessels, in the RPE, and in all layers of the neuroretina (Figure 6.1C,F). Choriocapillaris was negative. Negative controls showed no staining, except for the peptide control, in which staining of the RPE monolayer was not totally blocked compared to retinal staining, indicating an aspecific component in the RPE staining. In all eyes with CNV, we found the IGF-IR protein in RPE cells, and in 9 out of 13 classifiable eyes, staining was seen in newly formed vessels (Table 6.2; Figure 6.2C,F,I). Eyes with PDR showed expression in the retina similar to normal eyes, and in the diabetic preretinal membranes endothelial cells from newly formed capillaries were positive (Figure 6.1I).

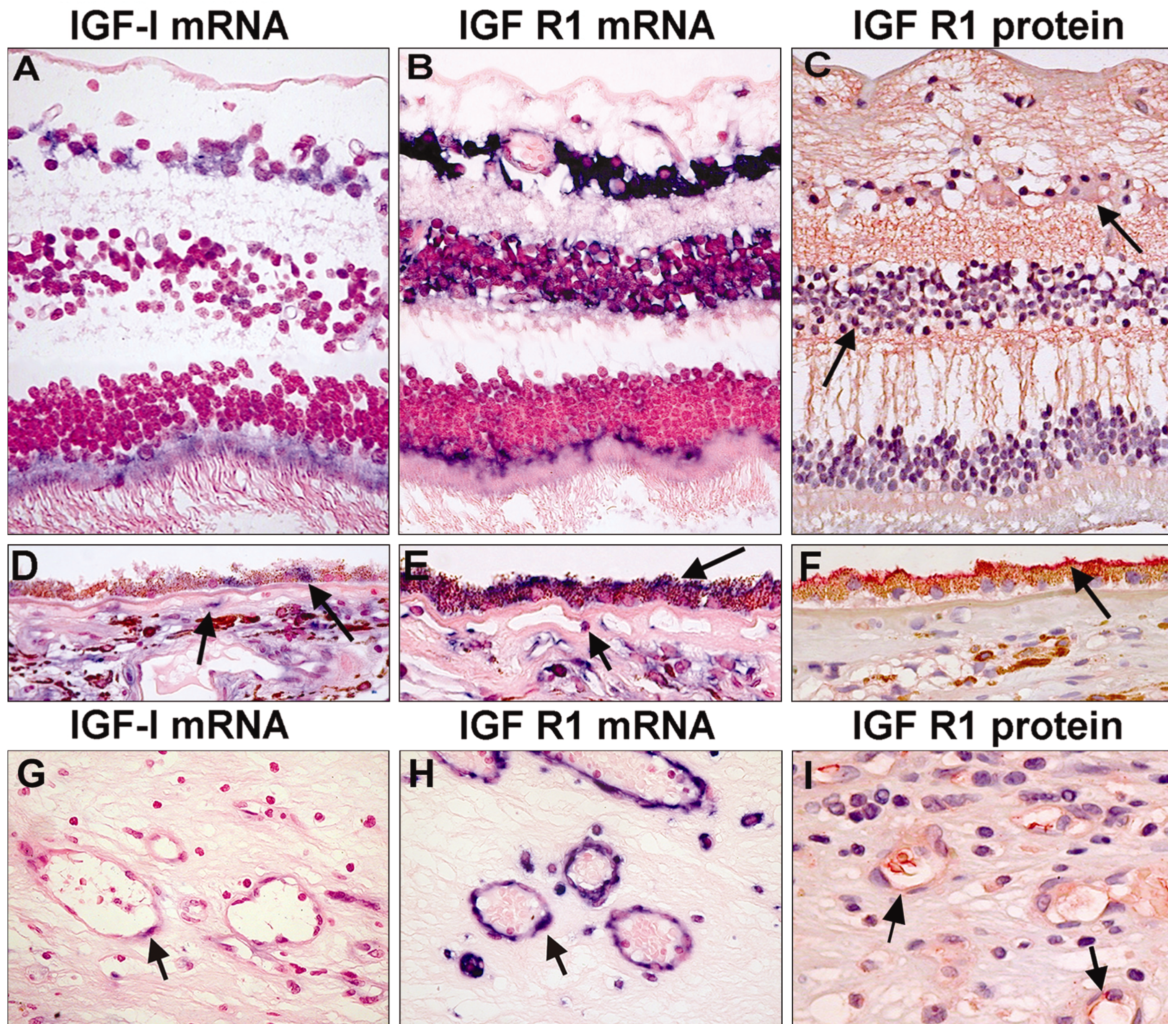


***Figure 6.1*** ***Localization of IGF-I and IGF-IR in the human eye and in eyes with diabetic proliferative retinopathy.*** *Expression of IGF-I mRNA (left column), IGF-IR mRNA (middle column) in paraffin-embedded tissue, detected by* in-situ *hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGF-IR protein (right column) in paraffin-embedded tissue, detected with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin. Short arrows indicate capillary endothelial cells, long arrows indicate RPE cells.* ***(A to F)*** *Posterior pole of normal eye (Co1) with IGF-I mRNA expression* ***(A, D)****; with IGF-IR mRNA expression* ***(B, E)****; and with IGF-IR protein expression* ***(C, F)****.* ***(G, H, I)*** *Diabetic preretinal membrane (DM2) with IGF-I mRNA expression* ***(G)****; with IGF-IR mRNA expression* ***(H)****; and with IGF-IR protein expression* ***(I)****. Original magnification X400*

*Statistics*

Grading of mRNA and IGF-IR protein expression was performed in a masked fashion by two authors (AL and CM), twice with a time interval of 7 weeks and once, respectively. For inter-observer variability, kappa was 0.75; for intra-observer variability kappa was 0.83.

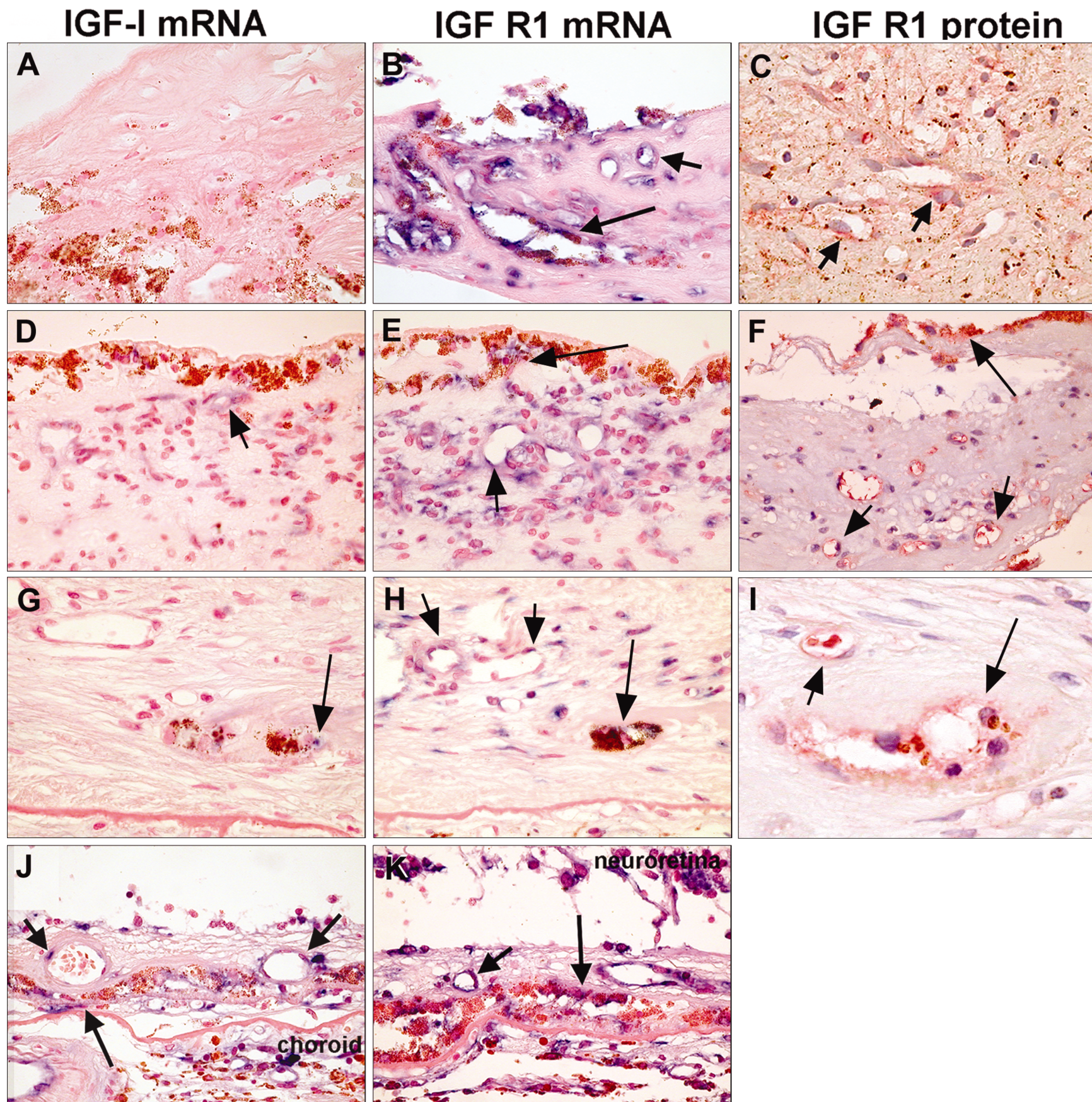


***Figure 6.2** **Localization of IGF-I and IGF-IR in eyes with neovascular AMD**. Expression of IGF-I mRNA (left column), IGF-IR mRNA (middle column) in paraffin-embedded tissue, detected by* in-situ *hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGF-IR protein (right column) in paraffin-embedded tissue, detected with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin. Short arrows indicate capillary endothelial cells, long arrows indicate RPE. **(A, B, C)** Surgically excised CNV (CNV2) with IGF-I mRNA expression **(A)**; with IGF-IR mRNA expression **(B)**; and with IGF-IR protein expression **(C)**. **(D, E, F)** Surgically excised CNV (CNV3) with IGF-I mRNA expression pression **(D)**; with IGF-IR mRNA expression **(E)**; and with IGF-IR protein expression **(F)**. **(G, H, I)** Sub-RPE CNV (CNV11) with with IGF-I mRNA expression **(G)**; with IGF-IR mRNA expression **(H)**; and with IGF-IR protein expression **(I)**. **(J, K)** Subretinal CNV (CNV9) with IGF-I mRNA expression **(J)**; and with IGF-IR mRNA expression **(K)**. Protein detection of IGF-IR with immunohistochemistry gave aspecific staining; therefore not shown. Original magnification X400.*

## DISCUSSION

In the normal human retina, we found IGF-I and IGF-IR mRNA expression throughout the neuroretina in the same pattern as described for other species.[261,262] Expression in human RPE was not described before *in-situ*, but was detected in cultured human RPE cells.[254-256] IGF-I stimulates differentiation and proliferation of vascular endothelial cells,[170] RPE cells[256] and neural retinal cells,[171,263] and is also neuroprotective.[263-265] Our finding of protein and receptor co-localization confirms a paracrine/autocrine function of IGF-I in the normal human retina.[261,266]
The observed hybridization signal of IGF-IR was much stronger than that of IGF-I. This might represent a real difference in expression, confirming the findings of higher IGF-IR expression in cultured human RPE.[254,255] On the other hand, intensity of staining is not a reliable quantitative criterion, and the IGF-IR probe might be more sensitive than the IGF-I probe.

In CNV secondary to ARM, we demonstrated mRNA expression of IGF-I and IGF-IR in vascular endothelial cells, in RPE cells and in fibroblast-like cells. Similarly, we found expression of IGF-I mRNA and its receptor in epiretinal membranes in eyes with PDR, including endothelial cells from newly formed capillaries and in fibroblast-like cells. This is in accordance with a previous diabetic retinopathy study, in which binding sites for IGF-I (receptors or binding proteins) were demonstrated in vessel walls as well as in cells in fibrous tissue in human diabetic epiretinal membranes.[258] In human and rat diabetic retinas, IGF-I mRNA levels are decreased,[259,267] but vitreal levels of IGF-I are elevated.[268-272] Our findings confirm that intraocular synthesis may contribute to these elevated concentrations.[271]
The pathogenesis of neovascular AMD involves the choroidal vasculature rather than the retinal vasculature as in diabetic retinopathy. *In vitro*, IGF-I stimulates the proliferation of choroidal endothelial cells.[174] Therefore, synthesis of IGF-IR and IGF-I in endothelial and RPE cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD, since both cell types appear to have an important role in this process.[1]
IGF-I participates in each step of ocular neovascularization.[170] It is involved in the degradation of basement membranes and extracellular matrix proteolysis, and in vascular endothelial cell migration and proliferation.[173] Intravitreous injection of IGF-I in animals produces preretinal neovascularization in rabbits[175] or microangiopathy resembling diabetic microangiopathy in pigs.[176] IGF-I also increases RPE cell migration and proliferation *in vitro*.[172] Furthermore, IGF-I induces upregulation of VEGF mRNA expression in RPE cells[156] and fibroblasts,[178] which in turn also stimulates endothelial cells.[156] Antagonism of IGF-IR suppresses retinal

neovascularization and reduces the retinal endothelial cell response to VEGF,[273] which allows for the hypothesis that IGF-I has a permissive role in VEGF-induced neovascularization.
Inhibition of IGF-I, achieved with somatostatin analogues,[177] may occur at different levels. Firstly, somatostatin inhibits the GH-IGF axis. Furthermore, somatostatin can inactivate the mitogenic potential of IGF-I directly by inactivating the phosphorylated, active form of IGF-IR.[170,274] Finally, somatostatin can act directly as an anti-angiogenic agent through binding to somatostatin receptors.[173] Therefore, somatostatin analogues may be an effective therapy for neovascular AMD.
IGF-I is recruited in normal wound repair.[275,276] This may partly explain the presence of IGF-I in CNV, because formation of the disciform lesion is regarded as normal wound repair.[1]

In conclusion, in this descriptive study we localized the expression of IGF-I mRNA and IGF-IR protein and mRNA in the normal eye. The co-localization of protein and receptor indicates an autocrine function of IGF-I in the human retina. Furthermore, we detected synthesis of both IGF-I and its receptor, and IGF-IR protein in ocular neovascular membranes of patients with AMD and diabetic patients. Since IGF-I participates in ocular neovascularization, synthesis of IGF-IR and IGF-I in vascular endothelial, RPE and fibroblast-like cells in CNV may point towards a role of this growth factor in the pathogenesis of neovascular AMD. The exact role of the IGF family in CNV formation and its possible therapeutic possibilities need to be established.

# CHAPTER 7

# INSULIN-LIKE GROWTH FACTOR-BINDING PROTEINS ARE EXPRESSED IN NEOVASCULAR AGE-RELATED MACULAR DEGENERATION

## ABSTRACT

**Purpose**: The insulin-like growth factor-I (IGF-I) protein is a growth promoting polypeptide that can act as an angiogenic agent in the eye. Earlier we detected IGF-I and type 1 IGF receptor in neovascular AMD. The diverse activities of IGF-I are mediated through binding and activation of the type I IGF receptor. IGF-I is usually bound to one of the six major IGF-binding proteins (IGFBPs). These complexes prolong half-life in the circulation and function as a reservoir for IGF-I. Furthermore most IGFBPs have actions that are independent of IGF-I binding. The purpose of our study is to localize the expression of IGFBP proteins and mRNA *in situ* in the normal human eye and in eyes with neovascular age-related macular degeneration (AMD).
**Methods**: Formalin-fixed, paraffin-embedded slides of 4 normal control eyes and 17 eyes with choroidal neovascularization (CNV) secondary to AMD were used. IGFBP mRNA was detected by *in-situ* hybridization with digoxigenin-labeled RNA probes, and IGFBP proteins were studied by immunohistochemistry.
**Results**: In the normal retina, we found mRNA of IGFBP-2 and IGFBP-4; mRNA of IGFBP-1 was not detected. IGFBP-1, -2, -3, -4 and -6 were immunolocalized throughout the eye in a spatially differentiated pattern. In eyes with CNV we localized mRNA of IGFBP-2 and -4 in CNV resulting from AMD, as well as most IGFBP proteins, in vascular endothelial cells, RPE cells and fibroblasts.
**Conclusions**: The finding of these members of the IGF family may indicate a role of the IGF family in the pathogenesis of neovascular AMD, since IGF-I is involved in angiogenesis. The functional role of the various IGF family members in AMD needs to be established.

