University of Alberta

Prevention and Treatment of Age-related Macular Degeneration (AMD)

by

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With great appreciation and love, I dedicate this work to my late Grandpa Al, who taught me from a very young age the importance of a good education and to "always finish what you start".

Abstract

Age-related macular degeneration (AMD) is the leading cause of Government-registered blindness in the elderly of the Western world and has two forms: wet and dry. No current AMD therapies are curative, and most are provided after retinal damage from the disease has already occurred (to preserve what is left of the retina). We have constructed a multi-factorial Phase II randomized, controlled clinical trial, titled: "Omega-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) nutritional supplementation to delay the progression of age-related macular degeneration (AMD): The OMEGAlberta Study". Each day, participants in the experimental arm of this study will receive 600mg DHA and 1200mg EPA, plus Vitalux AREDS antioxidant formula. Based on the physicochemical properties of DHA, EPA, and Vitalux, our aim is to delay the 5-year incident rate of progression of intermediate dry AMD to wet AMD. Several tests will be performed, not only to quantify the incident rate of progression of AMD, but also to gain insight of the physiological mechanisms behind the supplements being provided. If the supplements are proven to delay AMD progression, this knowledge should be implemented by changes in health services and policy relating to public education and the treatment of AMD.

Preface

An application to start a clinical trial in Canada must go through many regulatory authorities before approval is granted. Clinical Research Organizations (CROs) form a multi-billion dollar industry by filing and maintaining clinical trials. Our lab has created an investigator-initiated clinical trial, but with limited sources of funding, we were unable to afford a CRO. Instead, we filed an application on our own, which was a tedious, time-consuming task without the backing of a CRO. To submit a quality application, we were required to have a clear understanding of the science behind age-related macular degeneration (AMD). Many obstacles got in the way of submitting a quality study for review, and we quickly realized that investigators who do not have experience submitting applications to regulatory authorities in health care need to start with a well-constructed protocol and utilize free resources offered by their university.

Acknowledgements

Many thanks go out to the members of my Supervisory Committee, the Sauvé lab, the U of A Department of Ophthalmology, Dr. James McCabe, and Dr. Bill Stell, who all with their invaluable support, have driven me to strive for excellence in my Graduate studies. Thank you to CIHR and the Faculty of Medicine and Dentistry Graduate Research Assistantship for funding my Masters research.

