Non-invasive monitoring of lipofuscin: an imaging technique predictive for age-related macular degeneration

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NON-INVASIVE MONITORING OF LIPOFUSCIN: AN IMAGING
TECHNIQUE PREDICTIVE FOR AGE-RELATED MACULAR
DEGENERATION

by

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DEGENERATION

ERIN FLYNN

ABSTRACT

This paper outlines the progression of age-related macular degeneration in the eye and discusses the diagnostic approaches and therapies used currently to treat this disease. Age-related macular degeneration has a complicated pathophysiology involving genetic and environmental factors. This paper focuses its attention on the role of lipofuscin accumulation in this disease. Lipofuscin in the eye refers to the bisretinoid products of the visual cycle. While lipofuscin accumulation is normal in healthy eyes, the excessive accumulation causes retinal dysfunction.

Lipofuscin accumulation has been linked heavily not only to age-related macular degeneration but also juvenile macular degeneration, retinitis pigmentosa, Best’s Villiform disease, and many others. New techniques in ophthalmic research have evaluated the role of lipofuscin accumulation in such retinal genetic diseases. This paper proposes an approach to apply techniques such as quantified autofluorescence imaging and high-powered liquid chromatography of bisretinoids in the eye to track the role of lipofuscin accumulation in the progression of age-related macular degeneration.
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INTRODUCTION

Age-related macular degeneration (“AMD”) is the number one cause of blindness in the developed world.¹ Risk factors include age, genetic variants, family history, smoking, cardiovascular disease, hypertension, obesity, and a diet low in omega-3 fatty acids and dark green leafy vegetables.²

There are two distinct types of AMD: dry and wet. Dry affects the majority of AMD patients (85%) while wet is less common although much more severe.³ Dry AMD occurs as a result of scarring on the macula, without either fluid or blood. Wet AMD occurs when blood vessels have grown in the macula. Over the past decade, drug therapy has become highly successful in managing wet AMD; the prognosis with respect to severe, dry AMD is far less promising.⁴

This paper will accomplish the following objectives:

(1) Consider the manifestation of AMD (i.e., what is AMD),

(2) Consider and analyze the causes and risk factors for AMD (i.e., what causes the malfunction that leads to the phenomenon);

(3) Consider and analyze the physiological transformation from dry to wet AMD;

(4) Discuss the diagnostic tools, techniques and limitations applicable to retinal analysis;
Assess A2E, a bisretinoid component of lipofuscin, as a biomarker for progression of AMD and its use in the diagnostic process for AMD based upon mouse study;

Consider treatment potential for AMD. This will review the current drug therapies for AMD and their efficacy and evaluate potential strategies to prevent progression to AMD; and

Consider the future of AMD research, therapies and potential for prevention (including gene therapy).

THE MANIFESTATION OF AMD

The progression of AMD begins with photoreceptor cells and the retinal pigment epithelium (RPE). To understand the progression of AMD, it is helpful to begin with a brief description of eye anatomy.

MAMMALIAN EYE ANATOMY

The mammalian eye consists of multiple layers. The first layer is the outer layer, consisting of the sclera and cornea; these layers protect the eye, contain its shape, and focus light to the back of the eye. The second layer consists of the uvea and iris.
These act as a vascular layer and pigmented layer. The pigment in this layer restricts light entry into the eye and also absorbs stray light photons within the eye to reduce scatter. Next is the lens, which is a non-neural, multi-layer, biconvex structure that changes shape, permitting the eye to focus on objects. The focused images are then formed on the retina. The final layer consists of the retina and optic nerve. These structures in the eye sense light and transmit messages to the brain as electrical impulses.


The retina consists of two types of photoreceptors, rods in the peripheral retina and cones located in the macula in the posterior pole. The fovea, a structure that is part of the macula, is the most sensitive part of the retina, being responsible for fine visual discrimination and color vision. Rods are responsible for dark adaptation and night vision. Input from rods and cones travels to the visual system via the optic nerves and
posterior visual fibers. Vision itself takes place in the brain. In contrast to the other structures of the eye, the retina and optic nerves are part of the brain.8

The macula is a small (2.5 millimeter) structure located where light at the center of gaze is focused in the back of the eye by the cornea and lens. The macula and its cones have the highest rate of oxygen consumption of any tissue in the body.9 This requires a circulation consisting of inner vessels derived from the central retinal artery and a separate outer circulation derived from the choriocapillaris, a dense capillary bed in the uvea. The latter (choroid) is the more significant source of macular nutrition because the macula is predominantly supplied by the central retinal artery; indeed the center of the macula, a region called the fovea centralis, contains only cones and is exclusively supplied by the choriocapillaris. A blood-ocular barrier exists for both retinal and choroidal circulations. Retinal blood vessels are comprised of endothelial cells with tight junctions. Choroidal vessels are separated from the retina by several layers of tissues known as Bruch’s Membrane and overlying retinal pigment epithelial (RPE) cells. These two layers of tissue share a fused basement membrane.10 The effect of the blood-ocular barrier is to allow into the retina and the eye only those molecules and oxygen which are necessary to the function of the eye. All other constituents of blood are excluded from the interior of the eye. This arrangement in the eye is analogous to the blood-brain barrier.11

Optimal retinal function requires tight control over flow of water, nutrients, oxygen and metabolic waste products into and out of the eye. The photoreceptor cells
shed their outer segments daily—which contain the waste products left over from the visual cycle, referred to as bisretinoids. Studies have linked the accumulation of bisretinoids to activation of the immune system and have shown the harmful effects these bisretinoids have on the function of photoreceptor and RPE cells. Several disorders adversely affect the degradation of these waste products and, accordingly, have the potential to impair vision or cause blindness. One such dysfunction is AMD.