## INTRODUCTION

Neovascular age-related macular degeneration (AMD) is characterized by choroidal neovascularization (CNV), in which newly formed vessels from the underlying choroid grow beneath the retinal pigment epithelium (RPE) and the retina. CNV may cause (sub) acute blindness because of bleeding or scar formation.[1] Although the morphology of angiogenesis in CNV secondary to AMD has been described in detail, the pathogenesis is still poorly understood.

Growth factors are acknowledged to play an important role in retinal neovascularizations. Vascular endothelial growth factor (VEGF), an endothelial specific mitogen, is regarded as one of the most important ocular angiogenic factors, especially under hypoxic circumstances.[166,251] Other angiogenic factors in ocular neovascularization include basic fibroblast growth factor, transforming growth factor-beta (TGF-β), platelet derived growth factor and insulin-like growth factor-I (IGF-I).[144,251]

In an earlier study we detected production of IGF-I and the type 1 IGF receptor (IGF-IR) in the normal eye and in eyes with neovascular AMD [manuscript submitted]. IGF-I is a growth promoting polypeptide that has mitogenic and differentiating effects on many cell types, among which ocular cell types.[171,172,174,252,253] In animal models, IGF-I is associated with ocular angiogenesis.[177,178]. Furthermore, *in vitro* IGF-I can act as a direct angiogenic factor on retinal endothelial cells,[173] or indirectly through increased VEGF gene expression in cultured RPE cells.[156] The diverse activities of IGF-I are mediated through binding and activation of IGF-IR. Intravascularly and in the extracellular space, IGF-I is usually bound to one of the IGF-binding proteins (IGFBP). Six major IGFBPs are described currently. Most IGFBPs have additional actions that are independent of IGF-I binding, including inhibition or enhancement of cell growth and induction of apoptosis (reviewed by Baxter[167]). IGF-I and the IGFBPs are mainly produced by the liver, however they are also synthesized locally by most tissues, where they act in an autocrine or paracrine manner.[168] In many situations on pathological growth, multiple components of the IGF system may be dysregulated.[169]

The purpose of this study is to explore mRNA and protein expression of the six IGFBPs *in situ* in normal human eyes and in eyes with CNV.

## MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

*Patients*

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. Four enucleated eyes without ischemic disease (enucleated for other reasons) were used to study the normal human eye. Term placental tissue was used as positive control.[277] Seventeen eyes (8 enucleated eyes, 4 donor eyes and 5 surgically removed subretinal neovascular membranes) of 16 patients with neovascular AMD were used. The clinical and histological diagnosis of the eyes and the classification of CNV are described in Table 7.1. The eyes were processed for routine diagnostic procedures by fixation in 10% buffered formaldehyde and were embedded in paraffin. Five-µm sections were prepared for *in-situ* hybridization and immunohistochemistry.

*RNA probes and antibodies*

The cDNA containing plasmids for human IGFBP-1 to 6 were kindly provided by Shunichi Shimasaki (Department of Reproductive Medicine, University of California, La Jolla, CA, USA). Digoxigenin-11-UTP labeled RNA probes were prepared according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) using T7 RNA polymerase for antisense and T3 RNA polymerase for sense probes.

Goat polyclonal antibodies against IGFBP-1 to 6 (respectively M-19; M-18; M-19; C-20; C18; M20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies against pankeratin (monoclonal mouse antibodies; 1:100 dilution); factor VIII (monoclonal mouse antibodies; 1:50 dilution) and glial fibrillary acidic protein (GFAP; polyclonal rabbit antibodies; dilution 1:200) were obtained from DAKO (Glostrup, Denmark). Monoclonal mouse antibodies against smooth muscle actin (SMA; 1:150 dilution) were obtained from Biogenex (San Ramon, CA, USA).

*In-situ hybridization*

Sections were deparaffinized with xylene and rehydrated. Before hybridization, the slides were incubated in the following solutions: 0.2 N HCL, 0.3% Triton X-100 in phosphate-buffered saline (PBS), RNase-free proteinase K (5 µl/ml for 20 minutes at 37ºC), 4% formaline in PBS, acetic anhydride in 0.1 M triethanolamine, and 2xSSC.

| No | Age/ sex | OD / OS | Clinical description | Histological classification CNV | | |
|---|---|---|---|---|---|---|
| | | | | subret/ sub-RPE | FV/FC | other characteristics |
| **Co1** | 83/M | OS | corneal ulcer | | | |
| **Co2** | 57/M | OS | recurrent conjunctival melanoma | | | |
| **Co3** | 42/M | OS | choroidal melanoma | | | |
| **Co4** | 69/F | OD | orbital metastasis, post irradiation | | | |
| **CNV1** | 79/M | u | surgically excised CNV | mixed | FV + FC | hemorrhage |
| **CNV2** | 79/F | u | surgically excised CNV | subretinal | FV + FC | hemorrhage |
| **CNV3** | 76/F | OD | surgically excised CNV | subretinal | FV | BLD |
| **CNV4** | 79/M | u | surgically excised CNV | mixed | FV + FC | BLD, hemorrhage |
| **CNV5** | 78/M | u | surgically excised CNV | sub-RPE | FV + FC | BLD, confluent soft drusen |
| **CNV6** | 72/M | OS | disciform MD | mixed | FV + FC | BLD, hemorrhage |
| **CNV7** | 86/M | OS | disciform MD, acute glaucoma | sub-RPE | FV + FC | BLD, hemorrhage, retinal detachment; posterior uveitis |
| **CNV8** | 91/M | OS | disciform MD, donor eye | mixed | FC | disciform MD, BLD |
| **CNV9** | 87/M | OS | disciform MD, donor eye | mixed | FV + FC | disciform MD, BLD |
| **CNV10** | 83/M | OD | painful eye, suspected uveal melanoma | mixed | FV + FC | ischemic retinal disease; disciform MD, BLD, hemorrhage |
| **CNV11** | 73/M | OS | disciform MD | subretinal | FV + FC | |
| **CNV12** | 73/M | OD | disciform MD, post irradiation | subretinal | FV | |
| **CNV13** | 82/M | OD | disciform MD | mixed | FC | confluent soft drusen |
| **CNV14** | 85/F | OS | post surgical endophthalmitis | subretinal | FV | endophthalmitis, uveitis |
| **CNV15** | 84/F | OD | expulsive hemorrhage preoperatively | subretinal | FV | hemorrhage |
| **CNV16** | 80/F | OS | disciform MD, hemorrhage, secondary glaucoma | mixed | FV + FC | BLD, hemorrhage |
| **CNV17** | 84/F | OS | disciform MD | mixed | FV + FC | BLD, hemorrhage |

*TABLE 7.1 DIAGNOSIS OF STUDIED EYES AND HISTOLOGICAL CLASSIFICATION OF CNV*

*OD = right eye; OS = left eye; FV = fibrovascular; FC = fibrocellular; PDR = proliferative diabetic retinopathy; u = unknown; MD = macular degeneration; BLD = basal laminar and linear deposits.*

The slides were preincubated in 50% formamide in 2xSSC at 37ºC. For hybridization, antisense and sense probes were diluted in hybridization solution (50% deionized formamide, 10% dextran sulphate, 4xSSC (IGFBP-2 and IGFBP-4) or 2xSSC (IGFBP-1), 1xDenhardt's solution, 1 μg/ml tRNA, 250 μg/ml herring sperm RNA) to a concentration of 400 ng/ml, and incubated at 68ºC for 30 minutes. The

hybridization solution was then layered onto the sections and hybridized overnight at 55ºC in a humid chamber. Posthybridization washes were performed at 45ºC for 30 minutes in the following solutions: 50% formamide in 2xSSC, 50% formamide in 1xSSC, 0.1xSSC. The slides were incubated with RNAse T1 (2 U/ml) in 2xSSC/1mM EDTA in 37ºC for 15 minutes and washed at 45ºC with 1xSSC and at room temperature with 2xSSC. The digoxigenin-labeled hybrids were detected by antibody incubation performed according to the manufacturer's prescription (Roche Diagnostics, Mannheim, Germany) with following modifications. A 1:1000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase was used for a 2.5 hour incubation overnight at 4ºC. Afterwards, an extra washing step of 0.025% Tween in Tris-buffered saline (pH 7.5) was introduced. For staining, sections were layered with detection buffer (0.1 M Tris-HC, 0.1 M NaCl, 0.05 M $MgCl_2$ pH 9.5) containing NBT (4-nitroblue tetrazolium chloride), BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (both from Vector, Burlingame, CA, USA) and 6% polyvinylalcolhol (m.w. 31.000-50.000, from Aldrich Chemical Milwaukee, WI, USA). The color reaction was performed in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Sections were washed in 10 mM Tris-HCl, 1 mM EDTA pH 8.0, counterstained with nuclear red solution, dehydrated with ethanol gradients and mounted. The slides were examined by light microscopy. Staining of the antisense probe was scored positive if the sense probe scored negative in the same region. Intensity of staining between slides could not be used as reliable quantitative criterion because of possible differences in fixation time.

*Immunohistochemistry*

The sections were deparaffinated and rehydrated. Antigen retrieval was performed for the IGFBP-6 and pancytokeratin antibodies (microwave-heating for 10 minutes), and for the factor VIII antibodies (pronase treatment for 20 minutes at 37°C). Immunohistochemistry with the IGFBPs was performed as follows. After blocking with 1,5% normal rabbit serum (Dako) in PBS for 30 minutes, the slides were incubated with the primairy antibodies (1:150 for IGFBP-1, IGFBP-2, and IGFBP-4; 1:100 for IGFBP-3 and IGFBP-6) overnight at 4°C. The sections were further incubated with biotinylated rabbit-anti-goat antibodies (Dako) for 1 hour, followed by alkaline phosphatase-labeled antibiotin (Dako) for 30 minutes. For immunohistochemistry with Factor VIII, SMA, GFAP and pankeratin, slides were blocked with normal goat serum in PBS (Dako, 1:10) for 15 minutes. The primairy antibodies were incubated for 1 hour at room temperature, followed by biotinylated multilink antibodies for 30 minutes. The avidin-biotin complex was visualized by incubation with new fuchsin (as a red chromogen) for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. Negative controls for immunohistochemistry included 1) omission of the

primary antibody and 2) preabsorbtion of the antibodies with a tenfold of the immunizing IGFBP peptide (Santa Cruz) for 4 hours.

## RESULTS

In two eyes with neovascular AMD, (Table 7.3: CNV 8 and CNV13) no mRNA could be detected with digoxigenin-labeled mRNA *in-situ* hybridization, regardless of the probe. All mRNA appeared to be lost in these eyes. These eyes were considered as 'not classifiable'.

*IGFBP-1*

The sense probe for IGFBP-1 mRNA showed light aspecific staining in the retina, and negative controls for IGFBP-1 immunohistochemistry showed no staining. In the normal eye (Table 7.2, Figure 7.1A,D), mRNA for IGFBP-1 could not be detected. IGFBP-1 protein was found in all nuclear and plexiform layers of the neuroretina, including photoreceptor inner segments, in the RPE at the basal side. Retinal and choroidal vessels stained positive, choriocapillary cells incidentally stained positive. Lens epithelium, non-pigmented epithelium of the ciliary body and corneal layers also stained for IGFBP-1 protein.

In eyes with CNV (Table 7.3, Figure 7.2A,D), faint staining for IGFBP-1 mRNA was only detected in one sample. All CNVs showed positive immunohistochemical staining in capillary endothelial cells, RPE and/or fibroblasts.

*IGFBP-2*

The sense probe for IGFBP-2 mRNA, and negative controls for IGFBP-2 immunohisto-chemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1B,E), we found IGFBP-2 mRNA in the ganglion cell layer, inner nuclear layer, photoreceptor layer and RPE. IGFBP-2 protein was found with higher intensity but in the same pattern as IGFBP-1, that is in all nuclear and plexiform layers of the neuroretina, including photoreceptor inner segments, in the RPE and in the choriocapillaris. Lens epithelium, non-pigmented epithelium of the ciliary body and corneal layers also stained for IGFBP-2 protein.

In CNV (Table 7.3, Figure 7.2B,E) IGFBP-2 mRNA was found in 9 out of 14 CNV, localized in endothelial cells, RPE or fibroblasts. Nearly all cellular components of CNV, including capillaries, RPE and fibroblasts, stained intensely positive for IGFBP-2 protein.

**TABLE 7.2 EXPRESSION OF IGFBP MRNA AND PROTEIN IN CONTROL EYES**

| | IGFBP-1 | | IGFBP-2 | | IGFBP-3 | | IGFBP-4 | | IGFBP-6 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | RNA | prot | RNA | prot | RNA | prot | RNA | prot | RNA | prot |
| **GGL** | - | ++ | ++ | + | | ++ | ++ | ++ | | + |
| **INL** | - | + | + | ++ | | + | ++ | + | | - |
| **ONL** | - | + | - | ++ | | - | + | - | | - |
| **PHR** | - | ++ | ++ | ++ | | ++ | ++ | ++ | | + |
| **RPE** | - | ++ | + | ++ | | ++ | ++ | ++ | | + |
| **ChC** | - | + | - | + | | - | + | + | | - |

*Expression of mRNA (RNA) and protein (prot) expression: - = no expression; + = moderate expression; ++ = intense expression. NFL = neural fiber layer; GGL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer; PHR = photoreceptor layer; RPE = retinal pigment epithelium; ChC = choriocapillaris. IGFBP-3 and –6 mRNA in-situ hybridization was not performed.*

*IGFBP-3*

mRNA for IGFBP-3 was not determined because of technical problems with the probe. Negative controls for IGFBP-3 immunohistochemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1F), we found IGFBP-3 protein in the ganglion cell layer, the plexiform layers, the photoreceptor inner segments and in the RPE. In the RPE, staining was localized to the basal side. Retinal and choroidal vessels were positive, while the choriocapillaris stained incidentally. Often intravascular fluid stained positive. Lens epithelium was negative, and the cornea and non-pigmented epithelium of the ciliary body showed only slight staining.

In CNV (Table 7.3, Figure 7.2F), variable protein staining was found in newly formed vessels and in fibroblasts. RPE cells stained positive in all CNV.

*IGFBP-4*

The sense probe for IGFBP-4 mRNA, as well as negative controls for IGFBP-1 immunohistochemistry, showed no staining. In the normal retina (Table 7.2, Figure 7.1C,G), we found IGFBP-4 mRNA in the neuroretina, the RPE and in the corneal layers. IGFBP-4 protein was detected in the nerve fiber layer, ganglion cell layer, inner nucelar layer, the photoreceptor inner segments and in the RPE. In the RPE, staining was localized to the basal side. Positive staining was also found in large choroidal vessels and retinal vessels. Lens epithelium was positive, as was corneal endothelium and non-pigmented epithelium of the ciliary body. Stroma of the choroid, iris and sclera stained intensely positive.

In CNV (Table 7.3, Figure 7.2C,G), IGFBP-4 mRNA was detected in endothelial cells, RPE, fibroblasts and other cells. IGFBP-4 protein staining was found mostly in RPE and fibroblasts, and to a lesser extend in vascular endothelial cells. The stroma of most CNV stained intensely.