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Abbreviations

A2E: N-retinylidene-N-retinylethanolamine
AA: arachidonic acid
ABCA4: ATP-binding cassette protein-4
AF: autofluorescence
AHFMR: Alberta Heritage Foundation for Medical Research
ALA: α-linolenic acid
AMD: age-related macular degeneration
ApoE: Apolipoprotein E
AREDS: Age-Related Eye Disease Study
ARMS2: age-related maculopathy susceptibility protein-2
bFGF: basic fibroblast growth factor
C2: Complement Component 2
C3: Complement Component 3
CEP: carboxyethylpyrrole
CFB: Complement Factor B
CFH: Complement factor H
CFI: Complement Factor I
cGMP: cyclic guanosine monophosphate
CIHR: Canadian Institutes of Health Research
CNV: choroidal neovascularization
CRO: Clinical Research Organization
CSNB Type I: Complete Congenital Stationary Night Blindness
CSNB Type II: Incomplete Congenital Stationary Night Blindness
CTA: Clinical Trial Application
DHA: docosahexaenoic acid
DIN: drug identification number
DIQ: Dietary Intake Questionnaire
DSMB: Data Safety Monitoring Board
E4-: non-supplemented ELOVL4 mice
E4+: supplemented ELOVL4 mice
EE: ethyl ester
ELOVL4: elongation of very long-chain fatty acid-4
EPA: eicosapentaenoic acid
ERG: electroretinogram
ETDRS: Early Treatment of Diabetic Retinopathy Study
Fat-1: fatty acid metabolism-1
ffERG: full-field electroretinogram
FGF-2: fibroblast growth factor-2
HC: Health Canada
HERO: Human Ethics Research Online
HIV: Human Immunodeficiency Virus
HREB: Human Research Ethics Board
HTRA1: high-temperature requirement A-1 serine protease
IGF-1: insulin-like growth factor-1
ISCEV: International Society for Clinical Electrophysiology of Vision
LF: lipofuscin fluorophores
mfERG: multifocal electroretinogram
MF#: Master File Number
mGluR6: metabotropic glutamate receptor-6
mII: metarhodopsin II
N1: first negative mfERG component
NACTRC: Northern Alberta Clinical Trials and Research Centre
NFκB: nuclear factor kappa-B
NHP: Natural Health Product
NIH: National Institutes of Health
NOL: No Objection Letter
NPD-1: neuroprotection-D1
NPN: Natural Product Number
NSERC: Natural Sciences and Engineering Research Council
OCT: optical coherence tomography
ONL: outer nuclear layer
OP: oscillatory potential
P1: first positive mfERG component
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PEDF: pigment epithelial derived factor
PI: phosphatidylinositol
PRS: Protocol Registration System
PS: phosphatidylserine
PSEAT: Protocol Safety and Efficacy Assessment Template
PUFA: polyunsaturated fatty acid
RAH: Royal Alexandra Hospital
RCT: Registered Clinical Trial
REB: Research Ethics Board
RGC: retinal ganglion cell
ROS: reactive oxygen species
RPE: retinal pigment epithelium
SDI: Secure Diagnostic Imaging
SSHRC: Social Sciences and Humanities Research Council
STGD: Stargardt`s Disease
STGD3: Stargardt-like dystrophy
TCPS: Tri-Council Policy Statement
TG: triglyceride
TGFβ: transforming growth factor-beta
TNFα: tumour necrosis factor-alpha
U of A: University of Alberta
VEGF: vascular endothelial growth factor
VLCFA: very long-chain fatty acid
WT-: non-supplemented wild-type mice
WT+: supplemented wild-type mice

Chapter 1: Formation of Age-related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of Government-registered blindness in the elderly of the Western world and has two forms: wet and dry (Klein *et al.*, 2004). Visual impairment due to AMD is predicted to rise from 0.62 to 1.6 million Americans over the next 40 years (Rein *et al.*, 2009). More specifically, AMD affects 2 million Canadians over 50 years of age (Cruess *et al.*, 2009). Estimates from studies conducted in different parts of the world suggest AMD prevalence is 1% in people age 65 to 74, 5% in people age 75-84, and 13% in people age 85 and older (van Leeuwen *et al.*, 2003). Due to the increasing prevalence of visual loss in the elderly population, approaches to delay the onset and progression of AMD are necessary to reduce this burden on society. The main risk factors for developing AMD are age (greater than 65 years old), smoking, and hyperlipidemia (van Leeuwen *et al.*, 2003; Tan *et al.*, 2007; Klein *et al.*, 2010), while there is also a predominant genetic component. The dry form represents 90% of all AMD cases and is characterized by central field vision loss with the accumulation of lipofuscin fluorophores (LF) in the retinal pigment epithelium (RPE). Dry AMD is diagnosed by combining a patient's history with a detailed ophthalmoscopic examination, color fundus photographs, and fluorescein angiography. Patient history should list reports of vision loss or abnormalities, systemic diseases, drug use (including smoking), and a pedigree showing family members with ocular disease. Fundus examination of dry AMD reveals drusen (deposits of protein-carbohydrate-lipid complexes of extracellular

material) between Bruch's membrane and RPE cells, and hyper- / hypo-pigmentation of the RPE (Anderson *et al.*, 2002). The wet form typically develops after the dry form and is diagnosed by choroidal neovascularization (CNV) under the RPE, usually presenting with subretinal hemorrhage, exudative retinal detachment, retinal edema, and / or fibrous scarring (Penfold *et al.*, 2001; Figure 1). This chapter will review the risk factors and pathophysiology behind the formation of AMD, which is necessary for constructing a rationale to support a proposed therapeutic intervention.