(2) AMD DYSFUNCTION

Photoreceptor cells in the retina create metabolic waste. Since these cells are the most metabolically active of the body, there is a relatively high volume of metabolic waste. Extracellular waste accumulation is normal in all mammals. The RPE (described above) is a part of the retina lying external to the neural retina and in direct contact with the outer segments of the photoreceptors. It serves many important functions: it provides oxygen and nutrients to the photoreceptors, absorbs stray light, recycles spent photopigment, and delivers metabolic waste from the photoreceptors to the capillary bed of the choroid. In healthy eyes, the RPE also manages bisretinoid accumulation without causing severe problems. In general, the RPE breaks down waste, excreting it back into circulation, and, with respect to those waste products that it can’t excrete, RPE sequesters and packages these wastes as drusen. Drusen come in different sizes and shapes, and consist of a heterogeneous group of cellular waste products, many of which produce inflammation.
Figure 2. Intermediates of the Retinoid Visual Cycle. "Visual cycle" by Krishnavedala - Own work.

With age, lipofuscin can accumulate—even in healthy subjects—as the RPE becomes less efficient at processing it. However, certain people are more susceptible to waste accumulation. In some cases, the ability of RPE to degrade lipofuscin break down and these individuals are more likely to show signs of dry, advanced AMD and scarring. When this happens, the RPE cells are compromised and the retina begins to die, leading to blindness. There are signs of disease at the retina but most markedly at the fovea. This leads to dry AMD, which can progress to wet AMD. However, the causes underlying the progression from dry to wet AMD aren’t known at this time.
Figure 3. Risk Factors and the Progression of Age-related Macular Degeneration. Figure generated by the author.

RISK FACTORS OF AMD

The risk factors relating to AMD are controversial. There appear to be several factors that contribute to its progression, including genetic predisposition, accumulation of lipofuscin, accumulation of drusen, local inflammation, and neovascularization (in wet form).\textsuperscript{20}

(1) GENETIC PREDISPOSITION

Genetics are an important factor that appears to contribute to AMD. Individuals with a family history of AMD are more likely to develop AMD than those with no family
history. This finding is consistent with genetic research relating to other retinal diseases. Some genes have been directly linked to retinal diseases with symptoms similar to AMD.

In studies, mutations associated with a gene that codes for the ATP-binding cassette transport protein in photoreceptor cells (gene is called ABCA4) have been linked to both dominant and recessive Stargardts disease. Stargardts is an inherited, autosomal recessive retinal dystrophy leading to blindness in the adult years. The mutation causes a dysfunctional ATP-binding cassette transporter protein. This protein rids the photoreceptor cells of the visual cycle waste products. Other genes such as ELOVL4, FBL-6, APOE, and SOD2 have been linked to retinal diseases with similar manifestations in the macula to AMD. Genes most closely associated to AMD tend to affect the immune system’s complement cascade. For instance, a mutation in the complement factor’s H (CFH) gene has been hypothesized to cause 50% of AMD cases. It seems genetic predisposition to AMD is most heavily dependent on mutations that alter or affect the body’s immune system; however, no genetic mutation has yet been proved to cause AMD.

(2) LIPOFUSCIN

As noted above, RPE cell dysfunction is an initial step of AMD progression. The RPE cells lie along the retina. Their function is vital to the health of the photoreceptor cells. Lipofuscin accumulates outside the photoreceptor cells in the visual cycle, and RPE cells work to phagocytize and transport these vitamin A derivatives.
In the healthy retina, photoreceptor cells capture photons and perceive light in what is called the retinoid visual cycle. This process involves an enzymatic pathway whereby the cells generate the “inactive” 11-cis-retinal chromophore of rhodopsin; rhodopsin is in its “active” form in darkness. The initial intermediate of this pathway, all-trans-retinal, is moved from the inner to the outer segment layer of the photoreceptor cells by an ATP cassette-binding transporter known as the ABCA4 transport or rim protein. All-trans-retinal is then converted enzymatically to all-trans-retinol or it is further oxidized to form the more reactive A2PE and its successor, A2E. All-trans-retinol will go on to form 11-cis-retinol to be recycled back into the cytoplasm of the photoreceptor cell and the retinoid visual cycle.

In healthy eyes, these intermediate molecules of the visual cycle are engulfed by phagosomes from the RPE in the outer segment layer, but some collect in the lysosomes of the RPE cells. These cells are unable to breakdown the intermediates, and the inadvertent products of the visual cycle make up what is known as RPE lipofuscin. Other cell types in the body generate lipofuscin in response to stress, but even healthy RPE cells accumulate lipofuscin. Though accumulation is related to age and light exposure, excessive amounts have been linked to retinal degenerative diseases such as rod-cod dystrophy, recessive Stargardt’s disease, Best vitelliform macular dystrophy, and AMD.

The inability of the RPE cells to metabolize these waste products leads to an accumulation of lipofuscin in the retina. Although associated with dysfunction of the RPE cells, environmental factors like exposure to visible and UVA light or high oxygen concentrations can exacerbate the accumulation.
levels in the eye can increase the accumulation of lipofuscin in the retina. While excessive amounts of lipofuscin in the eye is linked to retinal disease, lipofuscin accumulates in healthy retinas and increases with age until it generally plateaus at around the age of 70 in normal patients. Furthermore, lipofuscin doesn’t include a single bisretinoid in the visual cycle but a host of vitamin A derivatives; however, one bisretinoid in particular (A2E), the result of two all-trans retinal reacting with ethanolamine, is understood to be its main component and is used as an indicator of lipofuscin levels and retinal degeneration in research.

(3) DRUSEN

Another component of AMD is the formation of drusen in the macula. Drusen are amorphous deposits of lipid, fatty proteins that occur in the retina—derived from waste that cannot be excreted by RPE. Drusen occur naturally in healthy eyes. However, excessive drusen can lead to AMD. Excessive lipofuscin in the retina has been linked to drusen formation—as residual lipofuscin accumulates and forms pockets of drusen in the eye.