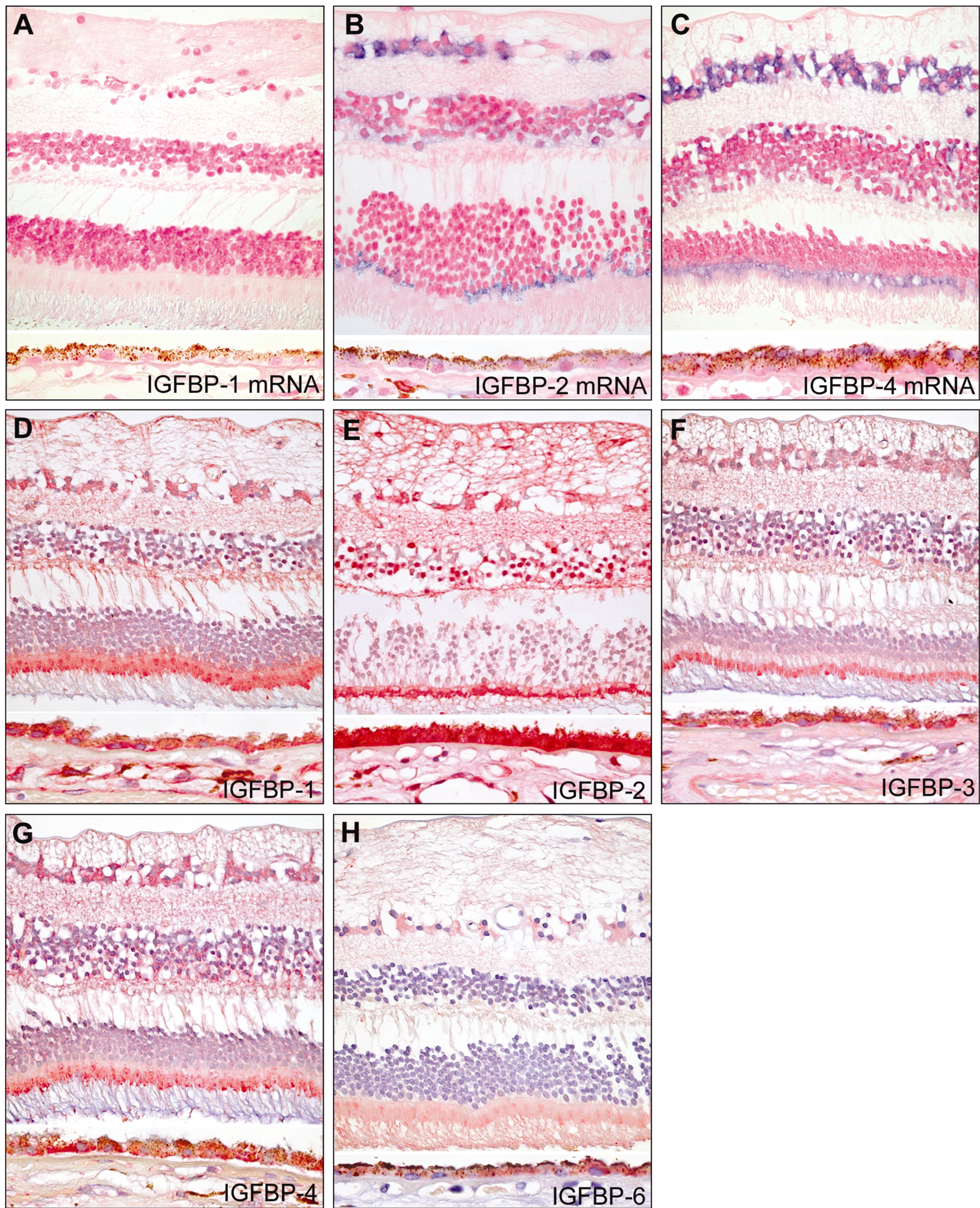


***Figure 7.1*** ***Localization of IGFBPs in the posterior pole of the human eye***. *Expression of IGFBP mRNA was detected in paraffin-embedded tissue by in-situ hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGFBP proteins was detected in paraffin-embedded tissue with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin.* ***(A)*** *IGFBP-1 mRNA****;*** ***(B)*** *IGFBP-2 mRNA;* ***(C)*** *IGFBP-4 mRNA.* ***(D)*** *IGFBP-1 protein;* ***(E)*** *IGFBP-2 protein;* ***(F)*** *IGFBP-3 protein;* ***(G)*** *IGFBP-4 protein;* ***(H)*** *IGFBP-6 protein. mRNA for IGFBP-3, -5 and -6 were not determined because of technical problems; neither was IGFBP-5 protein. Original magnification X400.*

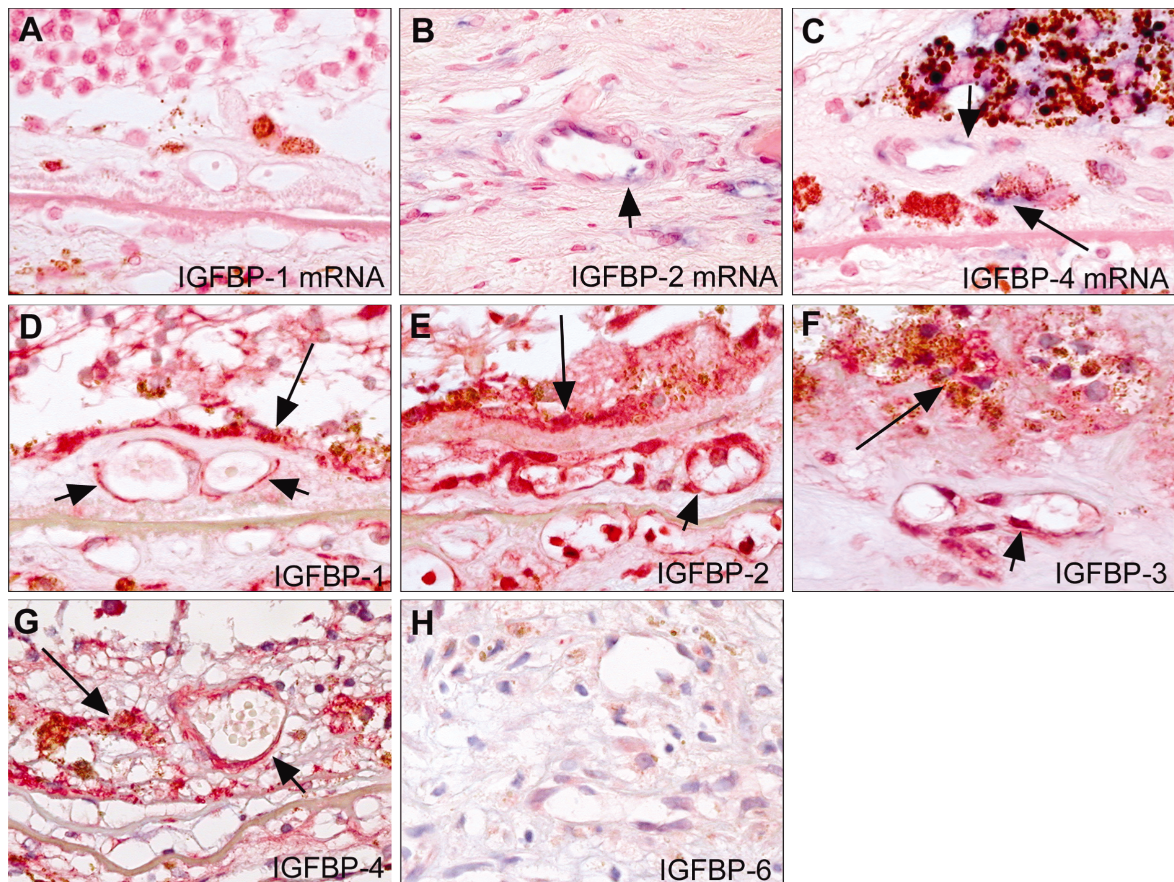


*Figure 7.2.* ***Localization of IGFBPs in CNV secondary to AMD****. Expression of IGFBP mRNA was detected in paraffin-embedded tissue by in-situ hybridization with digoxigenin-labeled probes, colored with (blue) NBT/BCIP, and counterstained with nuclear red. Expression of IGFBP proteins was detected in paraffin-embedded tissue with polyclonal antibodies, and visualized with an alkaline phosphatase detection system using a red chromogen and counterstained with hematoxylin.* ***(A)*** *IGFBP-1 mRNA in CNV11;* ***(B)*** *IGFBP-2 mRNA in CNV 11;* ***(C)*** *IGFBP-4 mRNA in CNV12;* ***(D)*** *IGFBP-1 protein in CNV11;* ***(E)*** *IGFBP-2 protein in CNV11;* ***(F)*** *IGFBP-3 protein in CNV1;* ***(G)*** *IGFBP-4 protein in CNV9;* ***(H)*** *IGFBP-6 protein in CNV2. mRNA for IGFBP-3, -5 and -6 were not determined because of technical problems; neither was IGFBP-5 protein. Original magnification X400.*

*IGFBP-5*

mRNA and protein for IGFBP-5 were not determined because of technical problems with the probe and the antibodies.

*IGFBP-6*

mRNA for IGFBP-6 was not determined because of technical problems with the probe. Negative controls for IGFBP-6 immunohistochemistry showed no staining. In the normal retina (Table 7.2, Figure 7.1H), we found little IGFBP-6 protein in the ganglion cell layer and the photoreceptor inner segments, and some staining in the RPE. Non-pigmented epithelium of the ciliary body showed slight staining.

In CNV (Table 7.3, Figure 7.2H) only some RPE and fibroblasts showed positive staining for IGFBP-6 protein.

*TABLE 7.3 MRNA ANP PROTEIN EXPRESSION OF IGFBPS IN NEOVASCULAR AMD*

| | | IGFBP-1 | | | | IGFBP-2 | | | | IGFBP-3 | | | | IGFBP-4 | | | | IGFBP-6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O |
| CNV1 | mRNA | ○ | ○ | ○ | ○ | ◍ | ◍ | ◍ | ◍ | | | | | ● | ◍ | ● | ◍ | | | | |
| | protein | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ○ | ◍ | ◍ | ○ |
| CNV2 | mRNA | ○ | ○ | ○ | ○ | ○ | ● | ◍ | ◍ | | | | | ◍ | ● | ⊘ | ⊘ | | | | |
| | protein | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ○ | ● | ◍ | ○ |
| CNV3 | mRNA | ⊘ | ○ | ○ | ○ | ◍ | ● | ◍ | ◍ | | | | | ● | ● | ● | ● | | | | |
| | protein | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ◍ | ● | ● | ● | ● | ○ | ◍ | ○ | ○ |
| CNV4 | mRNA | ⊘ | ○ | ○ | ◍ | ● | ◍ | ● | ◍ | | | | | ● | ● | ● | ● | | | | |
| | protein | ● | ● | ● | ● | ● | ● | ● | ● | ◍ | ● | ◍ | ◍ | ● | ● | ● | ● | ○ | ◍ | ◍ | ◍ |
| CNV5 | mRNA | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | | | | | ○ | ◍ | ◍ | ◍ | | | | |
| | protein | ● | ● | ⊘ | ⊘ | ● | ● | ⊘ | ● | ◍ | ● | ◍ | ● | ◍ | ● | ⊘ | ◍ | ○ | ○ | ○ | ◍ |
| CNV6 | mRNA | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | | | | | ○ | ○ | ○ | ○ | | | | |
| | protein | ○ | ● | ○ | ◍ | ○ | ● | ○ | ○ | ○ | ◍ | ○ | ○ | ◍ | ● | ◍ | ◍ | ○ | ○ | ○ | ○ |
| CNV7 | mRNA | ○ | ○ | ○ | ○ | ◍ | ◍ | ◍ | ○ | | | | | ● | ◍ | ● | ⊘ | | | | |
| | protein | ◍ | ● | ◍ | ◍ | ● | ● | ● | ● | ◍ | ● | ◍ | ◍ | ⊘ | ⊘ | ⊘ | ⊘ | ○ | ○ | ○ | ○ |
| CNV8 | mRNA | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | | | | | ⊘ | ⊘ | ⊘ | ⊘ | | | | |
| | protein | ● | ◍ | ● | ● | ● | ● | ● | ● | ○ | ● | ● | ● | ● | ● | ● | ● | ○ | ◍ | ○ | ○ |
| CNV9 | mRNA | ○ | ○ | ○ | ○ | ● | ● | ● | ● | | | | | ◍ | ● | ◍ | ⊘ | | | | |
| | protein | ● | ● | ● | ● | ● | ● | ● | ● | ◍ | ◍ | ◍ | ◍ | ● | ● | ● | ◍ | ○ | ◍ | ○ | ○ |

| | | IGFBP-1 | | | | IGFBP-2 | | | | IGFBP-3 | | | | IGFBP-4 | | | | IGFBP-6 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O | EC | RP | FB | O |
| **CNV10** | **mRNA** | ○ | ○ | ○ | ○ | ◐ | ◐ | ○ | ○ | | | | | ◐ | ◐ | ◐ | ◐ | | | | |
| | **protein** | ◐ | ● | ◐ | ◐ | ● | ● | ● | ● | ○ | ○ | ◐ | ◐ | ○ | ◐ | ◐ | ◐ | ○ | ○ | ○ | ○ |
| **CNV11** | **mRNA** | ○ | ○ | ○ | ○ | ◐ | ● | ● | ○ | | | | | ⊘ | ⊘ | ⊘ | ⊘ | | | | |
| | **protein** | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ◐ | ● | ● | ● | ◐ | ○ | ○ | ○ | ○ |
| **CNV12** | **mRNA** | ○ | ○ | ○ | ○ | ◐ | ◐ | ○ | ○ | | | | | ◐ | ● | ◐ | ◐ | | | | |
| | **protein** | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ◐ | ● | ◐ | ◐ | ○ | ○ | ○ | ○ |
| **CNV13** | **mRNA** | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ | | | | | ⊘ | ⊘ | ⊘ | ⊘ | | | | |
| | **protein** | ● | ● | ● | ● | ● | ● | ● | ● | ◐ | ◐ | ◐ | ◐ | ○ | ● | ◐ | ○ | ○ | ○ | ○ | ○ |
| **CNV14** | **mRNA** | | | | | | | | | | | | | | | | | | | | |
| | **protein** | ◐ | ◐ | ◐ | ○ | ◐ | ◐ | ◐ | ○ | ○ | ◐ | ○ | ○ | ○ | ◐ | ⊘ | ⊘ | ○ | ◐ | ◐ | ○ |
| **CNV15** | **mRNA** | | | | | | | | | | | | | | | | | | | | |
| | **protein** | ◐ | ◐ | ◐ | ○ | ◐ | ◐ | ◐ | ○ | ○ | ◐ | ○ | ○ | ○ | ◐ | ◐ | ⊘ | ⊘ | ⊘ | ⊘ | ⊘ |
| **CNV16** | **mRNA** | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | | | | | ◐ | ○ | ○ | ◐ | | | | |
| | **protein** | ● | ● | ● | ○ | ● | ● | ● | ◐ | ◐ | ◐ | ◐ | ◐ | ◐ | ● | ◐ | ◐ | ○ | ○ | ○ | ○ |
| **CNV17** | **mRNA** | ⊘ | ○ | ⊘ | ○ | | | | | | | | | | | | | | | | |
| | **protein** | ◐ | ● | ● | ◐ | ● | ◐ | ● | ● | ○ | ● | ◐ | ○ | ○ | ◐ | ◐ | ○ | ○ | ◐ | ○ | ○ |

*Categories of expression:* ○ *= no expression;* ◐ *= 1 to 50% of cells;* ● *= 51 – 100% of cells. EC = endothelial cells; RP = retinal pigment epithelium; FB = fibroblast-like cells; O = other cell types.* ⊘ *= not classifiable; open spaces indicate not performed*

## DISCUSSION

In the normal retina, including RPE, we localized mRNA expression of IGFBP-2 and 4. The localization is consistent with earlier findings of *in-situ* studies.[278,279] In contrast, in adult rat retina, mRNA was not detected for IGFBP-2 and IGFBP-4.[261] This difference can be explained by difference in species, or in different sensitivity of used techniques.
We did not detect IGFBP-1 mRNA in the normal eye, consistent with earlier findings.[261,280] However, the presence of IGFBP-1 mRNA can not be excluded in the eye, since in general only a part of total mRNA is detected with digoxigenin-labeled mRNA *in-situ* hybridization in paraffin-embedded material.[281]
IGFBP proteins were found in a spatially differentiated pattern in the normal human eye. IGFBP-2 was detected in a more extensive pattern than described by Miyamura and coworkers.[279] This can partly be explained by the shorter intervals between enucleation and fixation of our material, because most specimens we studied were surgically enucleated eyes or excised CNV, while Miyamura used donor eyes with postmortem times ranging from 4 to 21 hours. Furthermore different techniques and different antibodies used could account for the difference in expression. IGFBP-3 was also found in intravascular fluid, consistent with the fact that IGFBP-3 is the most abundant IGFBP in serum, and binds more than 95% of the IGF.[282] The circulating IGF-I/IGF-BP complexes limit access of IGF-I to specific tissues and to the IGF receptor and also function as a reservoir for IGF-I. Intravascularly, the IGF-I/IGFBP3 dimer forms a complex with the acid-labile subunit, resulting in a prolonged half-life of several hours. When released from this complex, IGF-I can enter target tissues with help of other binding proteins.[168]
Little IGFBP-6 was detected in the normal eye. This could be explained by the higher affinity of IGFBP-6 to IGF-II than to IGF-I.[283] IGF-II expression in the eye is low,[278] and IGF-II is important in fetal growth but has no effect on postnatal somatic growth.[284]
The function of IGFBPs in the normal eye is still unclear. The presence in the outer retina suggests a role in the maintenance of normal physiology of the retina.[266]
In CNV secondary to ARM we found mRNA of IGFBP-2 and IGFBP-4, and IGFBP-1, -2, -3, -4 proteins, and rarely IGFBP-6 protein localized in endothelial cells, RPE cells and fibroblasts. Earlier we detected IGF-I and IGF-IR in neovascular AMD. Since IGF-I has the capability of stimulating angiogenesis in the eye,[170] its presence could indicate a function in the pathogenesis of neovascular AMD. The presence of IGFBPs strengthen this conclusion, since IGFBPs are major modulators of IGF-I function. The interaction of IGF-I with one of the six major IGFBPs generally blocks receptor activation.[167] Besides inhibition of IGF-I action, some of the IGFBPs

