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. 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. These processes involve the
cumulative effects of extrinsic influences and an intrinsic molecular program of cellular aging.\textsuperscript{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.\textsuperscript{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 possess their own genetic material and that they only have a limited arsenal of DNA repair processes makes them especially vulnerable to oxidative damage.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 thereof, for instance degenerative diseases and malignant tumours, respectively.
Apoptosis is an active process, that is usually dependent on protein synthesis 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). 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, 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. The removal of redundant cells in the developing mouse retina occurs by apoptosis in the various retinal layers in time waves.

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. Apoptotic cells have also been identified in late stages of AMD, both in the neovascular form as well as in the geographic form. It is suggested that apoptosis is one of the pathways of photoreceptor degeneration in AMD.

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. 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. Bcl-x is another apoptosis protective protein and is predominantly present in postnatal neural tissues and adult rat retina. Overexpression of Bcl-2 or Bcl-x delays photoreceptor cell death in a mouse model of retinal degeneration, although conflicting reports exist.

Fas (CD95) receptor mediates apoptosis when triggered by its ligand, Fas-ligand (FasL) or by agonistic antibodies. 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. 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\textsuperscript{64} by inducing apoptosis of endothelial cells. The \textit{p53} tumor suppressor protein is involved in the control of the cell cycle, and is associated with apoptosis in various cell types, especially following DNA damage.\textsuperscript{65} DNA damage induces \textit{p53} to stop the cell cycle allowing the DNA damage to be repaired. If the damage is beyond repair, \textit{p53} activates the apoptotic program. The \textit{p53} product is thought to play a dynamic role in the process of apoptosis due to retinal ischemia.\textsuperscript{66,67} In a mouse model of retinal degeneration, absence of \textit{p53} delays photoreceptor cell loss.\textsuperscript{68} However, also \textit{p53}-independent apoptosis has been described in studies on retinal degeneration.\textsuperscript{69}

The proteins \textit{Jun} and \textit{Fos} are proto-oncogenes.\textsuperscript{70} There is evidence that both \textit{Fos} and \textit{Jun} are involved in apoptosis of various cell types, including neurons\textsuperscript{71} and other retinal cell types.\textsuperscript{41} In both differentiated and undifferentiated retinal cells, expression of c\textit{-Jun} is correlated with apoptosis, preceding the morphological and biochemical characteristics of apoptosis.\textsuperscript{41} c\textit{-Fos} deficient mice are protected against light-induced photoreceptor apoptosis.\textsuperscript{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.\textsuperscript{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,\textsuperscript{1,72,73} giving rise to other signs of deterioration, such as deposits in Bruch's membrane and RPE and associated photoreceptor cell death.\textsuperscript{1} The RPE dysfunction may be due to imperfections in the cell's digestive mechanisms,\textsuperscript{1} or to
oxidative stress due to free radical chain reactions.\textsuperscript{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.\textsuperscript{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.\textsuperscript{74} Other authors assume choroidal circulatory abnormalities to be the primary event in the pathogenesis of ARM.\textsuperscript{75} Vascular insufficiency could lead to insufficient removal of waste products from the outer retina and to a disrupted supply of oxygen and nutrients.\textsuperscript{76} Friedman proposes that AMD is the result of atherosclerotic changes in chorioidal vasculature and deposition of lipids in Bruch’s membrane.\textsuperscript{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.\textsuperscript{78,79} Studies in support of this theory demonstrated choriocapillary atrophy,\textsuperscript{80} reduced choroidal arteries and reduced choroidal blood flow in ARM patients.\textsuperscript{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,\textsuperscript{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.\textsuperscript{83,87} Also other depositions in Bruch’s membrane such as cholesterol,\textsuperscript{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.\textsuperscript{90} In the case of ABCR-mediated retinal degeneration, the defected rod rim protein causes RPE dysfunction because of excessive lipofuscin accumulation.\textsuperscript{91,92} This theory is strengthened by the detection of mutations in the ABCR gene in some patients with AMD,\textsuperscript{9} although the detected mutations may simply reflect polymorphisms found in normal healthy people.\textsuperscript{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\textsuperscript{95} and Kliffen.\textsuperscript{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 fundoscopically seen as amorphous deposits with indistinct borders, usually larger than 63 \(\mu\)m 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 \(\mu\)m), 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.](image)


**Basal deposits**

Two types of basal deposits are distinguished by electron 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. 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, 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. Both basal linear deposits and soft drusen provide a cleavage plane within Bruch’s membrane which may facilitate the ingrow of choroidal neovascularization. Basal laminar and linear deposits (BLD) are detectable by light microscopy (Figure 2.3C) but are clinically only detectable by secondary changes of RPE. Basal deposits are positively associated with early ARM lesions and may be a significant indicator of progression to late AMD. The origin of basal deposits is unclear. Several authors suggest that these deposits are released from the RPE via the basal plasma membrane.

**Deterioration of Bruch’s membrane**

With advancing age, the thickness of Bruch’s membrane increases, expanding between the choriocapillary vessels, the so-called intercapillary pillars (Figure 2.3D). Further changes include hyalinization, densification and calcification. 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.

**Retinal Pigment Epithelium abnormalities**

Lipofuscin accumulates in the RPE with age as a byproduct of photoreceptor outer segment phagocytosis. Further RPE changes in ARM include attenuation of the RPE overlying drusen and BLD (Figure 2.3B), RPE atrophy, hypertrophy, hyperplasia and pigment clumping. RPE hyper-pigmentation and hypo-pigmentation are significant independent risk factors for the development of exudative AMD.