There are two types of drusen: hard and soft. Hard drusen is common in the elderly population and doesn’t correspond to AMD development—these are generally small (1-63 microns in diameter) and spread apart in the retina. More concerning is soft drusen. Soft drusen is larger than 125 microns in diameter or between 63 and 125 microns in diameter with a visible thickness. While soft drusen can occur in non-AMD retinas (commonly observed in individuals over 60), the link between drusenogenesis and AMD is closely related to the activation of the immune system in AMD. It is notable that
AMD is rarely diagnosed in the absence of soft drusen.\textsuperscript{40} Size, number, and appearance of drusen determine the risk for AMD development in retinas. Soft, large, and/or clustered drusen is associated with choroidal neovascularization symptomatic of wet AMD.\textsuperscript{41}

![Figure 4. Soft Drusen Pictured in Color Fundus and Fluorescein Angiography Images. Images above were obtained from a case with Doyne’s Honeycomb Dystrophy. This disease is associated with a single mutation in the EFEMP1 gene. This gene codes for an EGF-containing fibrillin-like extracellular matrix protein 1, fibulin 3 which is present in the fused basal lamina of the RPE and the Bruch’s membrane. Here, the accumulation of drusen has coalesced and initiated an inflammatory state. The right most image is a fluorescein angiography of the eye pictured on the left. This fluorescein angiography shows the resulting CNV, the small vessels and the leaking blood. Images provided by Dr. Thomas E. Flynn M.D.](image)

Drusen accumulates between the basal side of RPE cells and Bruch’s membrane in the retina (Figure 4). Bruch’s membrane separates the retina (RPE and photoreceptor cells) from the primary capillary bed of the choroid. This barrier consists of two extracellular collagenous layers, the basement membranes of both Bruch’s and the RPE.\textsuperscript{42} Bruch’s membrane has 5 layers. Extending from interior to exterior of the eye, these are: first, basement membrane of RPE, second, inner collagenous zone, third, central band of elastic fibers, fourth, outer collagenous zone, and, fifth, choriocapillaris basement membrane. Transport of nutrients and oxygen to the retina and movement of metabolic waste products from photo-receptors to the choroid occurs through Bruch’s membrane.
The deposition of the RPE cells’ membranous debris or extracellular material into this barrier makes up drusen.\textsuperscript{43}

Drusen accumulation leads to the vision loss that is symptomatic of AMD. Formation of drusen initially causes changes in color vision, contrast sensitivity, visual acuity within the central visual field, and spatiotemporal contrast sensitivity leading to vision loss. These drusen deposits cause the photoreceptor cells associated with the overlying RPE cells to die and create an interruption of the RPE cell monolayer.\textsuperscript{44} Current research hypothesizes that drusen directly causes damage to the photoreceptor/RPE cells but also indirectly causes damage due to its activation of the immune system and associated inflammation.

Drusen in AMD patients contains large amounts of proteins involved in the body’s complement system along with RPE cell-remnants.\textsuperscript{45} These proteins consist of dendritic cells (potent antigen presenting cells which are recruited solely by signals, chemokines and cytokines, of the immune system), tissue inhibitor of metalloproteinase 3, immunoglobulins (antibody complexes), MHC class II antigens, factors of the complement cascade (most notably, the complement factor H associated with the CFH gene and the Y402H mutation noted above), and terminal pathway components (including the membrane attack complex—MAC; C5b-9). The factor C5b-9 is created by the immune cells to attack pathogens but is also detrimental to the RPE, photoreceptor, and choroidal cells.\textsuperscript{46}

In the past, researchers believed that because many components of drusen were synthesized primarily in the liver, the components had accumulated there from the
choroidal vasculature. However, now it is understood that the RPE cells create the accumulations found in drusen. The RPE cells have been shown to have a large amount of the mRNAs associated with the drusen components such as apolipoprotein E (ApoE), complement factors (C3, C5, and C9), and vitronectin (Vn). It was found that the injured RPE accounts for the majority of this complement factors and immune proteins found in drusen.

It has been hypothesized that the drusen (initially an accumulation of RPE cell membrane debris) leads to AMD. This is due to the inflammation caused by the drusen. Some inflammatory components found in drusen are classic acute phase reactant, but most are components of the complement cascade or inhibitors of the membrane attack pathway of complement. Here, acute refers to the initial non-specific reaction of the immune system to a threat; drusen normally contains components that provoke a specialized immune response. This suggests that the development of drusen occurs over a period of years and develops into a state of chronic inflammation—never allowing the system to return to a state of tolerance. Lipofuscin, in addition to RPE cell components and immune system-associated proteins, has also been observed within small, early drusen, and some hypothesize that the presence of lipofuscin leads to RPE dysfunction and the development of AMD.

(4) NEOVASCULARIZATION

Another process involved in AMD is choroidal neovascularization (CNV). This is the major cause of severe vision loss in patients with AMD. Choroidal neovascularization
is the creation of new and abnormal blood vessels originating from the choroidal blood vessels and growing through Bruch’s membrane into the space beneath the RPE monolayer. This may cause serum or blood to collect below the RPE, which can cause distortion of the macula and the introduction of a scotomata, a blind spot in the visual field. CNV is the major cause of vision loss of AMD. The new vessels from CNV bleed and form dense macular scars. Vessels associated with CNV are curled, weak, and leaky.

With the highest oxygen consumption per unit weight of all human tissues, the retina is extremely sensitive to damage and stress. It is vascularized by two independent circulatory systems: the choroid and retinal vessels. The retinal circulatory system provides oxygen and nutrients to the inner two-thirds of the retina while the outer third of the retina, which lies adjacent to Bruch’s membrane, is avascular but relies on the choroidal circulation for its nutrients and oxygen.

Endothelial cells lining the blood vessels in the healthy eye don’t react to neovascular or pro-angiogenic stimuli, and endothelial cell proliferation normally doesn’t occur in the retinal vessels. For angiogenesis to occur, there must be an over-activity of pro-angiogenic signaling (as in any system). The process of neovascularization involves an interplay between stimulators and inhibitors in which the stimulators win out.