(IGFBP-1, IGFBP-5 and mostly IGFBP-3) have paradoxical stimulatory effect on IGF activity.[167] Furthermore, some IGFBPs have actions that are independent of IGF-RI binding. IGFBP-1 stimulates cell migration through interaction with the $\alpha_5\beta_1$-integrin (fibronectin receptor).[285] IGFBP-3 can inhibit the growth of cells independent of IGF-IR,[286] and induces apoptosis.[287] IGFBP-3 has a number of ligands in addition to IGF-I, such as plasminogen and certain cell-surface and matrix components.[167] Finally, at least some IGF-R1 independent actions of IGFBP-3 and IGFBP-5 are assumed to be mediated through signaling receptors located on the plasma membrane of target cells, of which the type V TGF-β receptor may be one.[288]

The influence of the various members of the IGF-I family on CNV needs to be established. Several authors suggest hypoxia or relative hypoxia as a stimulus in the pathogenesis of neovascular AMD.[74,144] Averbukh and coworkers showed that both hypoxia and relative hypoxia may cause IGF system stimulation in the retina of a neonatal rat model, through upregulation of IGF-IR and IGFBPs.[289] This stimulation may result in neovascularization, suggesting that the IGF system may play an important role in angiogenesis induced by relative tissue hypoxia.[289]

In conclusion, we localized mRNA of IGFBP-2 and -4 in CNV resulting from AMD, as well as most IGFBP proteins. The mRNA and proteins were detected in newly formed vessels, in RPE and in fibroblasts. Together with the finding of IGF-I and its receptor in CNV membranes, the presence of these members of the IGF family may indicate a role of the IGF family in the pathogenesis of neovascular AMD. The functional role of the various IGF family members in the pathogenesis of AMD needs to be established.

# CHAPTER 8

## SOMATOSTATIN RECEPTOR 2A EXPRESSION IN CHOROIDAL NEOVASCULARIZATION SECONDARY TO AMD


### ABSTRACT

**Purpose:** The growth of ocular neovascularization is regulated by a balance between stimulating and inhibiting growth factors. Somatostatin effects angiogenesis by inhibiting the growth hormone/insulin-like growth factor axis and also has a direct anti-proliferative effect on human retinal endothelial cells. The purpose of our study is to investigate the expression of somatostatin receptor (sst) subtypes and particularly sst subtype 2A ($sst_{2A}$) in normal human macula, and to study $sst_{2A}$ in different stages of age-related maculopathy (ARM), because of the potential anti-angiogenic effect of somatostatin analogues.

**Methods:** Sixteen eyes (10 enucleated eyes, 4 donor eyes and 2 surgically removed choroidal neovascular (CNV) membranes) of 15 patients with eyes at different stages of ARM were used for immunohistochemistry. Formaldehyde-fixed paraffin-embedded slides were incubated with a polyclonal anti-human $sst_{2A}$ antibody. mRNA expression of five sst subtypes and somatostatin was determined in the posterior pole of 3 normal human eyes by reverse transcriptase-polymerase chain reaction.

**Results*:*** The immunohistochemical expression of $sst_{2A}$ in newly formed endothelial cells and fibroblasts-like cells was strong in fibrovascular CNV membranes. mRNA of sst subtypes 1, 2A and 3, as well as somatostatin, was present in the normal posterior pole; sst subtypes 4 and 5 were not detectable.

**Conclusions***:* Most early-formed CNV in ARM express $sst_{2A}$. We confirmed the presence of mRNA of sst subtype 2A in normal human macula, and demonstrated that also subtype 1 and 3, as well as somatostatin, are present. $Sst_{2A}$ receptors bind potential anti-angiogenic somatostatin analogues such as octreotide. Therefore, somatostatin analogues may be an effective therapy in early stages of neovascular AMD.

## INTRODUCTION

Age-related maculopathy (ARM) is the major cause of blindness in people over 65 years of age in the Western world. The prevalence of ARM is up to 14% in people more than 85 years.[3] Late stages of ARM, also called age-related macular degeneration (AMD), include geographic atrophy and neovascular macular degeneration. The neovascular form is characterized by choroidal neovascularization (CNV) and is responsible for 80% of cases of severe vision loss.[3] These numbers will increase because of the increasing age of the population. In CNV, newly formed vessels from the underlying choroid grow beneath the retinal pigment epithelium (RPE) and the retina.[251] Although the morphology of angiogenesis in CNV secondary to AMD has been described in detail, the pathogenesis is still poorly understood. A balance between a number of stimulating and inhibiting growth factors regulates the growth of neovascularization.[251] Vascular endothelial growth factor (VEGF), an endothelial specific mitogen, is regarded as one of the most important ocular angiogenic factors, especially in ischemic disease.[144,145,148,159,251,290,291] Other regulating growth factors include fibroblast growth factors (FGFs), transforming growth factor-β (TGF-β) and insulin-like growth factor-I (IGF-I). Most of these growth factors are shown to be upregulated in a diversity of cells (RPE, fibroblasts, capillary endothelial cells) involved in CNV.[145,159,160,189,190,246,247]
Recently, it has been shown in a transgenic mouse model that inhibition of growth hormone (GH), mediated by IGF-I, can inhibit ischemia-induced retinal neovascularization in vivo.[14] GH secretion is inhibited by somatostatin and somatostatin analogues. Systemic treatment with a somatostatin analogue diminished the level of ocular neovascularization in mice.[177]
Somatostatin binds with high affinity to 5 subtype receptors ($sst_1$ to $sst_5$). These receptors were identified in various animal retinas.[292-294] The exact role of a direct receptor-mediated effect by somatostatin analogues is still unknown. To date, information about $sst_2$ receptor expression in CNV is not available, and until now sst subtype expression has not been described in normal human retina.
The purpose of our study was to investigate the expression of somatostatin receptor 2A ($sst_{2A}$) in different stages of ARM, and the expression of sst subtypes and somatostatin in normal human macula.

## MATERIALS AND METHODS

The study was performed according to the tenets of the Declaration of Helsinki. Enucleation or surgical excision of subfoveal CNVs was performed after obtaining informed consent of the patient.

*Patients*

All eyes were retrieved from the files from the Ophthalmic Pathology Department of the University Hospital of Rotterdam. Sixteen eyes (10 enucleated eyes, 4 donor eyes and 2 surgically removed subretinal neovascular membranes) of 15 patients with eyes at different stages of ARM were used for immunohistochemistry. The description of each eye is given in Table 8.1. Eight eyes (of 7 patients) had clinical diagnoses of AMD. In 8 other eyes, ARM was diagnosed histopathologically according to the following criteria: Early stages of ARM (n=3) were characterized by the presence of basal laminar deposits, basal linear deposits (BLD), soft drusen, and thickening of Bruch's membrane.[112] Neovascular AMD (n=12) was classified as sub-RPE CNV, subretinal CNV (between neuroretina and RPE) or mixed sub-RPE and subretinal CNV.[120,295] Photoreceptors, Bruch's membrane and BLD were helpful in the orientation of the specimens.[120] Sub-RPE CNV and mixed CNV, or subretinal CNV in elderly patients in the presence of BLD or soft drusen were classified as CNV secondary to AMD.[120] In CNV, we recorded the presence of fibrovascular or fibrocellular tissue, hemorrhage, vascular endothelium, BLD and RPE.[120] One eye was classified as non-neovascular (geographic) AMD. Eight enucleated eyes without ARM (donor eyes or enucleated for other reasons) were used as controls (Table 8.2). The eyes were processed for routine diagnostic procedures by fixation in formaldehyde and were embedded in paraffin.

*Immunohistochemistry*

Rabbit anti-human $sst_{2A}$ polyclonal antibody (R2-88) was kindly provided by Dr. A. Schonbrunn (Department of Integrative Biology and Pharmacology, University of Texas Houston Medical School, USA). The antibody was raised against a 22-amino acid peptide located at the C-terminal region of the $sst_2$ receptor. The $sst_{2A}$ antibody had been characterized and tested before by Western blot analysis and peptide binding.[296,297] Mouse monoclonal antibody against smooth muscle actin (SMA) was obtained from Biogenex (San Ramon, CA, USA) and mouse monoclonal antibody against macrophages (CD68) from Dako (Glastrup, Denmark). Five μm sections were prepared. The sections were deparaffinated, rehydrated and (for $sst_{2A}$ and CD68)

*TABLE 8.1 PATIENT MATERIAL AND $SST_{2A}$ RECEPTOR EXPRESSION IN EYES WITH ARM*

| No. | Age/sex | OD/OS | Clinical description | Histological classification | $Sst_{2A}$ expression* | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Preexistent tissue | | | Neovascular tissue | | | |
| | | | | | | | | FV | | FC | |
| | | | | | RPE | CC | CH | EC# | FBL | EC# | FBL |
| **ARM1** | 85/M | OS | necrotising sclerokeratomalacy | early ARM: BLD | ++ | 0 | ++ | . | . | . | . |
| **ARM2** | 98/F | OS | corneal ulcer | early ARM: confluent soft drusen | ++ | + | ++ | . | . | . | . |
| **ARM3** | 96/F | OD | staphyloma, suspected ciliary body melanoma | early ARM: BLD; glaucoma; corneal ulcer | ++ | 0 | + | . | . | . | . |
| **ARM4** | 77/M | OS | neovascular glaucoma | nonneovascular AMD; early geographic atrophy; occlusion central retinal artery; ischemic retinal disease | ++ | 0 | + | . | . | . | . |
| **CNV1** | 79/M | U | surgically excised CNV | mixed CNV, FV and FC, hemorrhage | NP | NP | NP | 37/48 | ++ | NP | 0 |
| **CNV2** | 79/F | U | surgically excised CNV | subretinal CNV, FV and FC, hemorrhage | NP | NP | NP | 15/18 | ++ | NP | ++ |
| **CNV6** | 72/M | OS | disciform MD | mixed CNV, BLD, FV and FC, hemorrhage | + | 0 | + | 28/50 | + | 0/7 | 0 |
| **CNV7** | 86/M | OS | disciform MD, acute glaucoma | sub-RPE CNV, BLD, FV and FC, hemorrhage; retinal detachment; posterior uveitis | ++ | + | ++ | NP | NP | 2/4 | ++ |
| **CNV8** | 91/M | OS | donor eye | disciform MD, mixed CNV, BLD, FC | NC | 0 | NC | . | . | 0/6 | + |
| **CNV9** | 87/M | OS | donor eye | disciform MD, mixed CNV, BLD, FV and FC | ++ | + | ++ | 11/16 | + | 3/5 | + |
| **CNV10** | 83/M | OD | painful eye, suspected uveal melanoma | ischemic retinal disease; disciform MD, mixed CNV, BLD, FV and FC, hemorrhage | ++ | 0 | + | 26/64 | ++ | 0/3 | ++ |
| **CNV11** | 73/M | OS | disciform MD | subretinal CNV, FC and FV | ++ | 0 | + | 13/15 | ++ | NC | + |
| **CNV12** | 73/M | OD | disciform MD, post irradiation | subretinal CNV, FV | + | 0 | ++ | 2/3 | + | . | . |
| **CNV13** | 82/M | OD | disciform MD | mixed CNV, confluent soft drusen, FC | + | 0 | 0 | . | . | 13/36 | 0 |
| **CNV14** | 85/F | OS | post surgical endophthalmitis | subretinal CNV, FV, endophthalmitis, uveitis | + | 0 | + | 2/2 | + | . | . |
| **CNV17** | 84/F | OS | disciform MD | mixed CNV, FV and FC, BLD, hemorrhage | + | 0 | + | 0/2 | + | NC | 0 |

**Categories of $sst_{2A}$ expression: 0 = 0 – 10% positive cells; + = 11 – 50% positive cells; ++ = 51 – 100% positive cells. #$Sst_{2A}$ expression in endothelial cells in CNV was quantitatively determined by counting the proportion of positive vessels in randomly selected sections. (MD = macular degeneration; mixed CNV = mixed subretinal and sub-RPE CNV; FV = fibrovascular CNV; FC = fibrocellular scar; BLD = basal laminar deposits; RPE = retinal pigment epithelium; CC = choriocapillaris; CH = choroidal vessels; CNV = choroidal neovascularization; EC = endothelial cells; FBL = fibroblasts-like cells. U = unknown; NC = not classifiable; NP = not present)*

**Table 8.2. Patient data and sst receptor subtype expression in normal eyes**

| No | Age/ sex | OD/ OS | Clinical description | Sst receptor subtype expression* (RT-PCR) | | | | | | | $Sst_{2A}$ expression† (Immunohistochemistry) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | $Sst_1$ | $Sst_{2A}$ | $Sst_3$ | $Sst_4$ | $Sst_5$ | SS14 | HPRT | RPE | CC | CH |
| **1** | 71/U | OD | donor eye | . | . | . | . | . | . | . | ++ | + | ++ |
| **2** | 51/M | OD | ciliary body melanoma | . | . | . | . | . | . | . | + | 0 | + |
| **3** | 78/M | OS | choroidal melanoma | . | . | . | . | . | . | . | ++ | 0 | + |
| **4** | 81/M | OS | tarsal squamous cell carcinoma | . | . | . | . | . | . | . | + | + | ++ |
| **5** | 42/M | OS | choroidal melanoma | . | . | . | . | . | . | . | ++ | 0 | ++ |
| **6** | 76/F | OS | choroidal melanoma | . | . | . | . | . | . | . | ++ | 0 | ++ |
| **7** | 57/M | OS | recurrent conjunctival melanoma | . | . | . | . | . | . | . | + | 0 | + |
| **8** | 60/M | OS | choroidal melanoma | . | . | . | . | . | . | . | ++ | 0 | ++ |
| **9** | 69/M | OD | ciliary body adenoma | + | + | + | - | - | + | + | . | . | . |
| **10** | 78/M | OS | spindle cell nevus | + | + | + | - | - | + | + | . | . | . |
| **11** | 26/M | OS | choroidal melanoma | + | + | + | - | - | + | + | . | . | . |

**Categories of sst subtype expression (RT-PCR): - = no expression, + = positive expression. †Categories of $sst_{2A}$ expression (immunohistochemistry): 0 = 0 – 10% positive cells; + = 11 – 50% positive cells; ++ = 51 – 100% positive cells. (SS14 = somatostatin; HPRT = hypoxanthine-guanine phosphoribosyl transferase; RPE = retinal pigment epithelium; CC = choriocapillaris; CH = choroidal vessels. U = unknown)*

microwave heated for 10 minutes. After the slides were blocked with normal goat serum (Dako, 1:10) for 15 minutes, they were incubated with the $sst_{2A}$ antibody (1:1000) or CD68 antibody (1:2000) overnight at 4°C, or with anti-SMA (1:150) for 1 hour at room temperature. The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both from Biogenex) for 30 minutes. The bound antibodies were visualized by incubating the sections with new fuchsin for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy. We determined the $sst_{2A}$ expression quantitatively in endothelial cells of CNV by counting the proportion of positive vessels in randomly selected sections. The total number of counted vessels was pooled, and the proportions of positive cells in fibrovascular and fibrocellular CNV were compared with $\chi^2$ analysis. For other tissue components, we semi-quantitatively graded $sst_{2A}$ expression in 3 categories: 0 (0 – 10% positive cells), 1 (11 – 50% positive cells) and 2 (51 – 100% positive cells). Negative controls for immunohistochemistry included 1) omission of the primary antibody, 2) use of an irrelevant antibody of the same isotype, and 3) preabsorbtion of the $sst_{2A}$ antibodies with the immunizing receptor peptide for 4 hours at a concentration of 3 μg/ml.