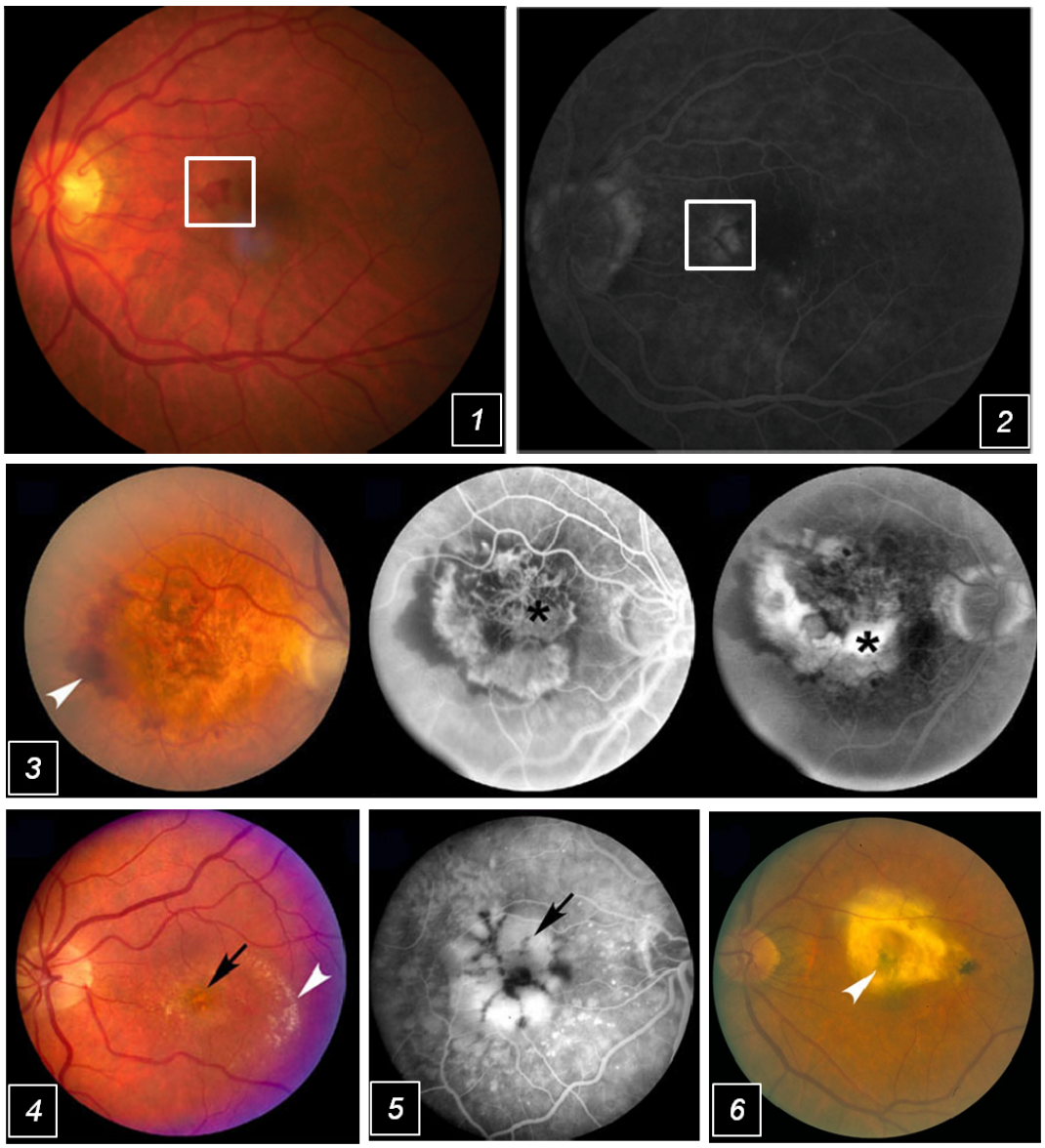

Figure 1: Wet AMD: 1) Color fundus photograph and; 2) Fluorescein angiogram showing an area of CNV (white box; adapted from Haynie, 2010); 3) Color fundus photograph of wet AMD, with corresponding early and late phase FA (arrow shows area of CNV; * denotes area of fluorescein leakage); 4) Color fundus photograph revealing retinal exudates (white arrow) and drusen in the macula (black arrow); 5) Pigment epithelial detachment caused by CNV (black arrow); 6) Disciform scar (white arrow) formed after retinal detachment from CNV (adapted from Hageman *et al.*, 2008).