Neovascularization in the retina occurs in response to lack of oxygen (hypoxia) or ischemia (lack of blood flow). A lack of oxygen or blood supply is derived from two factors. First, as the body ages, the RPE and other agents in the eye become less efficient at conducting oxygen to the photoreceptor cells. Second, there can be other problems in
the body (beyond the eye) that slow the transmission of oxygen and blood. For instance, high blood pressure, problems with circulation and heart/lung function can impair the optimal transmission of blood and/or oxygen. These issues can be brought on or exacerbated by environmental issues, such as smoking.\textsuperscript{54} The impairment of hemoglobin oxygenation by carbon monoxide from inhaled cigarette smoke leads to tissue hypoxia. Other compounds in cigarette smoke may also act to stimulate the immune system in ocular tissues.

Local inflammation is also implicated in the creation of new blood vessels. Neutrophils, macrophages, mast cells, and microglia are capable of releasing pro-angiogenic factors like vascular endothelial growth factor (VEGF).\textsuperscript{55} VEGF and other pro-angiogenic factors released by the immune cells and distressed RPE cells signal to the endothelial cells to proliferate and create new blood vessels.

\section*{Physiological Transformation from Dry to Wet AMD}

There is a high incidence of dry AMD—roughly 85\% of AMD is dry, as compared to 15\% of wet AMD. However, among patients who suffer severe vision loss due to AMD, the percentages are roughly reversed—nearly 90\% of AMD patients experiencing severe vision loss have wet AMD.\textsuperscript{56} All wet AMD starts with dry AMD; once the disease has progressed from dry to wet, it never reverses to dry again. Due to the prevalence of vision loss associated with wet AMD, it is important to understand the progression of the disease.
The change from dry to wet AMD appears to be initiated by CNV across Bruch’s membrane and the RPE monolayer. The initiation of CNV is primarily caused by one or more of three main factors: VEGF stimulation, damage to Bruch’s membrane, and activation of macrophages. With advancing age, the Bruch’s membrane thickens.\(^5^7\) This thickening compromises and decreases the ability of the nutrients and oxygen to diffuse to the RPE cells.\(^5^8\) It should also be noted that this age-related thickening of Bruch’s membrane linearly coincides with the accumulation of lipofuscin in the retina.\(^5^9\) This thickening is hypothesized to be due to degeneration of the overlying RPE. In effect, RPE becomes less effective at degrading waste in its lysosomes with age. Due to Bruch’s membrane thickening, RPE cells are isolated from their source of nutrition. This, in turn, causes an increase of collagen and mineralized deposits in the elastic lamina (which correlate to increasing lipid content in Bruch’s membrane).\(^6^0\)

Changes due to RPE and Bruch’s thickening lead to a decrease in hydraulic conductivity that makes it more difficult for nutrients and oxygen to diffuse from the choroidal vascularization to the RPE cells. This stress causes the RPE cells to release VEGF, a pro-angiogenesis signal, as a stress response to initiate growth of the endothelial cells lining the choroid’s vessels.\(^6^1\) Furthermore, the change in thickness makes it more difficulty for RPE cells to dispose of waste. However, CNV doesn’t occur without compromise of Bruch’s membrane.\(^6^2\)

In the healthy retina, the Bruch’s membrane separates the choroidal vessels from the RPE and helps transport water, nutrients, and macromolecules between the RPE and choroid. Only a weakened Bruch’s membrane will allow the growth of new vessels in
AMD. In AMD donor eyes where one eye was affected and the other unaffected by CNV, the affected eyes contained a calcification and gaps in the Bruch’s membrane. Further, eyes with neovascular AMD exhibit thinner and more interrupted elastic layers (EL). EL is the layer between the collagenous zones, the two extracellular membranes of the RPE and Bruch’s membrane meet.

It should be stressed that advanced AMD can evolve in two different ways. One is geographic atrophy and the other is CNV. Geographic atrophy is when the soft drusen coalesce and clusters together. These clusters form islands of photoreceptor cells that die and lead to scarring and vision loss. (There is no current treatment for geographic atrophy so this is beyond the scope of this paper.) CNV leads to wet AMD, but in advanced dry AMD, CNV doesn’t occur. Rather, the RPE cells die, and macular scarring occurs. In advanced dry AMD, there may be Bruch’s membrane damage and/or calcification, but it doesn’t lead to CNV.

One last component that initiates CNV is the activation of the immune response and the aggregation of macrophages in the Bruch’s membrane. Several factors have been characterized whose dysfunction leads to a weakening of the Bruch’s membrane and allows the distressed RPE cells to not only release pro-angiogenic factors but activate an immune response. Three adult-onset, autosomal dominant diseases share retinal degeneration similar to that observed in AMD: Sorsby fundus dystrophy, late-onset retinal degeneration (LORD), and malattia leventinese-Doyne honeycomb retinal dystrophy (ML-DH).
Researchers have isolated the mutations causing these disorders. The anti-
angiogenic factors include: TIMP-3 in Sorsby’s, CTRP-5 in LORD, and EFEMP1 in ML-
DH. TIMP3 is a tissue inhibitor of metalloproteinases, while CTRP-5 and EFEMP1 are
extracellular matrix proteins. Mutations in these genes leave the shared basal lamina of
the RPE and Bruch’s membrane vulnerable. Gaps and damage to Bruch’s membrane
observed in these diseases share a similar pathway to that seen in wet AMD, and all three
have the potential to advance to CNV.

In Sorsby and LORD, the basal lamina of the RPE fills up with lipid deposits and
the RPE cells atrophy. The thickened basal lamina deposit is due to the accumulation of
O-binding lipid. In LORD eyes, drusen deposits contain EC, UC, and apoB within the
basal laminar of the RPE. (EC, UC and apoB are proteins involved in the immune
response.)\(^{69}\) Due to the shared phenotypes with wet AMD, the thickening of the basal
laminar deposit has been implicated as a major cause in CNV. Though it was initially
speculated that the lipids found in basal laminar deposit were LDL cholesterol deposits,
current research suggests that these are native lipoproteins caught in transit from the RPE
to the choricocapillaries.\(^{70}\)

In ML-DH, drusen is observed distributed radially throughout the retina along
with fatty deposits located around the optic nerve, in the back of the eye.\(^{71}\) In wet AMD,
the drusen deposits are located exclusively in the macula. These disorders provide some
insight into the factors that malfunction during AMD’s progression from dry to wet. It
seems that the deposition of fats in the Bruch’s membrane hinders movement of solutes
between the RPE and choroid and increases the risk of RPE stress and/or detachment.\(^{72}\)
Oxidation of these lipids activates an inflammatory response, overexpression of VEGF from the distressed RPE, that leads to CNV in wet AMD. Studies have shown macrophage aggregation in and around these gaps in Bruch’s membrane. After infiltration, the macrophages only increase damage to the membrane and the RPE’s basal lamina as they release their own metalloproteinases and collagens to access and phagocytose the fatty deposits. In this way, activation of the immune response and clustering of phagosomes to further compromise Bruch’s membrane is necessary for the initiation of CNV in AMD.