*RT-PCR*

In order to study the mRNA expression of sst subtypes in normal human eyes, posterior poles from three eyes (Table 8.2) were dissected directly after enucleation. A sample of about 0.2 $mm^2$ located in the macula, including RPE, choroid and sclera, was snap frozen in liquid nitrogen. RT-PCR was performed as described before[298] but with different primers (Table 8.3).

Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the $polyA^+$ mRNA preparation (because the sst genes are intronless), the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the hypoxanthine-guanine phosphoribosyl transferase (HPRT) specific primers served as positive control for the quality of the cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the sst receptor subtypes, 0.1 to 0.001 μg of human genomic DNA, representing approximately 30.000 to 300 copies of sst-template, was amplified in parallel with the cDNA samples. As a positive control for the PCR of HPRT and somatostatin cDNA, aliquots of a cDNA sample known to contain somatostatin and HPRT mRNA were amplified, because these primer pairs did enclose introns in the genomic DNA.

**TABLE 8.3 PRIMERS USED FOR RT-PCR ANALYSIS**

| receptor | primer | sequence (5' –3')* | product size (base pair) |
|---|---|---|---|
| **$sst_1$** | forward | ATGGTGGCCCTCAAGGCCGG | 318 |
| | reverse | CGCGGTGGCGTAATAGTCAA | |
| **$sst_{2A}$** | forward | GCCAAGATGAAGACCATCAC | 414 |
| | reverse | GATGAACCCTGTGTACCAAGC | |
| **$sst_3$** | forward | CCAACGTCTACATCCTCAACC | 314 |
| | reverse | TCCCGAGAAGACCACCAC | |
| **$sst_4$** | forward | ATCTTCGCAGACACCAGACC | 321 |
| | reverse | ATCAAGGCTGGTCACGACGA | |
| **$sst_5$** | forward | CGTCTTCATCATCTACACGG | 226 |
| | reverse | CCGTCTTCATCATCTACACGG | |
| **SS14** | forward | GATGCTGTCCTGCCGCCTCCAG | 349 |
| | reverse | ACAGGATGTGAAAGTCTTCCA | |
| **HPRT** | forward | CAGGACTGAACGTCTTGCTC | 413 |
| | reverse | CAAATCCAACAAAGTCTGGC | |

*The sequences of the primers for $sst_1$ were derived and adapted from Wulfsen et al.,[41] for $sst_5$ from Kubota et al.,[42] and all others were designed by use of the Primer3! software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and the appropriate GenBank entries. (SS14 = somatostatin; HPRT = hypoxanthine-guanine phosphoribosyl transferase)*

## RESULTS

*Immunohistochemistry*

In normal retina (n=8) we found strong $sst_{2A}$ expression in the inner plexiform layer (IPL) and moderate expression in the outer plexiform layer (OPL), the cellular membrane of the inner nuclear layer (INL) (Figure 8.1A), and the RPE. RPE stained most frequently at the apical side in a membranous pattern (Figure 8.1B), which was also noted in tangentially cut sections. Thick-walled choroidal vessels stained mostly positive, whereas chorio-capillaris only sporadically (Table 8.1). In negative controls, no staining was detected.

In the eyes with early ARM (n=3), $sst_{2A}$ expression of the neuroretina, choroidal vessels and choriocapillaris was similar to normal controls (Table 8.1). The RPE stained positive in all cases. BLD were negative (Figure 8.1C).
In eyes with neovascular AMD (n=12), Bruch's membrane and BLD did not show $sst_{2A}$ expression (Table 8.1). The choriocapillaris showed focal expression in only two eyes. Approximately 50 to 75% of thick-walled choroidal vessels stained positive, which was similar to normal controls. The CNV, both surgically excised and in enucleated eyes, could be subdivided in three groups, according to the activity of neovascularization. [120] The first group consisted of fibrovascular tissue with inflammatory cells, fibroblast-like cells and sparse fibrosis (n=2). The second group

consisted of fibrocellular scar tissue (n=2), and the third group consisted of a mixture of both fibrovascular and fibrocellular tissue (n=8). In the CNV, monolayers of pigmented cells adjacent to BLD were scored as RPE cells. Approximately half of these morphologically RPE cells showed $sst_{2A}$ expression. The expression of $sst_{2A}$ in newly formed endothelial cells was strong in fibrovascular membranes. Similarly, $sst_{2A}$ was strongly expressed in endothelial cells of mixed fibrovascular and fibrocellular membranes (Figure 8.1D,E,F). Fibroblast-like cells and macrophages stained strongly positive in young membranes and less strongly in older scars (Figure 8.1D,E,F,G). Little or negative staining was observed in old hypocellular scars (Figure 8.1G). Expression of endothelial cells in fibrovascular membranes (61.5%) was found statistically significant more often than in fibrocellular membranes (29.5%; $\chi^2$ analysis, $p<0.001$). Staining in CNV was considered specific, because peptide blocking significantly decreased staining of all structures mentioned.

In one eye with a mixed fibrovascular and fibrocellular membrane (eye number CNV10), we found positive staining of myofibroblasts in a hypercellular area of the underlying choroid in the posterior pole. This area also stained positive with antibodies directed against SMA and CD68, confirming the presence of myofibroblasts and macrophages.

In the eye with nonneovascular AMD, the staining pattern was similar to control tissue. The RPE stained positive. No staining was seen in the choriocapillaris, and vessels in the choroid were mostly positive.

*RT-PCR*

RT-PCR analysis of 3 posterior poles, including retina, RPE, choroid and sclera, revealed that mRNA encoding for $sst_1$, $sst_{2A}$, $sst_3$ and somatostatin is expressed in the posterior pole of normal human eyes. No mRNA encoding for $sst_4$ or $sst_5$ was detected (Figure 8.2, Table 8.2).

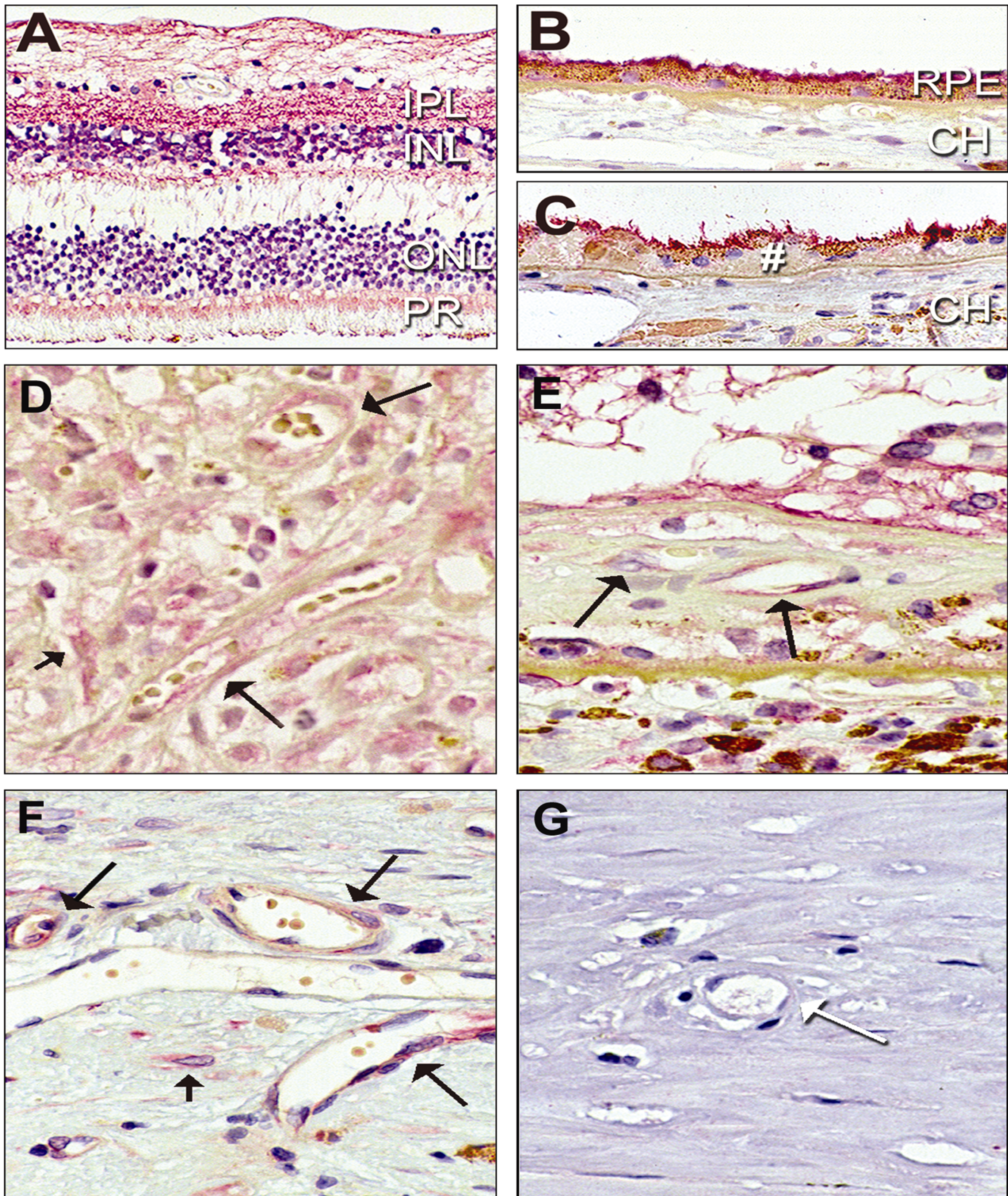


***Figure 8.1*** ***Immunolocalization of*** $sst_{2A}$ *in posterior pole of normal eyes and eyes with different stages of ARM. Immunohistochemistry was performed on paraffin-embedded tissue, and visualized with an alkaline phosphatase detection system using a red chromogen.* ***(A)*** *Positive staining in normal neuroretina, with strong* $sst_{2A}$ *expression in the inner plexiform layer (IPL) and moderate expression in the outer plexiform layer and the cellular membrane of the inner nuclear layer (INL).* ***(B)*** $sst_{2A}$ *staining of normal RPE, showing the membranous staining pattern on the apical side.* ***(C)*** $sst_{2A}$ *staining of an eye with early ARM, showing negative staining BLD and soft drusen (#).* ***(D through G)*** $sst_{2A}$ *staining of CNV in eyes with ARM.* ***(D)*** *Surgically excised fibrovascular CNV (eye CNV1), with many positive fibroblast-like cells.* ***(E)*** *Fibrovascular CNV (eye CNV12).* ***(F)*** *Mixed fibrovascular and fibrocellular CNV (eye CNV11). Long arrows: positive endothelium of newly formed vessels; short arrows: positive fibroblast-like cells.* ***(G)*** *Staining of a fibrocellular CNV (eye CNV 13) with negative endothelial cells (white arrow) and fibroblast-like cells. ONL, outer nuclear layer; PR, photoreceptor layer; RPE, retinal pigment epithelium; CH, choroids; BM, Bruch's membrane; NR, overlying neuroretina. Original magnification* ***(A)*** *×200;* ***(B through G)*** *×400.*

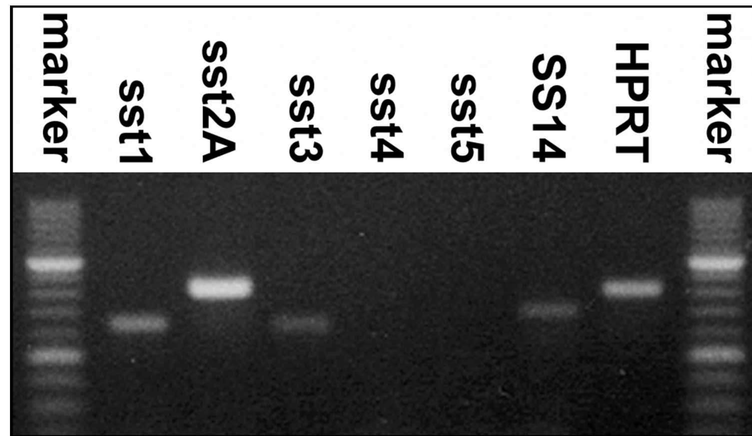


***Figure 8.2 Expression of sst receptor subtype mRNA** in the posterior pole of a normal human eye, detected by RT-PCR. $sst_1$, $sst_{2A}$, and $sst_3$ were detected. Signals for $sst_4$ and $sst_5$ were too low to detect or absent. mRNA for somatostatin (SS14) was also detected. HPRT was used as internal control. Marker, 100 bp.*

## DISCUSSION

In the present study normal human eyes and eyes with early and late stages of ARM express $sst_{2A}$. The localization of $sst_{2A}$ expression in the neuroretina is consistent with findings in rabbit[292] and rat[293] retina and reflects the assumed physiological neuromodulator function of somatostatin.[299,300] In early stages of ARM, the choroidal vasculature and neuroretinal tissue stained identically with control tissue. We found no expression of $sst_{2A}$ in BLD or drusen, which is in contrast with findings for other angiogenic growth factors such as VEGF.[144]
In eyes with neovascular AMD, we found strong expression of $sst_{2A}$ in endothelial cells and fibroblast-like cells in early CNV. The expression of $sst_{2A}$ in newly formed capillaries was abundant in fibrovascular CNV membranes. Similarly, in the active component of mixed fibrovascular/fibrocellular CNV, $sst_{2A}$ was strongly expressed in endothelial cells. Grant and co-workers demonstrated the presence of somatostatin receptors on cultured human retinal endothelial cells.[173] They showed a direct inhibitory action of a somatostatin analogue on proliferation of these endothelial cells. Therefore, the angiogenic cells of CNV membranes may be capable of receiving angiogenic inhibition, directly receptor mediated or indirectly via inhibition of GH and IGF-I by somatostatin. In mice retina, somatostatin analogues have an inhibitory effect on neovascularisation.[177] Somatostatin analogues, such as the long-acting octreotide, which binds to somatostatin receptor subtypes 2 and 5, are used as experimental treatment in neovascular eye diseases such as diabetic retinopathy.[301-303]
We found strong $sst_{2A}$ expression in fibroblast-like cells and macrophages in fibrovascular CNV and in intrachoroidal myofibroblasts. $Sst_{2A}$ staining in

myofibroblasts may be due to cross-reactivity to myosin,[304] but macrophages have been shown to express $sst_{2A}$.[305] Macrophages and choroidal fibroblasts are thought to be one of the main sources of VEGF in the early stage of the disease.[155,247,290] Both macrophages and choroidal fibroblasts are also capable of releasing other angiogenic factors such as tumor necrosis factor-α (TNF-α) and IGF-I.[306] Somatostatin analogues have been shown to inhibit the release of macrophage and monocyte products such as TNF-α, interleukin (IL)-1β, IL-6 and IL-8 in vitro,[307,308] although there are also conflicting data.[309] The functional role of somatostatin with regard to the angiogenic factor synthesis and release has to be established.

In the overlying neuroretina of eyes with CNV, we found no obvious change of $sst_{2A}$ expression and localization in comparison to normal eyes. This is in contrast to VEGF expression in neuronal tissue, which is upregulated under hypoxic circumstances.[144,148] This may indicate that the function of somatostatin on neuronal tissue is not influenced by hypoxic retinal disease. However, some care should be taken when interpreting these results, because they are semi quantitatively determined. It has recently been shown in a transgenic mice model that inhibition of GH, mediated by IGF-I, can inhibit ischemia-induced retinal neovascularization in vivo, but it does not reduce hypoxia-induced VEGF mRNA or protein levels. It was postulated that GH / IGF-I and VEGF may have distinct functions in the control of angiogenesis: VEGF may control acute oxygen regulation, whereas IGF-I may control neovascularization on the basis of availability of nutrients for cell division.[177] Our findings support the hypothesis that somatostatin and VEGF have distinct functions in the control of angiogenesis.

We confirmed local synthesis of $sst_{2A}$ in the macula of normal human eyes with RT-PCR. We also demonstrated the expression of mRNA encoding for sst subtypes 1 and 3. In rats, $sst_2$ appeared to be the major subtype in the retina, but all other subtypes were expressed in retina and posterior pole as well.[294] Differential expression of sst has also been described previously in the immune system.[310] We also found mRNA expression of the neuropeptide somatostatin in the human macula. Production of somatostatin in the retina has been shown in rats with Northern blot hybridization and mRNA in-situ hybridization.[311-313] The production of both somatostatin and its receptors simultaneously suggests an autocrine action of somatostatin in the human retina.