A. Grading of Age-related Macular Degeneration

Dry AMD is graded into four clinical categories adapted from the Age-Related Eye Disease Study (AREDS; AREDS Research Group, 2000). Patients with Category 1 AMD have less than five small-size drusen (classified as 63μm or smaller) in the retina. Patients are classified with Category 2 (mild AMD) if they have any one of the following factors: pigment abnormalities, greater than five small-size drusen, or single / non-extensive intermediate-size drusen (classified as 63-124μm). Category 3 (intermediate AMD) involves any one of these following factors: extensive intermediate-size drusen, one or more large-size drusen (classified as larger than 125μm), or non-central geographic atrophy. Category 4 (advanced AMD) is characterized by central geographic atrophy that leads to visual acuity worse than 20/32. Category 4 typically progresses to Category 5, which is exudative (wet) macular degeneration.

Alternatively, a more detailed grading system (now considered the gold standard) can be followed using the AREDS 9-step scale (Table 1; Figure 2; AREDS Research Group, 2001a; Davis *et al.*, 2005). The AREDS 9-step scale measures drusen type, size, and area, hyper- / hypo-pigmentation of the RPE, and geographic atrophy (Figure 3). The overall level of AMD severity determined by this scale is based on drusen area and amount of geographic atrophy. Measurements of pigmentary changes, largest drusen size, and predominance of soft, indistinct drusen are graded separately and form the basis of a simplified 5-step scale to determine progression statistics of generalized AMD severities (Figure 4). The 5-step scale assigns each eye one risk factor for the presence of

one or more large drusen (greater than or equal to 125µm, which is approximately equal to the width of an average large vein at the disc margin) and one risk factor for the presence of any pigment anomaly. Note that the smallest clinically detectable drusen range from approximately 25-30µm (Sarks *et al.*, 1999). The risk factors are summed from both eyes to yield a number from 0-4, which corresponds to a risk of 0.5% (0 factors), 3% (1 factor), 12% (2 factors), 25% (3 factors), and 50% (4 factors), of developing wet AMD within five years (AREDS Research Group, 2005). Although it is not practical to use these two scales in the clinic, they provide researchers with the necessary tools to quantify small changes in AMD progression.

Table 1: AREDS 9-step AMD grading scale (adapted from Davis *et al.*, 2005)

Grade	**Drusen Area**	**Geographic Atrophy**
0	**None, Q*, or $<C_0$**	**None**
1	**$\geq C_0$ but $<C_1$**	**Q***
2	**$\geq C_1$ but $<C_2$**	**$<I_2$**
3	**$\geq C_2$ or $<I_2$**	**$\geq I_2$ or $<O_2$**
4	**$\geq I_2$ or $<O_2$**	**$\geq O_2$ or <0.5DA**
5	**$\geq O_2$ or <0.5DA**	**≥0.5DA and <1.0DA**
6	**≥0.5DA and <1.0DA**	**≥1.0DA and <2.0DA**
7	**≥1.0DA**	**≥2.0DA**
8	**Cannot grade**	**Cannot grade**

Grade	Largest Drusen Size	Increased Pigment	Depigmentation	Predominance of Soft Indistinct Drusen
0	None	None	None	None
1	Q*	Q*	Q*	Q*
2	$<C_0$	$<C_0$	$<I_2$	Present, but not predominant
3	$\geq C_0$ or $<C_1$	$\geq C_0$ or $<C_1$	$\geq I_2$ or $<O_2$	Predominant in 1 of 3 zones
4	$\geq C_1$ or $<C_2$	$\geq C_1$ or $<C_2$	$\geq O_2$ or <0.5DA	2 of 3 zones
5	$\geq C_2$	$\geq C_2$ or $<O_2$	≥0.5DA and <1.0DA	3 of 3 zones
6	NA	$\geq O_2$	≥1.0DA and <2.0DA	NA
7	NA	Unrelated to AMD	≥2.0 DA	NA
8	Cannot grade	Cannot grade	Cannot grade	Cannot grade