CURRENT/DEVELOPING DIAGNOSTIC TECHNOLOGY AND TOOLS USED TO DETECT AND MONITOR AMD

In the past decade, there has been extensive research in understanding, diagnosing and, ultimately, treating AMD. In particular, as scientists and practitioners have become more knowledgeable about the role of lipofuscin, there has been a great deal of activity aimed at quantifying and tracking the accumulation of lipofuscin in the retina for degenerative disorders. This has led to the refinement of diagnostic tools, methods and technology, which has been instrumental in helping researchers understand and diagnose the progression of retinal disease, including AMD.

In the discussion that follows, the paper will outline these technologies/techniques and explore their current efficacy and potential in AMD research.

(1) Fluorescein Angiography

Imaging has become a crucial tool in assessing the state of the retina and progression of retinal disease. The most basic modality is color fundus photography,
which produces high quality color photographs for diagnosis and future follow-up of retinal disease. Closely related to this is Fluorescein Angiography (See Figure 4), which employs a modified fundus camera with excitation and barrier filters.\textsuperscript{76} Fluorescein (a dye) is injected intravenously into patient’s arm vein and sequential photographs are taken capturing the flow of fluorescein through the retinal and choroidal circulation.\textsuperscript{77} This dye is a xanthene derivative with an absorbance spectrum of 450-490 nanometers and an emittance of 520-530. This molecule binds to plasma proteins—mostly albumin, a carrier protein in the blood—and red blood cells. The pattern of fluorescence allows practitioners to diagnose and follow the progression of vascular diseases of the retina, RPE and choroid. These modalities have been in wide spread use since the late 1950’s and early 1960’s.\textsuperscript{78}

(2) Indocyanine Green Angiography

A related imaging technique is Indocyanine Green Angiography which employs intravenously-injected ICG. ICG is a tricarbicyanine derivative with an absorbance of 799 to 850 nanometers and an emittance of 830 to 840 nanometers. It binds best to globulins, large molecular complexes with proteins. This technique images different vascular structures within the choroid and retina from those visualized by Fluorescein Angiography. This is because the dye used in fluorescein angiography flows better through the smaller retinal vessels and can pass between the fused basal lamina of the RPE/Bruch’s membrane while ICG binds to larger molecules and shows up much better in the choroidal circulation. For this reason, fluorescein angiography images retinal and
subretinal vasculature while ICG is used for underlying choroid. However, ICG imaging technique is generally used in conjunction with fluorescein angiography but its use is limited by its expense, patient side effects, and uneven access to the dye. ICG Angiography has been in use since the 1990’s.\textsuperscript{79}

These techniques allow early visualization of abnormal blood vessel growth in the retina and choroid but have the limitation of being invasive, expensive and having no quantitative value in assessing disease progression. These techniques have proven uneven in their ability to predict a high risk for future progression from dry to wet AMD.\textsuperscript{80}

(3) Quantitative Fundus Autofluorescence Imaging

A newer technique, Quantitative Fundus Autofluorescence (QAF) Imaging is a non-invasive approach in measuring lipofuscin accumulation in the retina.\textsuperscript{81} In this approach, a confocal scanning laser ophthalmoscope emits a 488 nanometer wavelength laser into the eye that reflects images from the retina and the choroid shown into the patient’s eye. No dye is used. The process enables practitioners and researchers to actually see the retina deteriorating. This approach is currently being used to diagnose and track drusen and CNV in AMD.\textsuperscript{82}

In quantitative fundus autofluorescence imaging, this same laser and imaging technique is utilized. In contrast to color photography, fluorescein and ICG angiography, QAF appears to offer a way of predicting progression of patients with AMD to advanced dry or wet disease.\textsuperscript{83} QAF imaging uses the double conjugated structure of lipofuscin’s components to quantify the overall brightness of the retina. The excitation maxima of
lipofuscin fluorophores vary from 440 to 510 nm, but they have similar emission maxima (600 nm), which matches that of fundus autofluorescence.\textsuperscript{84} The confocal scanning laser ophthalmoscope is outfitted with an internal reference to compare laser power and detector gain during imaging. Immediately after a laser bleaches the bisretinoids, a picture is generated, and a computer program analyzes the brightness of mean grey levels throughout the fundus to yield a value, which consists of a ratio between the grey value of the fundus and reference. This analysis takes into account laser strength, degree of pixilation, artifacts, and any eye movement to yield a value of brightness.\textsuperscript{85} This brightness relates to lipofuscin accumulation.\textsuperscript{86}

Quantitative fundus autofluorescence imaging has been performed on mouse models, cell cultures, and patients to non-invasively track the accumulation of lipofuscin. Papers have shown that RPE lipofuscin accumulation, shown by increased fundus autofluorescence, precedes geographic atrophy in the retina.\textsuperscript{87} Other studies done on Abca4 null mice show hyperautofluorescence in areas correlating to a thickening of the Bruch’s membrane due to basal laminar deposits.\textsuperscript{88} A recent 2014 study done on mice modeled after Retinitis Pigmentosa (RP) illustrated the accumulation of lipofuscin fluorophores in areas of atrophying and sick photoreceptor cells, and, in a 2012 study, mice induced with an experimental retinal detachment also showed bright hyperfluorescent rosettes.\textsuperscript{89} Clinically, elevated autofluorescence has been associated with scotomas associated with acute macular neuroretinopathy or from rings observed in the fundus of RP patients.\textsuperscript{90} In recessive Stargardts, studies have shown that the accumulation of lipofuscin precedes RPE cell atrophy and vision loss.\textsuperscript{91} The location of
lipofuscin in the retina can foreshadow retinal dysfunction before RPE and photoreceptor cell death or a compromised Bruch’s membrane.  