From our findings we conclude that the $sst_{2A}$ receptor in choroid and retina of early ARM and nonneovascular AMD is localized similar to normal controls. In eyes with CNV, the $sst_{2A}$ receptor is strongly expressed in the fibrovascular phase of CNV, as well as in intrachoroidal myofibroblasts. mRNA of sst subtypes 1, 2A and 3, as well as mRNA of somatostatin are expressed in the macula of the normal human eye. The functional role of somatostatin with regard to the synthesis and release of angiogenic

factors has to be established. Because of the sst expression in CNV, somatostatin analogues may be an effective therapy in early stages of neovascular AMD.

## ACKNOWLEDGEMENTS

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# CHAPTER 9

# RADIOTHERAPY OF NEOVASCULAR AGE-RELATED MACULAR DEGENERATION; A CLINICAL AND PATHOLOGICAL STUDY


## ABSTRACT

**Purpose:** Radiotherapy has recently been employed to treat patients with neovascular macular degeneration in order to prevent severe visual loss. Radiotherapy affects the evolution of neovascular macular degeneration directly by endothelial toxicity, leading to capillary closure, and/or indirectly through its attenuating effects on the inflammatory response, mediated by macrophages and other inflammatory cells.
**Methods**: In this study we describe the histopathologic findings in a patient with neovascular age-related macular degeneration (AMD) in both eyes whose right eye was treated with radiotherapy (5 times 2 Gy) 3 years before he died. The eyes were enucleated and investigated by light microscopy. Additionally, immunohistochemical investigation with antibodies against CD34 and CD68 was performed to identify patent endothelial cells and macrophages.
**Results:** Both eyes showed neovascular AMD consisting of mixed fibrocellular and fibrovascular membranes. Capillaries in both the choriocapillaris and the neovascular membrane were patent in both eyes. Macrophages were present in the choroidal neovascularizations of both eyes. Neither preexistent choroidal, intraretinal, nor neovascular vessels showed increased wall thickness as sign of radiation damage.
**Conclusion:** No radiation-related histopathologic effect could be demonstrated 3 years after radiation therapy in this patient with AMD.

## INTRODUCTION

Neovascular age-related macular degeneration (AMD) typically causes a decreased central vision over a short period of time when located subfoveally. This disease is a major cause for visual loss in the elderly population.[3,129] To date the only treatments proven to be successful are subfoveal laser photocoagulation[212] and photodynamic therapy,[213] although both therapies are less effective in patients with occult subfoveal choroidal neovascularization (CNV). Radiotherapy is one of many experimental treatments. Varying results have been published,[314-324] but a recently performed pooled analysis of different studies indicated that radiotherapy may only act to slow or delay the progress of the disease.[216] The mechanism of the effect of ionizing radiation on CNV is not known in detail. Radiotherapy affects the evolution of neovascular macular degeneration directly by endothelial toxicity, leading to capillary closure,[325] and/or indirectly through its attenuating effects on the inflammatory response, mediated by macrophages and other inflammatory cells.[325-327]
Complications of radiotherapy for AMD include radiation retinopathy,[321,328] optic neuropathy,[321,328] cataract[317,318,323] and, recently described, radiation-associated choroidal neovasculopathy after low-dose radiotherapy.[329,330]

This is the first report on histopathological findings after radiotherapy for macular degeneration so far. These findings may help to understand the effect of radiotherapy.

## MATERIALS AND METHODS

*Case Report*
A 67-year-old man was examined in 1990 because of vision loss. Fluorescein angiography showed neovascular AMD in both eyes. Photocoagulation treatment was applied to both eyes, temporally of the fovea. Afterwards, his visual acuity (VA) was 20/40 in his right eye (OD) and 20/20 in his left eye (OS) (Snellen vision), with a low hypermetropic correction. Ophthalmoscopically, the lesions had dried. In May 1991, VA was 20/24 OD and had dropped to 20/240 OS because of a large choroidal neovascular membrane. No additional treatment was instituted. In 1993, patient was referred to our hospital because of a decreased vision in his right eye. His VA was 20/80 OD and 20/200 OS. Fluorescein angiography showed recurrent lesions with early hyperfluorescence at the margins of the old scars. The right eye showed a classical CNV (Figure 9.1A,C), the left eye a classical CNV with occult components (Figure 9.1B,D). Because the patient fixated just at the point of leakage no laser

treatment was given. Instead, he was treated with radiotherapy to his right eye, with a dose of 10 Gy delivered in 5 fractions (Figure 9.2). By 1 month after the treatment patient's VA had dropped further to 20/160 OD. The last clinical examination took place in September 1996; his VA was 20/240 ODS with grade 3 nuclear cataract. On ophthalmoscopy, flat fibrovascular lesions were seen without exudation. In March 1997, the patient died of coronary heart disease at the age of 73 years. Autopsy was permitted and performed within 6 hours. The eyes were removed for histological examination.

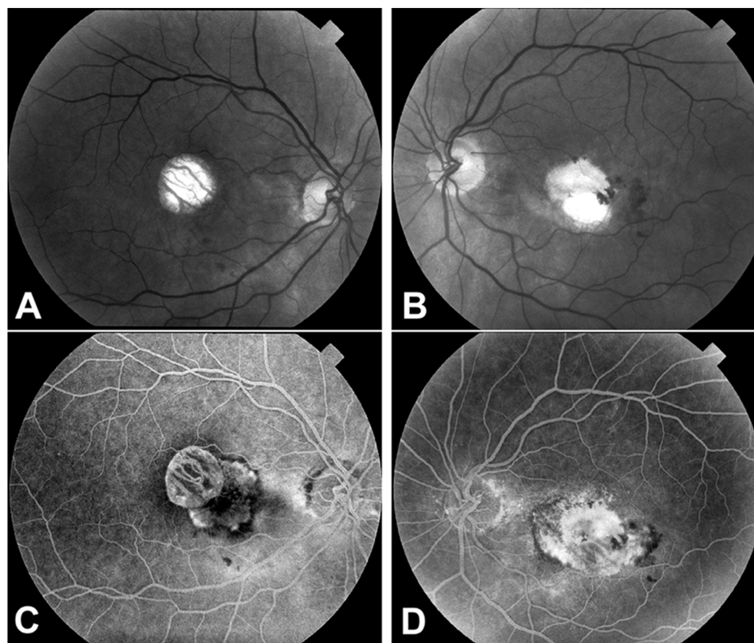


***Figure 9.1 Fluorescein angiography 8 months before radiotherapy*** ***(A,C)*** *Fluorescein angiography of the macular region of the right eye.* ***(A)*** *Red free picture showing areas of atrophy, laser scarring and neovascularization.* ***(C)*** *Early fluorescein angiography of the right eye, showing early hyperfluorescent lesions at the margins of the old scar.* ***(B,D)*** *Fluorescein angiography of the macular region of the left eye.* ***(B)*** *Red free picture showing areas of laser scarring and neovascularization.* ***(D)*** *Early fluorescein angiography, showing a mixed classical and occult lesion with early hyperfluorescence at the margins of the old scar.*

*Dosimetry*

The patient was treated with radiotherapy to his right eye with a dose of 10 Gy with 16 MeV electrons, in 5 fractions applied with sparing of the lens. The field size was 4 x 4 $cm^2$ at 100 cm SSD (source skin distance) using a Houston collimating system on a Siemens Mevatron KD-2 linear accelerator. The dose to the macula was calculated using an electron pencil beam model implemented in the Cadplan planning system. The contours are obtained from a CT image. Figure 9.2 shows the calculated isodose pattern. The macula is enclosed by the 100% isodose (2 Gy per fraction).

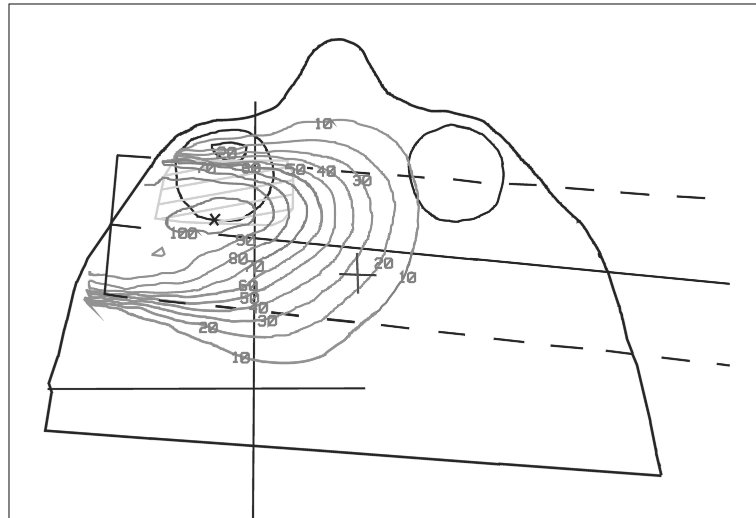


***Figure 9.2.*** *The dose distribution of the 16 MeV electron beam of 4 x 4 cm². The macula (x) is enclosed by the 100% isodose (2 Gy per fraction).*

*Material preparation and immunohistochemistry*

From each enucleated eye, a horizontal tissue block including the macula was excised and horizontally divided in 2 parts. One part was fixed by immersion in formalin for 24 hours and embedded in paraffin. The other part was frozen for other purposes. Five-µm thick paraffin sections were cut and stained for light microscopy with PAS, hematoxylin and eosin, and Mallory. A two-dimensional map was constructed from study of serial sections of both eyes (Figures 9.3A and 9.4A). For immunohistochemistry, monoclonal mouse antibodies against CD34 were obtained from Biogenex (San Ramon, CA, USA) and monoclonal mouse antibodies against CD68 from Dako (Glastrup, Denmark). Immunohistochemical staining was performed as described before.[248] In short, the sections were deparaffinated and rehydrated, and (for CD68) microwave-heated for 10 minutes. After the slides had been blocked with normal goat serum (Dako, 1:10) for 15 minutes, they were incubated with the CD68 antibodies (1:2000) overnight at 4°C, or with CD34 antibodies (1:20) for 1 hour at room temperature. The sections were further incubated with biotinylated multilink antibodies for 30 minutes, followed by alkaline phosphatase-labeled antibiotin (both Biogenex) for 30 minutes. The complex was then visualized by incubating the sections with new fuchsin (as a red chromogen) for 30 minutes in the dark. The slides were counterstained with Mayer's hematoxylin, mounted and examined by light microscopy.

## RESULTS

*Histopathologic examination*

The irradiated right eye (Figure 9.3B to F) showed a subretinal mixed fibrocellular and fibrovascular membrane (Figure 9.3B). The CNV comprised still identifiable RPE and subretinal basal laminar deposits (grade 3[112]). There was extensive loss of photoreceptors at the macular region. Vessels from the choriocapillaris could be demonstrated traversing Bruch's membrane (Figure 9.3D). Immunohistochemistry with CD34, a monoclonal antibody against endothelial cells, showed patent vessels in the choriocapillaris and in the neovascular membrane (Figure 9.3E). Staining with antibodies against macrophages (CD68) showed many macrophages in the CNV and in the underlying choroid (Figure 9.3F). Neither preexistent choroidal, retinal or neovascular vessels showed increased wall thickness as sign of radiation damage. Next to the CNV, a region of laser scarring was seen, with loss of neuroretina, choriocapillaris and choroidal structures (Figure 9.3B).

The left eye (Figure 9.4B to E) showed a large dome-shaped mixed fibrocellular and fibrovascular membrane, with mixed sub-RPE and subretinal areas (Figure 9.4B). Immunohistochemistry with CD34 demonstrated patent vessels in the choriocapillaris and in the neovascular membrane (Figure 9.4E). Staining with antibodies against CD68 showed many macrophages in the CNV and in the underlying choroid (not shown). The overlying neuroretinal layers were disorganized and atrophic. The CNV was partly overlying a region of laser scarring, with loss of choriocapillaris and choroidal structures (not shown).

## DISCUSSION

In this patient with bilateral neovascular AMD we showed that, 3 years after 10 Gy radiotherapy to the posterior pole of his right eye, choriocapillaries and neovascular capillaries were still patent and macrophages were present. The dose given is at the low end of the range of presently applied protocols.[216] There were no histologic signs of radiation effect on the preexistent vascular walls. This may be due to the relatively low dose of radiation or to the reversibility of minor damage.

Radiotherapy affects the evolution of neovascular macular degeneration by endothelial toxicity, resulting in narrowing or occlusion of blood vessels.[325-327] Endothelial cells are moderately sensitive to radiation and vessels may reveal manifestations of radiation injury months to years later.[325,331-333] In CNV, microvessel and endothelial cell loss

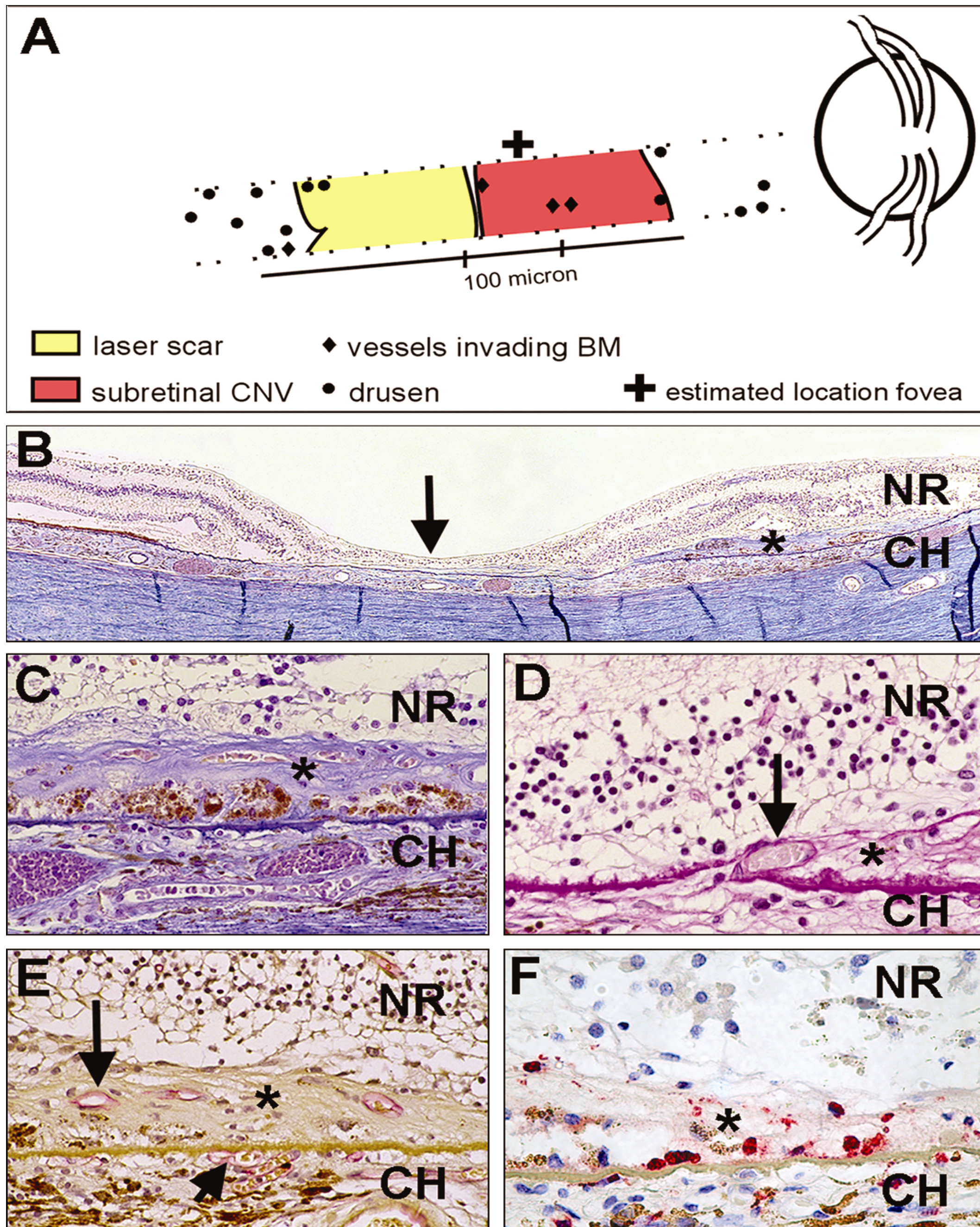


***Figure 9.3 Irradiated right eye.*** ***(A)*** *Two-dimensional reconstruction map showing size, shape and location of selected histopathologic features of the examined part of the macular region.* ***(B)*** *Histologic composition of macular region, showing a CNV membrane (*). A region of laser scarring is seen (arrow), with total atrophy of neuroretina, choriocapillaris and disturbance of choroidal structures (Mallory, original magnification x100).* ***(C)*** *Detail of CNV showing subretinal, fibrovascular region with intact choriocapillaris. (Mallory, original magnification x400).* ***(D)*** *At the margin of the CNV a vessel from the choriocapillaris traverses Bruch's membrane (arrow) (PAS, original magnification x400).* ***(E)*** *Immunohistochemical staining with antibodies against CD34, a marker for endothelial cells, with a red chromogen. Patent choriocapillaries (short arrow), as well as patent neovascular capillaries (long arrow) in the CNV are seen (original magnification x400).* ***(F)*** *Immunohistochemical staining with antibodies against CD68, a marker for macrophages, with a red chromogen shows many macrophages in the CNV as well as in the choroid (original magnification x400). * = choroidal neovascularization; NR = neuroretina; CH = choroid.*