*when the grader is at least 50%, but less than 90%, sure that the abnormality is present
DA = disc area; NA = not applicable; Q = questionable; C = central; I = inner; O = outer

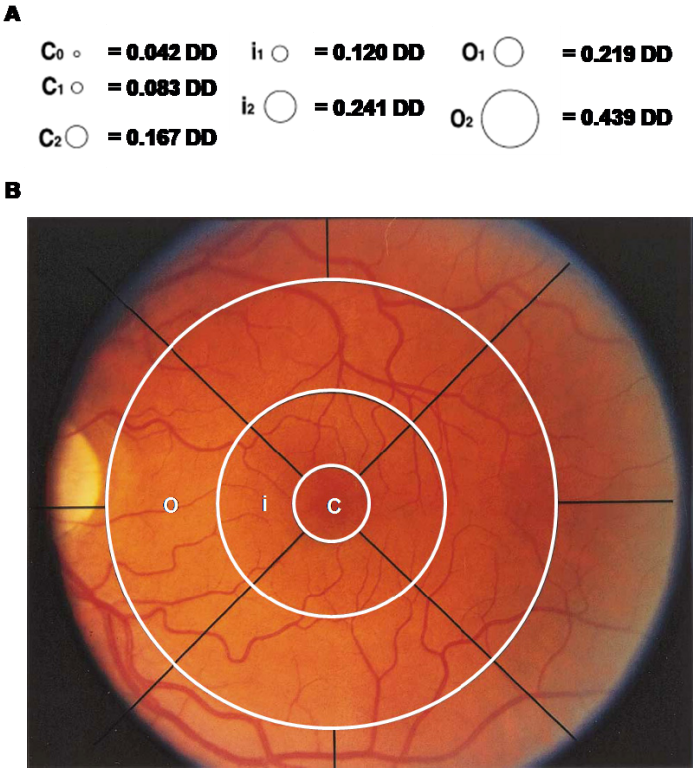

Figure 2: Fundus zones corresponding to Table 1: A) Area measurements for each zone of an average-sized fundus (C: central, i: inner, O: outer); B) Overlay of the 3 zones on a photograph of a healthy fundus (DD = disc diameter; adapted from AREDS Research Group, 2001a).

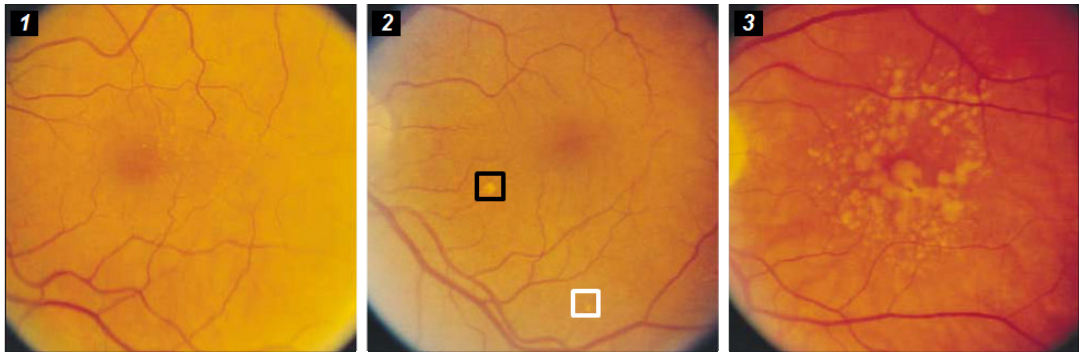

Figure 3: Example fundus photographs of left eyes, graded by the AREDS 5-step and 9-step AMD severity scales (assume right eye is healthy): 1) Healthy fundus: 5-step grade = 0; 9-step grade = 0; 2) Representative eye containing one risk factor: the drusen in the black box is classified as "large", whereas the drusen in the white box falls below the criteria to be classified as "large": 5-step grade = 1; 9-step grade = 3; 3) Representative eye containing two risk factors: large drusen and pigment abnormalities: 5-step grade = 2; 9-step grade = 7 (adapted from AREDS Research Group, 2005).