**Figure 5.** Autofluorescence Image of Geographic Atrophy in AMD. Unlike a fluorescein angiography, the metabolism of the vessels isn’t shown, but a diagnostician can see the accumulation of lipofuscin (light grey areas) and the presence of lipofuscin granules commonly seen in lesions located around the macula. Also areas of geographic atrophy are striking—seen here in the center over the patient’s fovea. Central RPE atrophy is common in AMD and Stargardt’s Disease also. Images provided by Dr. Thomas E. Flynn M.D.

Much research has been performed on genetic retinal diseases with this approach to illustrate the changes of lipofuscin during the disease’s onset. Many computer programs have been created to compare quantitative fundus autofluorescence values generated from different patients, institutes, or imaging equipment. QAF has been done since the late 1990’s.

(4) High Performance Liquid Chromatography Measurement of A2E

High Performance Liquid Chromatography (HPLC) is an invasive technique performed on donor eyes, mouse models, and cell cultures to measure the amount of A2E in the eye. Lipofuscin consists of different bisretinoids. Researchers have just begun to understand how these waste products can turn toxic through photodegradation and photoxidation. Photodegradation breaks down all the lipofuscin fluorophores (A2E among
them) to molecular fragments; some of these fragments are dicarbonyls that react with and damage protein to form products that incite the inflammation pathway and are numerous in drusen deposits. Studies have shown that A2E is less prone to oxidation than the other fluorophores (so it is more resistant to change its structure and not be detected during chemical analysis). Studies have shown that oxidized A2E doesn’t accumulate with age like the other fluorophores although A2E on its own accumulates and is a good reflection of lipofuscin in the retina. This makes the measurement of A2E fairly straightforward—as its chemical structure is more resistant to changes due to visible or UV light exposure.

By itself, A2E in the retina has been shown to incite complement activation and inhibit the autophagic pathway. (Autophagy refers to the programmed cell death without an inflammatory response—i.e. apoptosis of sick cells rather than necrosis.)

The measurement of A2E through HPLC analysis has been linearly correlated with quantitative autofluorescence values. This approach allows for an invasive chemical analysis of lipofuscin to correlate with quantitative autofluorescent imaging values.

(5) Optical Coherence Tomography

Optical Coherence Tomography (OCT) allows practitioners and researchers to track the thickness and morphology of the five retinal layers, RPE, Bruch’s membrane and the choroid and its vessels. This technique allows both anatomical and quantitative assessment of changes in the retina and choroid. OCT uses a similar approach as does an ultrasound and provides a picture of the membranes and their relative thicknesses. OCT
uses a scanning laser exciter and detector that sweeps extremely thin slices of retina and choroid. This rapid scanning of the retina allows a three-dimensional picture of the retina and choroid to be reproduced in real time.\textsuperscript{100} The laser is used analogously to the way sound waves are used in ultrasound to produce an image. The extremely small wavelength of laser and improvements in detection and computer power are allowing imaging of structures at almost a single cell level. The newest OCT machines permit imaging of individual arterial, venular, and capillary vessels at any given moment. This modality called OCT angiography permits users to evaluate and track blood vessel growth in the eye without injection of expensive and risky dyes.\textsuperscript{101} Again, no predictive value for OCT or OCT angiography has yet been demonstrated.

Current research on mouse models has correlated an increase in A2E accumulation and quantitative autofluorescence imaging values (i.e. increased retinal brightness) with a thinning of the photoreceptor cell layer.\textsuperscript{102} OCT provides a non-invasive and efficient way of tracking the degeneration of the retina—while imaging and HPLC analysis has shown an increase in lipofuscin accumulation to this degeneration.

Before the advent of OCT, researchers relied on histology to track retinal thickness, but OCT allows for the tracking of retinal disease in a mouse model over the course of its life.

(6) Conclusion

Standard imaging techniques, including color photography, Fluorescein and ICG angiography, OCT and OCT angiography permit clinicians and researchers to accurately distinguish dry from wet AMD and assess the success or failure of therapeutic
interventions (discussed below). However, none of these imaging modalities offers a predictive capacity for quantitatively assessing risk of progression of disease. Based upon current research and technology, it appears that QAF is the most promising, non-invasive technique for assessing the progression of AMD and other retinal diseases and abnormalities. In addition, mouse studies should be pursued to consider the progress of lipofuscin accumulation over time.

TREATMENT THERAPIES FOR AMD

Two decades ago, a diagnosis of wet AMD meant imminent blindness. While there were surgical procedures that were used, the success and prognosis was poor. Over the past decade, wet AMD has become treatable, with vision improvement achieved in the vast majority of cases.

This section will discuss the alternative therapies used for AMD, beginning with the disastrous surgical experiments, and culminating with the current injection protocol that has led to the improvement and retention of eye sight by thousands of elderly patients. Currently, there are four approaches to treating AMD: thermal laser, photodynamic therapy, intraviteal anti-VEGF therapy, and investigational platelet-derived growth factor inhibitor.

(1) Surgical Approaches

The first approach used to combat AMD was surgical. In the United States, a procedure was developed that essentially took out the membrane. However, while it
succeeded in removing the damaging accumulated lipofuscin, it also took out the RPE which led to the death of the retina. This strategy was quickly abandoned. In Germany, a scientist developed another surgical approach, in which a surgeon went into the eye and surgically repositioned the retina. This lengthy, aggressive and meticulous surgery proved only minimally successful and was similarly abandoned.

(2) Thermal Laser Therapy

After surgical alternatives were discredited, and before the development of intravitreal, anti-VEGF treatments, some practitioners used laser therapy as a way to treat AMD. The theory was that a thermal laser could burn and kill tissue affected by CNV to prevent vessel growth. No specific wavelength of laser shows an advantage above others. Treatment produced a white retina causing necrosis of the retina, RPE, and choroid. Bleeding or hemorrhage of the choroid can occur but very rarely.