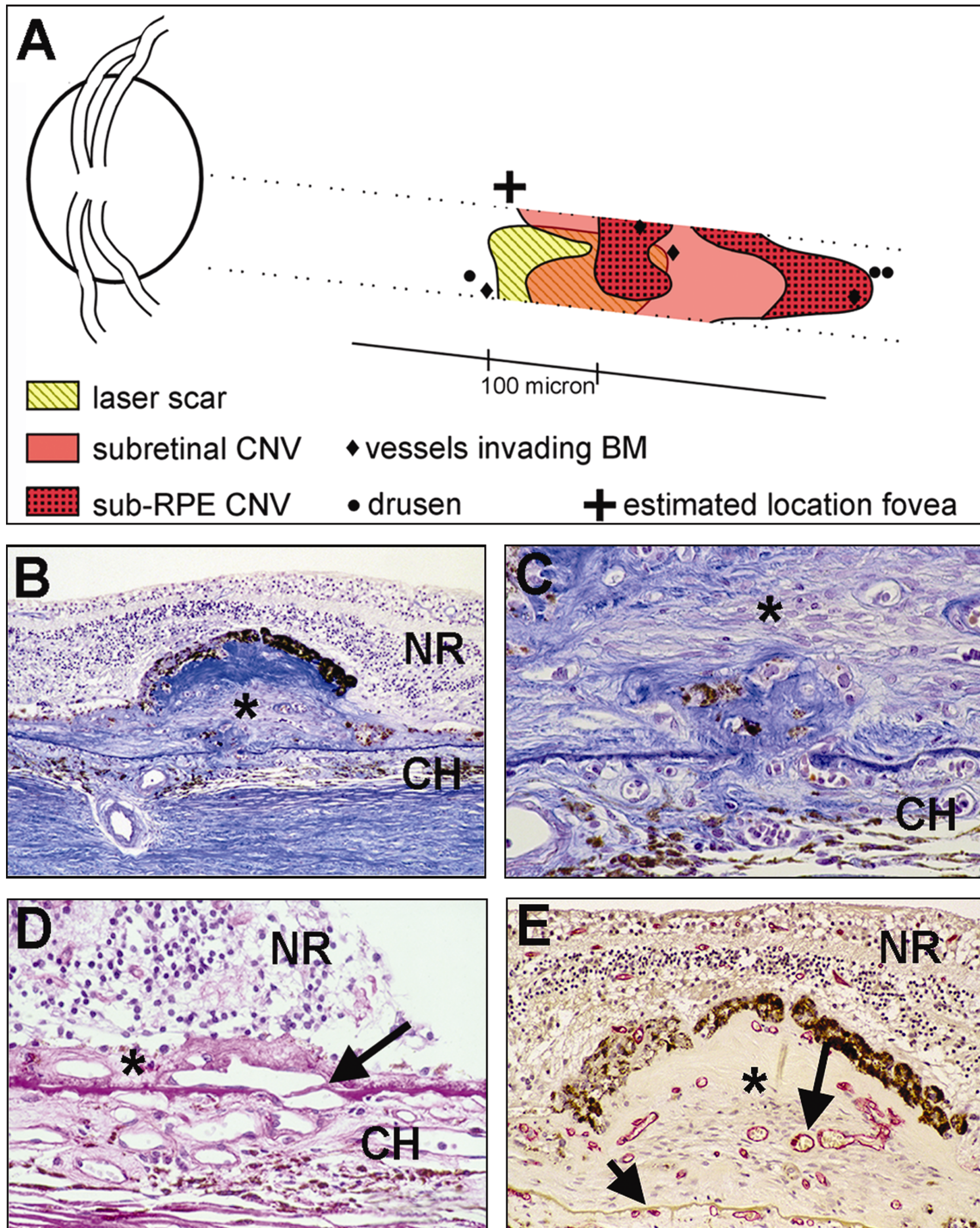


***Figure 9.4 Fellow left eye. (A)*** *Two-dimensional reconstruction map showing size, shape and location of selected histopathologic features of the examined part of the macular region.* ***(B)*** *Histologic overview of a dome-shaped CNV membrane with a large sub-RPE region. The overlying retina is disorganized. (Mallory, original magnification x200).* ***(C)*** *Detail of CNV showing intact choriocapillaris and vessels traversing Bruch's membrane (Mallory, original magnification x400).* ***(D)*** *At the margin of the CNV a vessel from the choriocapillaris traverses Bruch's membrane (PAS, original magnification x400).* ***(E)*** *Immunohistochemical staining with antibodies against CD34. Patent choriocapillaries (short arrow), as well as patent neovascular capillaries (long arrow) in the CNV are seen (original magnification x400). * = choroidal neovascularization; NR = neuroretina; CH = choroid.*

occurs about a year after irradiation.[325] Moreover, CNV membrane regression is not found until 6 months or more after radiotherapy, independent of the dose administered.[315] Since radiotherapy in our patient was performed three years before

histologic examination, it appears valuable to assess possible vascular damage as morphologic parameter for radiation damage.
A more rapid effect of ionizing radiation on neovascular macular degeneration is to be expected through its attenuating effects on the inflammatory response, mediated by macrophages and other inflammatory cells.[326,327] In our patient macrophages were similarly present in both eyes in the CNV as well as in the underlying choroid, three years after irradiation of the right eye. Therefore, it appears unlikely that the presence of these macrophages can be attributed to an immediate effect of the irradiation.
The histopathologic effect of radiotherapy has not been documented in cases of human macular degeneration so far. Miyamoto et al.[334] studied the histologic appearance of rabbit eyes with experimental CNV 4 weeks after a single fraction of 20 Gy of focal X-irradiation. The degree of vascular formation and the number of vascular endothelial cells in the subretinal membrane of the irradiated eyes were less than in those of control eyes. However, the pathogenesis of experimental CNV in rabbit eyes may not be identical to that of CNV in AMD. Furthermore, a single fraction of 20 Gy has different effects on choroidal endothelial cells than a fractionated dose.[335]
Clinical trials on radiotherapy show a probable benefit with higher doses.[314,317,318] Other clinical studies demonstrate similar results between treated and controls after lower-dose radiotherapy and longer follow-up.[319,322,324] In a pooled analysis of data from independent centers, fraction size was not found responsible for variation in visual outcome.[216] Our results are in concordance with the findings of little effect of low dose radiotherapy on CNV at longer follow-up.
With low-dose radiotherapy, few side effects are to be expected. However, recently a vasculopathy has been described, developing within months after low-dose radiotherapy (10 to 20 Gy),[329,330] called radiation-associated choroidal neovasculopathy.[330] The affected patients appeared to have a particularly poor visual prognosis. Our patient does not appear to belong to the (still poorly described) subset of patients who develop the vasculopathy.
The histologic significance of the findings on fluorescein angiography must be interpreted with care, because fluorescein angiography was not performed after radiotherapy. However, the clinical findings were well documented at regular intervals between fluorescein angiography and the last ophthalmoscopy and no obvious changes were recorded. At the last ophthalmoscopy, a flat fibrovascular lesion was seen without exudation, which is in accordance with the histologic findings.
In conclusion, no radiation-related histopathologic effect could be demonstrated 3 years after radiotherapy (10 Gy) in this patient with neovascular AMD.

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# Chapter 10

## General considerations and Future prospects

It is still not clear whether ARM is an exaggeration of the normal aging process, or a fundamentally different disease entity. Photoreceptor cell loss appears to occur in both instances.[26,90] Our finding of apoptosis in the aging retina can be explained by Wallace's theory of aging and disease, in which cells die at reaching a threshold by both genetic and environmental damages.[24] The time of onset could be influenced by genetic factors, or by environmental factors. Apoptosis can be initiated by multiple stimuli. In ARM, apoptosis is most likely the common pathway of cell death, resulting for instance from cellular damage, growth factor withdrawal or other stimuli. If one cell is damaged beyond repair, the apoptotic program is activated in order to prevent damage to the surrounding tissue. In this view, the finding of apoptotic cell death in specimens with ARM may state more about the quality and quantity of damage than about the general way of cell death in ARM. In my opinion, without restoring RPE function, the effect of anti-apoptotic modalities in the treatment of ARM will be limited. Anti-apoptotic interventions could leave severely damaged, probably non-functional retinal cells.[336]
The RPE seems to play a central role in the pathogenesis of ARM. Protective and shielding qualities are allocated to the RPE.[64,133] With aging, the RPE may lose some of these qualities, allowing neovascularization. Fas-ligand, expressed on the RPE, is proposed to be one of those protective factors, inducing apoptosis of proliferating vascular endothelial cells.[64] We showed in the study on Fas-ligand that the RPE does not have a decreased Fas-ligand expression with age. Furthermore, we demonstrated that Fas-ligand expression on RPE cells in sub-RPE CNV is similar to the expression in subretinal CNV, in which the vessels grow through the RPE into the subretinal space . These results make Fas-ligand less likely to be a major suppressive factor of the RPE, in case a CNV already has developed.

Malfunctioning RPE, age-related thickening of Bruch's membrane, BLD and other factors may eventually lead to neovascular AMD, possibly by relative hypoxia of the retina. In 1948, Michaelson proposed the presence of a diffusible biochemical "factor X" in the eye that was capable of inducing angiogenesis in diabetic retinopathy. The last two decades, numerous growth factors have been acknowledged in the

pathogenesis of neovascular retinal disease. VEGF has appeared to play a central role in the process of ocular angiogenesis. However, the precise mechanism of VEGF in the complex interaction of the different angiogenic growth factors in AMD has not been elucidated so far. Other angiogenic growth factors seem to play additional roles. In this thesis, we detected the presence of the Insulin-like Growth Factor (IGF) family in neovascular AMD. It is established that IGF-I has angiogenic properties in ocular vascular endothelial cells.[173] Therefore it is possible that IGF plays a role in the pathogenesis of neovascular AMD. While VEGF may control angiogenesis by acute oxygen regulation, IGF-I might do so on the basis of availability of nutrients.[177] IGF-I is recruited in normal wound repair,[276,337] that may partly explain the presence of IGF-I in CNV, because formation of the disciform lesion is regarded as normal wound repair.[1,74,119,120,275,276] On the other hand, IGF-I may function as a trophic factor for the normal vascular system.[338] With increasing age and consequently decreasing IGF-I levels,[339] the vascular endothelial cells may experience a decreased protective effect of IGF-I,[338] resulting in vascular insufficiency and thus further hypoxia in the outer retina, increasing the chance of angiogenesis.

For AMD the exact role of the IGF family and its possible therapeutic properties are still unclear. In order to study the role of IGF-I the individual IGF family members should be quantified in CNV and surrounding retinal tissue and related to values in normal tissue. Focusing on the dynamic role of the IGF family in CNV formation, it is mandatory to develop CNV in models of transgenic mice over- and underexpressing IGF-I, IGF receptor type 1 and the various IGFBPs.

Somatostatin and analogues such as octreotide seem to be candidates for inhibition of ocular angiogenesis.[177] They inhibit the secretion of growth hormone in the hypopituitary. Somatostatin seems to have further repressing effects on angiogenesis such as downregulation of VEGF in RPE cells[274] and anti-proliferating effects on vascular endothelial cells,[173,174,181] possibly mediated by somatostatin receptors.[173] These effects can be used as a tool to treat neovascular AMD. We demonstrated somatostatin receptor subtype 2A, which has high affinity for octreotide, in neovascular AMD. Therefore, local treatment could also be an option. Two further effects attributed to somatostatin can be of help in order to improve visual acuity in patients with neovascular AMD. Firstly, the drainage effect on macular edema of somatostatin,[340] and secondly the excitation of neuronal cells,[300] which could be directly associated with an increase of visual acuity. A randomized controlled phase II trial using octreotide in patients with neovascular AMD is currently under study.[183]

In view of the current assumption that angiogenic growth factors act in concert, anti-angiogenic treatment of patients with neovascular AMD addressing only one growth factor may be overruled by other growth factors. Therefore, it is likely that in the future a combination of pharmaceuticals mediating different growth factors will be applied as a therapy.

The view of the author on the pathogenesis of ARM is reflected in a schematic illustration in Figure 10.

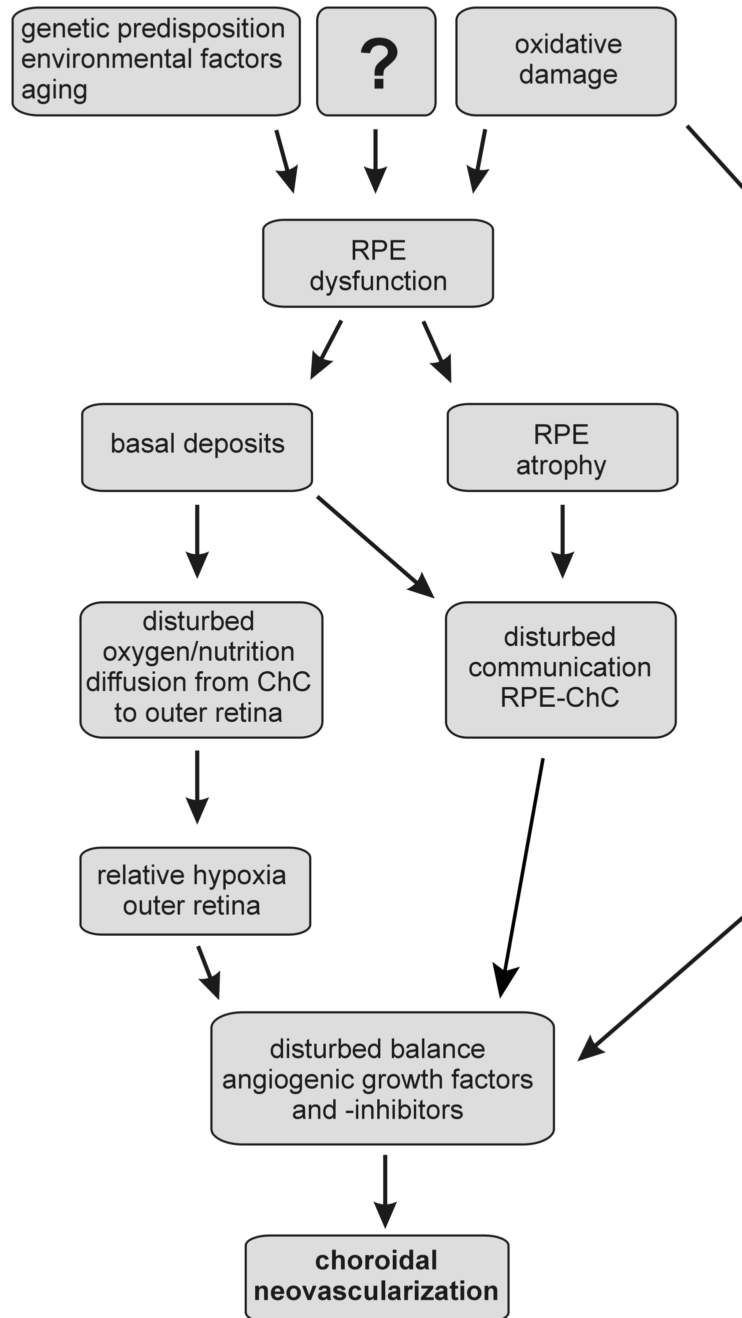


***Figure 10. Simplified schematic illustration of pathogenesis of ARM****, leading to choroidal neovasculari-zation.*

A major drawback of the research on neovascular AMD on paraffin-embedded human eyes is the scarcity of material. Patients with ARM rarely donate their eyes at autopsy for further research. Furthermore, eyes donated for corneal transplantation purposes rarely have signs of advanced ARM in the posterior segment. Most studies on human material are therefore performed on surgically removed (small) subretinal membranes.