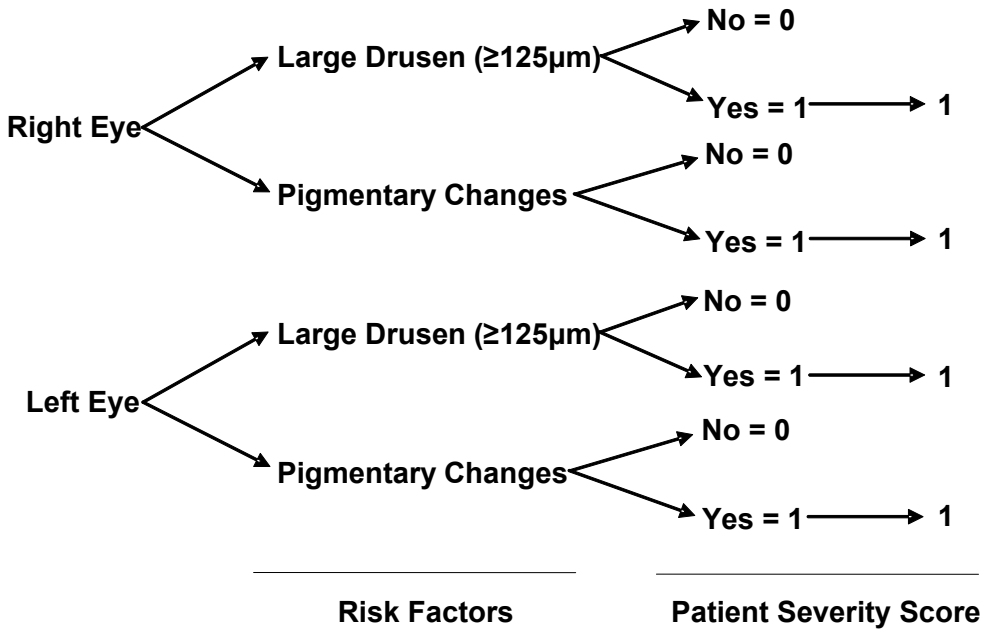

Figure 4: AREDS 5-step simplified AMD severity grading scale (adapted from AREDS Research Group, 2005)

B. Genetics and Risk Factors of Age-related Macular Degeneration

AMD is a multi-factorial disease, with several genes implicated as risk factors. AMD polymorphisms have been found in the following genes: 1) *ABCA4*; 2) *ARMS2/HTRA1*; 3) *APOE* and; 4) *C2*, *C3*, *CFB*, *CFH* and *CFI*. The effects of these mutations are summarized:

1. When a photon hits a rhodopsin molecule (G-protein coupled receptor) in a rod-photoreceptor disc, 11-*cis* retinal is isomerised to *all*-trans-retinal, which provokes the rearrangement of rhodopsin into metarhodopsin II (mII) and initiates the phototransduction cascade with the activation of the G-protein: transducin. The ultimate outcome of this cascade is a drop in the intracellular concentration of cyclic guanosine monophosphate (cGMP), leading to the closure of cGMP-dependent cyclic nucleotide-