Excessive laser treatment comes with a risk of vision loss because the laser light energy kills the cells of the retina (therefore, it is recommended that burns be inflicted far from the macula), and treatment near the optic nerve can cause tissue necrosis. It is suggested that laser therapy be performed 100-200 microns from the optic nerve. This treatment runs the risk of permanent vision loss, and recurrence rates of the CNV are high because the RPE surrounding the burn release the same VEGF and signal distress. Studies have shown that no more than 15% of wet AMD patients derived any benefit from thermal laser therapy, and all patients lost vision after receiving the laser. A criterion for treatment was that patients should lose less vision with the laser treatment
than if their wet AMD were left untreated. Currently thermal laser therapy as a treatment for AMD isn’t recommended.

(3) Photodynamic Therapy

Until 1999, no treatment was as effective as thermal laser therapy against CNV until the advent of photodynamic therapy with verteporfin. In photodynamic therapy, a photosensitizing drug, verteporfin, is intravenously injected into the patient, and a low-intensity (to minimize damage) infrared laser is emitted on the site of CNV to induce a photochemical reaction with the drug. This activates the circulating drug to injure adjacent cells and allows physicians to direct the attack on the new endothelial cells lining the choroidal vasculature. Despite targeting new growth in the choriocapillaries, anti-VEGF intravitreal injection still has been known to cause long-term macular scarring and central retinal damage accompanied by central vision loss in numerous cases.

The selective destructiveness of the photodynamic therapy made it more efficient than thermal laser therapy. Although retinal and choroidal tissue surrounding the CNV might be minimally disturbed, the RPE, choroid, and overlying sensory retina maintained their function. Rates of vision loss were much lower than those of AMD patients treated with thermal laser therapy.

Recurrence rates of CNV in photodynamic therapy were much lower than those experienced in thermal laser therapy, but patients needed to avoid bright light for 2 days after treatment before it could be ensured that the dye wouldn’t be activated to harm the tissue. Photodynamic therapy could prevent sight loss better than thermal laser therapy; however, it couldn’t be expected to improve or increase visual acuity that the patient had
already lost. The therapy also necessitated multiple treatments over a period of years—for instance, a patient could be expected to have three treatments in the first year and two in the second.114

(4) Intravitreal Anti-VEGF Therapy

Both thermal laser therapy and photodynamic therapy couldn’t restore sight lost and were only approved for the treatment of CNV if the sight maintained would be better than if the condition were left untreated. Both techniques provided limited benefit to patients and neither was used broadly by practitioners.115 However, the prognosis for treatment changed dramatically with the advent of anti-VEGF Therapy which was introduced nearly a decade ago. Anti-VEGF Therapy would ultimately prove successful in restoring sight lost by CNV.

In the early stages of this therapy, pegaptanib sodium was injected into patients. This oligonucleotide binds to an isoform of vascular endothelial growth factor. This treatment wasn’t beneficial for CNV which had turned to scar, but early trials showed that it made a difference in 15% of cases in which the CNV was in an initial stage or hemorrhagic.116 However, the treatment needed to be done repeatedly, and this increased the occurrence of side effects—like endophthalmitis, a bacterial infection of the retina introduced by needle carrying skin bacteria into the eye—on patients. Some trials showed 8% of patients in with induced endophthalmitis lost 6 or more lines of visual acuity.117

The introduction of ranibizumab as anti-VEGF therapy yielded much better trial results than pepaptanib sodium. Ranibizumab was a recombinant monoclonal antibody containing both mouse- and human-derived segments; this drug inhibits VEGF. This drug
is injected intravitreally (into the vitreous humor of the eye) on a monthly basis. Visual acuity improved (10.7 letters in the ranibizumab group compared to a loss of 9.8 letters in the pegaptanib sodium group after twenty-four months). Repeated injections of ranibizumab included local ocular adverse effects (AEs), ranging from subconjunctival or vitreous hemorrhage and vitreous floaters to more serious like endophthalmitis. This is because VEGF inhibitors can stray into the general circulation and affect wound healing or prevent the formation of new blood vessels—which could be detrimental to a patient who had suffered from ischemia. AMD patients suffer high rates of cardiovascular disease, and anti-VEGF therapy is risky without close monitoring. However, there was no evidence that administration of ranibizumab increased systolic or diastolic blood pressure. Higher rates of hypertension were reported in patients undergoing photodynamic therapy (8.4%) than those under ranibizumab (6.4%). Because the drug contains mouse-derived proteins, some studies observed patients with antibodies formed to ranibizumab, but instances are rare and patients’ blood should be monitored for antibodies.

Drugs inhibiting angiogenesis have been introduced to treat CNV since ramibizumab. A cheaper drug derived from similar antibody as that of ramibizumab, bevacizumab, was introduced into the market and showed similar (if slightly less impressive) success and low risk to the original antibody. In 2006, another anti-VEGF drug, aflibercept, was introduced to treat CNV. This recombinant fusion protein binds to VEGF receptors 1 and 2. In addition to treating wet AMD, aflibercept is also used to treat colorectal cancer. Repeated administration of aflibercept on a monthly basis yielded
equivalent results to ranibizumab.\textsuperscript{122} It should be noted that treatment with ranibizumab is the “gold standard” of anti-VEGF therapy, and newly introduced drugs’ antiangiogenic properties are compared to the original antibody’s patient outcomes in trials.