In addition, a study performed on paraffin embedded material of neovascular AMD is static research. However, the CNVs we studied reflect different stages of the development of the disease, with the assumption that a mixed or subretinal membrane (which in AMD often is a part of a mixed membrane) is a progression of a sub-RPE membrane into the subretinal space.[120]
Because of the scarcity of human material, in vitro models like co-cultures[341] and collagen gels[192,341] can be used. However, in order to test the hypothetical models of the pathogenesis of ARM, a dynamic approach with animal models is mandatory. Currently there is no efficient suitable animal model of ARM available. Only some animals have the macular anatomy that is comparable to the human macula, such as the non-human primates, as we described in Chapter 4. Rhesus monkeys (or *Macaca mulatta*) show changes similar to early ARM such as drusen, but end stage AMD rarely occurs in these animals.[231] It may be hypothesized that the RPE characteristics are different in monkeys, or that the richly pigmented choroid contains more antioxidants than human choroid. In black people, having a more pigmented choroid, features of early ARM are common, but advanced AMD is infrequent, compared to Caucasians.[342]
Mice have the advantage of fast aging, but the drawback of mouse models of CNV is the absence of a macula. In order to produce CNV in animals, retinal damage is induced by for instance laser treatment. However, this may not represent 'normal' conditions in which ARM develops in humans. In another animal model of CNV, VEGF expression in the RPE is upregulated.[161-164] Still in these models RPE probably functions normally, thus not all aspects of ARM are addressed. Additionally, in experimental CNV, budding capillaries are rapidly enveloped by proliferating RPE, followed by an involution of new vessels.[133] This could explain the self-limiting disease that often occurs in animal experiments of CNV. The ideal animal model for neovascular AMD should be a fast aging animal with a macula, and a dysfunctional RPE, since this seems to be critical in the pathogenesis of AMD.

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# SUMMARY

Age-related maculopathy (ARM) is a severe threat to the visual ability of people over 65 years of age. ARM involves the central part of the retina where visual acuity is highest. In the late stages of ARM, called age-related macular degeneration (AMD), photoreceptor cells gradually disappear. The disease may be complicated by new vessels growing beneath the retina, called 'wet' or neovascular AMD. The causes and pathogenesis of this eye disease are not clear yet and unraveling slowly, thanks to many studies on this subject. Knowledge about these issues could eventually lead to therapies, and probably even more important, to strategies that prevent the disease from occurring. The purpose of this thesis was to study several of many molecular changes that occur during the development of ARM.

In the **first part (Chapters 1 - 3)** the clinical, microscopical and molecular characteristics of ARM are described and theories of possible mechanisms responsible for the development of ARM are discussed. Questions about the role of cell death during the aging process of the retina are outlined. The major part includes the role of angiogenic factors in the development of neovascular, or 'wet', AMD, in which abnormal vessels grow through the barriers of normal anatomy, towards the retina to form a choroidal neovascularization (CNV). Many so-called growth factors that play a role in CNV are identified up to now, but others need to be investigated.

With advancing age, the thickness of the retina decreases. Little is known about the way cells disappear during this process. In the **second part** of this thesis **(Chapter 4)** one way of cell death is studied, that is apoptosis, which can be viewed as a cell suicide program, present in all cells of the body. In this study, apoptosis was studied in the macula of rhesus monkeys of different ages. It was found that apoptotic cells were present at all ages, with an increase in the oldest monkey eyes, while the thickness of the retinal outer nuclear layer decreased with increasing age. The apoptosis-modulating proteins Bcl-x, Fas and Fas-ligand were expressed equally at all ages. These findings indicate that apoptosis in the primate macula occurs at all ages at similar rate, possibly increasing in the oldest age group, and may account for the decreasing thickness of the primate macula with age.

Dysfunctional retinal pigment epithelium (RPE) appears to play a central role in ARM, in combination with other factors eventually leading to neovascular AMD. The role of Fas and its natural ligand Fas-ligand (FasL) has been acknowledged in the process of angiogenesis. Fas and FasL induce apoptosis in T-lymphocytes but are also expressed on non-lymphoidal tissue. In the eye Fas-FasL interactions appear to be an important mechanism for the maintenance of immune privilege by inducing apoptosis

of invading lymphocytes. Recently, FasL expressed on RPE cells has been suggested to inhibit the growth and development of subretinal neovascularization. In **Chapter 5** a study is described in which FasL expression was investigated in the aging RPE and in early and late stages of ARM. FasL expression in RPE was not related to age or to the presence of early ARM. Furthermore, FasL expression in RPE was similar in subretinal and sub-RPE CNV. Thus, it appears to be unlikely that FasL expressed on RPE controls the extension of CNV from sub-RPE to subretinal.

In the **third part** of this thesis, several growth factors are studied that could be involved in the pathogenesis of neovascular AMD. In **Chapters 6 and 7** the Insulin-like Growth Factor family is investigated. IGF-I is a peptide that stimulates growth and differentiation of almost all cell types. The effects of IGF-I are regulated by the binding to six IGF-binding proteins (IGFBPs). Most of these IGFBPs have additional actions that are independent of IGF-I binding, including stimulation of cell growth and induction of apoptotic cell death. IGF-I is known to participate in each step of neovascularization. Therefore, the presence of IGF-I, its receptor (IGF-IR), and IGFBP-1 to -6 was examined in eyes with neovascular AMD at protein level and at mRNA level, which is an indication of the protein production in a cell. IGF-IR, little IGF-I, and most of the IGFBPs were shown in various cell types of CNV, both at protein and mRNA level. These results may point towards a role of this growth factor family in the pathogenesis of neovascular AMD. The functional role of the various IGF family members in AMD needs to be established.

It is becoming clear that a balance between stimulating and inhibiting growth factors regulates the growth of ocular neovascularization. Somatostatin reduces newly formed vessels by inhibiting the growth hormone/insulin-like growth factor axis and also has a direct anti-proliferative effect on various cell types involved in angiogenesis. In **Chapter 8** is demonstrated that most early-formed CNV in eyes of patients with AMD express $sst_{2A}$, which is a receptor for somatostatin. The $sst_{2A}$ receptor binds potential anti-angiogenic somatostatin-analogues like octreotide. Therefore, somatostatin analogues may be an effective therapy in early stages of neovascular AMD.

In **Chapter 9** an experimental treatment for neovascular AMD is discussed. Radiotherapy has recently been employed to treat patients with neovascular macular degeneration in order to prevent severe visual loss. In this study the histopathological findings are described of a patient with neovascular AMD in both eyes, who was treated with low-dose radiotherapy 3 years before he died. No radiation-related histopathologic effect could be demonstrated following radiation therapy in this patient.

In **Chapter 10** the findings of the studies described are considered in view of the current knowledge. Problems encountered are discussed and a theoretical model on the pathogenesis of ARM is outlined. Suggestions for future research are made and the characteristics for the ideal animal model for research on ARM are discussed.

# SAMENVATTING VOOR NIET-DESKUNDIGEN

Ouderdoms-gerelateerde macula degeneratie (OMD) is de belangrijkste oorzaak van blindheid bij mensen ouder dan 65 jaar. In OMD is het centrale deel van het netvlies, genaamd macula of gele vlek, aangetast. In de macula is juist de gezichtsscherpte het hoogst. In de late stadia van de ziekte verdwijnen de lichtgevoelige cellen van het netvlies langzamerhand. Een veelvoorkomende complicatie van OMD is de groei van vaatjes onder het netvlies, wat 'natte' of 'neovasculaire' OMD heet. De oorzaken en het ontstaan van OMD zijn nog niet duidelijk en worden langzaam ontrafeld, dankzij vele studies die naar dit onderwerp worden verricht. Kennis omtrent de oorzaken en ontstaan van deze oogziekte kunnen leiden tot nieuwe behandelingen en, mogelijk nog belangrijker, tot vormen van preventie. Het doel van dit proefschrift was het onderzoeken van enkele van de vele moleculaire veranderingen die zich voordoen tijdens de ontwikkeling van OMD.

In het **eerste deel (Hoofdstuk 1, 2 en 3)** worden de klinische, microscopische en moleculaire kenmerken van OMD beschreven. Theorieën over mogelijke mechanismen die tot OMD leiden worden besproken. De rol van celdood tijdens het verouderingsproces van het netvlies wordt besproken. Het grootste deel gaat over de rol van groeifactoren in het ontstaan van neovasculaire OMD. Deze groeifactoren stimuleren vaatjes om dwars door de grenzen van de normale anatomie heen te groeien tot onder het netvlies. Daarbij wordt een litteken gevormd, dat choroidale neovascularizatie (CNV) wordt genoemd. Er zijn al veel groeifactoren geïdentificeerd, die een

rol spelen in het ontstaan van CNV, maar andere moeten nog onderzocht worden.
Met toenemende leeftijd vermindert de dikte van het netvlies. Er is nog niet veel bekend over de manier waarop cellen verdwijnen bij dit proces. In het **tweede deel** van dit proefschrift (**Hoofdstuk 4**) wordt een manier van celdood bestudeerd, te weten apoptose. Apoptose kan gezien worden als een soort zelfmoordprogramma, aanwezig in alle lichaamscellen. Door apoptotische celdood kunnen cellen verdwijnen zonder dat het omliggende weefsel hier nadeel van ondervindt. In dit onderzoek bestudeerden wij de aanwezigheid van apoptose in de macula van rhesus aapjes van verschillende leeftijd. We vonden een zeer gering aantal apoptotische cellen in het netvlies van aapjes van alle leeftijden, met een toename ervan in de oudere aapjes. De dikte van het netvlies nam af met toenemende leeftijd. De aanwezigheid van enkele eiwitten die invloed hebben op apoptose, namelijk Bcl-x, Fas en Fas-ligand, bleven gelijk bij alle leeftijden. Deze bevindingen kunnen er op wijzen dat apoptose in het netvlies van apen van alle leeftijden voorkomt, met mogelijk een toename in de oudste leeftijdsgroep. Tevens tonen wij aan dat apoptose het proces kan zijn waardoor het netvlies dunner wordt gedurende het verouderingsproces.
Onder het netvlies ligt een gepigmenteerde cellaag, het retinale pigment epitheel (RPE). Het niet goed functioneren van het RPE lijkt een centrale rol te spelen in het ontstaan van OMD. In combinatie met andere factoren kan dit slecht functioneren uiteindelijk leiden tot neovasculaire OMD. De apoptose-regulerende eiwitten Fas en Fas-ligand spelen een rol in het proces van vaatnieuwvorming. Recent is voorgesteld, dat de aanwezigheid van Fas-ligand op RPE cellen vaatnieuwgroei onder het netvlies tegenhoudt door de groeiende vaatjes aan te zetten tot apoptotische celdood. Het zou mogelijk kunnen zijn dat Fas-ligand verminderd aanwezig is op RPE cellen van oudere personen en daardoor de vaatnieuwvorming niet meer

tegen kan gaan. In **Hoofdstuk 5** onderzochten wij dit door het vóórkomen van Fas-ligand op het RPE in het verouderende netvlies te bepalen. Wij vonden dat de aanwezigheid van Fas-ligand hetzelfde was in alle leeftijdsgroepen, evenals in groepen met en zonder OMD. Verder was de aanwezigheid in het RPE hetzelfde in CNV die onder het RPE groeiden als in CNV die door het RPE heen onder het netvlies groeiden. Daarom lijkt het niet waarschijnlijk dat Fas-ligand op het RPE een belangrijke onderdrukker is van de doorgroei van het CNV tot onder het netvlies.

In het **derde deel** van dit proefschrift bestudeerden wij enkele groeifactoren die mogelijk een rol spelen in het ontstaan van neovasculaire OMD. In **Hoofdstuk 6 en 7** wordt de Insulin-like Growth Factor-familie onderzocht. Insulin-like Growth Factor-I (IGF-I) is een eiwit dat lijkt op insuline en dat vrijwel alle lichaamscellen kan aanzetten tot groei. Als IGF-I gebonden is aan een van zes IGF-bindende eiwitten (IGFBPs), functioneert deze niet. Deze IGFBPs vormen een soort reservoir voor IGF-I dat niet direct nodig is. Ook hebben de meeste IGFBPs een aanvullende rol waar IGF-I binding niet voor nodig is, waaronder het stimuleren van celgroei en aanzetten tot apoptose. IGF-I beïnvloedt alle stappen van het proces van vaatnieuwvorming. Daarom bestudeerden wij de aanwezigheid van IGF-I, zijn receptor IGF-IR en IGFBP-1 t/m –6 in ogen met neovasculaire OMD. We vonden IGF-IR, weinig IGF-I en vrijwel alle IGFBPs in diverse celtypen van CNVs. Deze resultaten kunnen wijzen op een rol van deze familie van groeifactoren in het ontstaan van neovasculaire OMD. De functie die de diverse IGF familieleden hierin hebben moet nog worden vastgesteld.
Niet alleen vaatstimulerende groeifactoren hebben invloed op het ontstaan van vaatnieuwvorming in het oog. Het lijkt eerder een (on-)balans te betreffen tussen groeistimulerende en groeiremmende factoren. Somatostatine is een groeifactor die vaatnieuwvorming

indirect remt door de afgifte van groeihormoon uit de hersenen te verminderen. Hierdoor komt weer minder IGF-I in het bloed vrij. Somatostatine heeft ook directe groeiremmende effecten op diverse cellen die bij vaatnieuwvorming een rol spelen. In **Hoofdstuk 8** toonden wij aan dat de meeste vaatjes in pasgevormde CNV in ogen van patiënten met OMD een receptor voor somatostatine bevatten. Deze receptor bindt tevens medicamenten die sterk op somatostatine lijken, zoals octreotide. Daarom zouden deze somatostatine-achtige medicamenten een interessante behandeling kunnen blijken voor pas gevormde neovasculaire OMD.

In **Hoofdstuk 9** bespreken we een experimentele behandeling van neovasculaire OMD. Bestraling kan vaatnieuwvormingen remmen en is de laatste jaren gebruikt met het doel verder gezichtsverlies bij sommige patiënten met neovasculaire OMD te voorkómen. In dit onderzoek bestudeerden wij onder de microscoop de ogen van een patiënt met neovasculaire OMD in beide ogen, die met een lage dosis was bestraald op een oog, drie jaar voor zijn overlijden. We konden geen effecten van de bestraling aantonen bij deze patiënt.

In **Hoofdstuk 10** worden de bevindingen van deze onderzoeken besproken in het licht van de huidige kennis van zaken. Problemen die we tegenkwamen worden aangestipt en een theoretisch model van het ontstaan van OMD wordt in een figuur geschetst. Suggesties voor verder onderzoek worden naar voren gebracht, waaronder de eigenschappen van het ideale diermodel voor onderzoek naar OMD.

# CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 14 januari 1968 in Wassenaar. Daar volgde ze het lager onderwijs en haalde ze in 1986 haar VWO/gymnasium beta diploma aan het Rijnlands Lyceum Wassenaar.

De studie geneeskunde volgde zij aan de Rijksuniversiteit Leiden. In 1993 werd het arts-examen behaald. Naast haar studie werkte ze onder andere als student-assistent op snijzaal. Ook verrichtte ze enkele jaren wetenschappelijk onderzoek aan de afdeling genetica van de Erasmus Universiteit Rotterdam. Verder deed ze onderzoek bij de afdeling anesthesie van de University of Utah Medical Centre in Salt Lake City, en bezocht ze ziekenhuizen in Gambia en Turkije in het kader van uitwisselings-programma's.

Eenmaal arts, werkte zij twee jaar als arts-assistent heelkunde in het Leyenburg Ziekenhuis te Den Haag, en vervolgens een jaar als arts-assistent radiotherapie in de Dr. Daniel den Hoed Kliniek in Rotterdam.

Van 1997 tot 2002 verrichtte ze promotie onderzoek aan de afdelingen oogheelkunde en pathologie van de Erasmus Universiteit Rotterdam (promotor Prof.dr. G. van Rij).

Vanaf april 2002 is zij in opleiding tot oogarts in het Erasmus MC (opleider Prof.dr. G. van Rij)

Antoinette is getrouwd en heeft drie kinderen.

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