**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. In the absence of neovascularization, geographical atrophy probably is the natural end-result of ARM.
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, 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. 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.

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. 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. 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. 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. 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. Neovascular AMD has a chronic inflammatory component. CNV contains chronic inflammatory cells such as macrophages and multinucleated giant cells which participate in the breakdown of Bruch's membrane and may provide an angiogenic stimulus for CNV. 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.
Classification of Choroidal Neovascularization

Clinically, two types of CNV are distinguished based on the pattern at fluorescein angiography: classic CNV and occult CNV. 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. Occult CNV can be visualized with indocyanine green videoangiography.

Histologically, two different types of CNV can be distinguished (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. 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\textsuperscript{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.\textsuperscript{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
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 degraded 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-
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.\textsuperscript{142,143} The provoking factors of angiogenesis in AMD are still unidentified. Hypoxia as a stimulus has been suggested by several authors.\textsuperscript{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\textsuperscript{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.\textsuperscript{119,142} Under physiological circumstances, VEGF has a low constitutive expression in the eye.\textsuperscript{146} In the RPE, this probably functions as a trophic factor in the maintenance of the endothelial cells of the choriocapillaris.\textsuperscript{119,146,147} VEGF is upregulated by multiple factors, including hypoxia,\textsuperscript{137,148-152} several growth factors such as fibroblast growth factors (FGFs),\textsuperscript{153,154} transforming growth factor-\(\beta\) (TGF-\(\beta\))\textsuperscript{155} and insulin-like growth factor-I (IGF-I),\textsuperscript{156} prostaglandins,\textsuperscript{157} alterations in the extracellular matrix,\textsuperscript{158} and also by oxidative stress.\textsuperscript{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.\textsuperscript{144,145,159,160} Animal models in which VEGF is overexpressed in the RPE show choroidal\textsuperscript{161-163} or intrachoroidal neovascularization.\textsuperscript{164} Blocking of VEGF receptor kinases causes dramatic inhibition of CNV under experimental circumstances,\textsuperscript{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\textsuperscript{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. 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. In many situations on pathological growth, multiple components of the IGF system may be dysregulated. IGF-I participates in each step of ocular neovascularization. It is involved in the degradation of basement membranes and extracellular matrix proteolysis, and in vascular endothelial cell migration and proliferation. IGF-I also increases RPE cell migration and proliferation in vitro. In the eye, IGF-I can act as a direct angiogenic factor on vascular endothelial cells of the retina and choriocapillaris, or indirectly through increased VEGF gene expression of cultured RPE cells. Intravitreous injection of IGF-I in animals produces preretinal neovascularization in rabbits or microangiopathy resembling diabetic microangiopathy in pigs. In mice, inhibition of IGF-I can decrease ischemia-induced retinal neovascularization. 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. 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]. 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, by inactivating the IGF-I mediated activation of IGF-IR, and by inhibiting VEGF expression in RPE cells. In addition, somatostatin and its analogues can inhibit angiogenesis directly, possibly by activation of somatostatin receptors located on capillary endothelial cells. 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. 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.
**Angiopoietin (Ang)-Tie2 system**

Tie2 is an endothelial cell-specific receptor which is thought to stabilize vascular integrity. Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are ligands for the Tie2 receptor. Ang2 is a natural antagonist for Tie2. 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. 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.

**Fibroblast growth factor (FGF)**

FGFs are a family of heparin binding proteins, with mitogenic, neurotrophic and angiogenic properties. In the macula, FGFs have a constitutive expression. 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. FGF has synergistic angiogenic activity with VEGF. In neovascular AMD, several FGFs have been identified and FGF is capable of inducing subretinal neovascularization in rabbits.

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

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

**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. 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, however also pro-angiogenic functions have been attributed to this cytokine. The angiogenic actions of TGF-β are indirect by modulating expression of other angiogenic factors such as VEGF, or by recruitment of inflammatory cells, which in turn produce positive regulators such as VEGF. In early ARM and in neovascular AMD, TGF-β expression is upregulated.
**Pigment Epithelium-Derived Factor (PEDF)**

PEDF is a neurotrophic factor and one of the most potent inhibitors of ocular angiogenesis, produced in the RPE. PEDF is probably responsible for the physiological avascularity of the cornea and vitreous. 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. PEDF inhibits aberrant blood vessel growth in mouse models of ischemia-induced retinopathy, and in experimental CNV. This angiogenesis inhibiting effect is thought to be caused by induction of apoptosis of activated endothelial cells.

**Angiostatin**

Angiostatin is a potent inhibitor of angiogenesis, selectively inhibiting endothelial cell proliferation. It is composed of an internal fragment of plasminogen. Angiostatin reduces neovascularization size in experimental rat CNV, and prevents retinal neovascularization in a mouse model of retinopathy of prematurity without affecting physiological angiogenesis. 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.

### 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. 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. It is unclear in these cases whether the laser treatment is inadequate or whether the recurrent CNV consists of a new neovascularization.
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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Angiogenesis inhibitor} & \textbf{Trials} & \textbf{Comments} \\
\hline
angiotatin & 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\textsuperscript{218} or integrins \\
antisense-oligonucleotides against VEGF & phase II multicentre trial & prevents translation of mRNA into proteins\textsuperscript{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 \\
\hline
\end{tabular}
\caption{Angiogenesis inhibitors possibly useful in neovascular AMD}
\end{table}