(5) Platelet-derived Growth Factor Inhibitor

A new therapy for CNV that has been introduced works by preventing platelet-derived growth factors from binding to their receptors; specifically, these new drugs for wet AMD target VEGF receptors on pericytes. One specifically, called Fovista, binds to a growth factor called PDGF-BB making it incompatible with the pericyte surface receptor PDGF-Beta.\textsuperscript{123} Pericytes are contractile cells that wrap around endothelial cells and reinforce the structure of capillaries and venules, and when unbound to blood vessels, they fall away from the endothelial cells.\textsuperscript{124} This makes the endothelial cells extremely vulnerable to anti-VEGF drugs. Platelet derived growth factor inhibitors are currently undergoing approval by the FDA and are in phase II of testing, but they have shown high success rates in early trials when used in concordance with anti-VEGF drugs.\textsuperscript{125}

(6) Importance of Early Detection in CNV

The majority of CNV therapy relies on early detection of CNV growth. Fluorescein angiography studies have shown a proliferation rate of 10-18 microns per day in the initial stages of CNV. It is important to identify CNV lesions before they form beneath the foveal center, before they affect the patient’s line of vision. It is suggested that anti-VEGF therapy in instances of early detection can yield better levels of visual acuity in the patient after treatment.

Mouse Model of Age-Related Macular Degeneration Study
Due to the importance of early detection in treatment of AMD, this paper proposes a study tracking lipofuscin accumulation and retinal degeneration on an AMD mouse model. The author of this paper worked on a study that shows the extraordinary potential for garnering data on AMD from mouse study and research. A description of the study follows.

(1) 2014 Mouse Study

In 2014, a study was undertaken to track the progression of mice that had gene knockouts for both Rdh8 and Abca4. In the past decade, Abca4 null mice have been used as a model of Stargardts Disease, a juvenile onset macular degeneration. Stargardts is much less complex than AMD to replicate in the laboratory because its onset is linked to a single gene mutation in the ATP-binding casette of photoreceptor cells. This mutation prevents photoreceptor cells from depositing waste outside of their cytoplasm to be degraded by RPE cells, and it manifests itself in very similar ways clinically. However, although the Abca4 gene knockout has been shown to lead to an accumulation of lipofuscin and photoreceptor death, this model doesn't show the same Bruch's membrane thickening and basal laminar deposits normally seen in Stargardts and AMD.

In the 2014 study, an extra mutation was added in the RDH8 gene. These genes (currently, there are four known) code for enzymes that reduce the waste products of the visual cycle before they are transported from the cytoplasm of the photoreceptor cells to be degraded by the lysosomes of the RPE cells. RDH8 specifically refers to an enzyme that reduces all-trans-retinal to all-trans-retinol with NADPH as a co-factor. This all-
trans-retinol is then transported out of the photoreceptor cell by the ATP-binding cassette transporter protein (coded by the Abca4 gene).

**Figure 7.** QAF Measurement in Mouse Models. A fundus image of a mouse is taken. Levels of brightness are measured and averaged around the optic nerve (shown in black in the middle) to give each eye a corresponding qAF value. High QAF values have been shown to correspond to high levels of A2E in the eye. These QAF values give a non-invasive means of measuring the lipofuscin levels in the eye. Figures generated by the author.

The 2014 study showed an earlier increased lipofuscin and A2E levels using both qAF and HPLC A2E analysis (see explanation of methods above). Photoreceptor death was preceded by heightened lipofuscin and A2E levels; in fact, 50% of photoreceptor thickness—measured by thinning of the outer nuclear layer in the retina through histology and OCT--occurred directly after a peak in lipofuscin and A2E was observed in the mice eyes.

The 2014 study also noted a sudden and dramatic decrease in lipofuscin and A2E levels to correspond to the majority of photoreceptor cell death.\(^\text{132}\) This mimics results seen in Abca4 null mice and the Crb null mice (modeled after retinitis pigmentosa which similarly correlates high levels of lipofuscin and A2E located in areas of dramatic retinal
atrophy).\textsuperscript{133} However, despite the decrease of A2E, QAF values remained elevated. The study attributes this to the presence of bright autofluorescent rosettes in the photoreceptor cells of the double knockout mice.

In addition, the thickening of Bruch's membrane which hadn't been observed in the Abca4 null mice manifested itself in the Rdh8/Abca4 null mice as rosettes of photoreceptor cells. Basal laminar deposits were correlated to these rosettes or semi-circles of photoreceptor cells facing inwards from the RPE.\textsuperscript{134} These rosettes have been discovered and studied in mouse eyes.\textsuperscript{135}

Previously, a study correlated hyperfluorescent granules to these rosettes in mice with surgically detached retinas. This 2014 study used a cryostat of the double knockout mice to show similarly hyperfluorescent granules in the center of these sick photoreceptor cells. OCT and histological analysis supported these claims.\textsuperscript{136}

The 2014 study proposed that these hyperfluorescent granules might be excessive lipofuscin that was concentrated in the photoreceptor cells, but it could give no indication as to why these clustered rosettes of photoreceptor cells--which covered 1/10 to 1/4 of the mouse macula--were especially hyperfluorescent. Another theory posited was that this hyperfluorescence was so concentrated because it had been phagocytosed by macrophages or microglial cells, and the photoreceptor cell rosettes were formed around these clusters of immune cells. The 2014 study emphasized the need for closer analysis of the rosettes which generally emerged after the peak and sudden decrease of lipofuscin. These clusters dramatically
increased after a majority of overall photoreceptor death and retinal thinning had occurred.\textsuperscript{137}

(2) Potential for Further Study with Respect to AMD Progression

Understanding the source of the hyperfluorescent rosettes might aid research in the pathway behind the immune system's role in Bruch's membrane thickening and the effects that make AMD so dramatic. The understanding of lipofuscin and A2E levels in the eye with regards to onset of AMD could also aid in early prevention or diagnosis in AMD.

CONCLUSION

Although much research has been done on the progression of genetic retinal diseases such as Stargardts, Retinitis Pigmentosa, and Best Villiform Disease in mice, very little has been done to observe the progression of lipofuscin in an AMD mouse model before and after the onset of AMD. Although lipofuscin is a known precursor and symptom of AMD, little is known about how its accumulation affects the progression of the disease.

If a study could track and understand patterns in accumulation before even the progression to dry AMD, this could benefit current therapy greatly. A study tracking lipofuscin in the retinas of the mice model before disease onset would permit researchers and physicians to gain a better understanding of the preceding patterns. Early detection is vital to the treatment and therapy of AMD.
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