gated channels, which hyperpolarizes the rods and stops the release of glutamate at their axon terminals. Inactivation of phototransduction in rods requires recycling of *all*-trans-retinal to 11-*cis* retinal via the retinoid cycle. The ATP-binding cassette protein-4 (ABCA4) is part of the retinoid cycle, responsible for energy-dependent transport of *all*-trans-retinal from the interior of rod outer-segment discs to the interdiscal space (Weng *et al.*, 1999), and is not present in RPE cells (Azarian and Travis, 1997). Mutations in ABCA4 have been implicated in AMD and are known to cause Stargardt's Disease (STGD), which is a recessive form of macular degeneration that occurs in childhood (Mata *et al.*, 2001). STGD has a similar phenotype to AMD, with loss of central vision, accumulation of LF in the RPE, and RPE atrophy in the macula (reviewed in Westerfeld and Mukai, 2008; Wu *et al*., 2009; reviewed in Westerfeld, 2010). Wiszniewski *et al.* (2005) found that ABCA4 mutations cause the protein to become dysfunctional (most likely due to misfolding) or prevent its expression in rod outer-segment disc membranes (due to mislocalization). Accumulated *all*-trans-retinal in the rod outer-segment discs, due to mutant ABCA4, is protonated to form N-retinylidene-phosphatidylethanolamine, which is then condensed and hydrolyzed to form N-retinylidene-N-retinylethanolamine (A2E; Mata *et al.*, 2000). The rod outer-segments, containing high concentrations of A2E, are phagocytosed by the RPE and A2E is subsequently sequestered within the cells (Weng *et al.*, 1999). Of note, phosphatidylserine (PS),

phosphatidylinositol (PI), and phosphatidylcholine (PC) are not protonated to form precursors of LF (Weng *et al.*, 1999). The relevance of these phospholipids is described in Chapter 3.

2. Polymorphisms of the age-related maculopathy susceptibility protein-2 (*ARMS2*) / high-temperature requirement A-1 serine protease (*HTRA1*) locus may affect functioning of the retinoic acid receptor-related orphan receptor-α, a cholesterol receptor (Silveira *et al.*, 2009). The *ARMS2*/*HTRA1* locus has been implicated for CNV in wet AMD, by causing an increase in pro-inflammatory cytokines (Andreoli *et al.*, 2009; Austin *et al.*, 2009).
3. Plasma Apolipoprotein E (ApoE) facilitates unloading of cholesterol and triglycerides (TGs) from plasma lipoproteins into peripheral tissues. In contrast, retinal ApoE protects the macula against nerve fiber loss and is synthesized in significant amounts by the RPE (Klaver *et al.*, 1998; Anderson *et al.*, 2001); therefore, dysfunctional ApoE could contribute to the central photoreceptor loss that is representative of AMD. ApoE is also a major constituent of drusen (Li *et al.*, 2006), which will be described in greater detail to follow.
4. A2E accumulation (Zhou *et al.*, 2006) and deposition of drusen is tightly linked with the complement cascade. Complement factor H (CFH) binds to and inhibits the complement cascade. The *Y402H* variant of this protein is unable to inhibit the complement cascade, resulting in an elevated immune response and subsequent drusen accumulation; CFH actually

becomes a component of these deposits, along with the activated complement factors (Hageman *et al.*, 2005; Lin *et al.*, 2008; Clark *et al.*, 2010). Complement Component 2 (C2), Complement Component 3 (C3), Complement Factor B (CFB) and Complement Factor I (CFI) have also been implicated in CNV (Francis *et al.*, 2009).

Many of the aforementioned polymorphisms hinder RPE function to eventually cause RPE cell death. The RPE forms the blood-retina-barrier, contains the pigment melanin (which minimizes light scatter), produces trophic factors that support photoreceptor survival and function, phagocytoses rod and cone outer-segments, recycles components of the retina for maintenance of proper phototransduction and homeostasis, and plays an integral role in transporting nutrients (reviewed in Strauss, 2005). Tight junctions between cells of the RPE form the blood-retina barrier, to prevent choroidal blood from flowing into the retina; entry of nutrients from the choroid depends on specific transporters along the basolateral membrane of the RPE. If juxtacellular junctions between RPE cells are damaged, the blood-retina-barrier is compromised, allowing uncontrolled entry of glucose, ions, and essential metabolites from the choroid. The diffusion of nutrients and gases through the RPE plays a crucial role in sustaining the aerobic metabolism of the outer (avascular) retina (Takata *et al.*, 1992; reviewed in Bok, 1993). After loss of RPE integrity, outer retina ischemia will eventually lead to the death of photoreceptors, which are cells confined solely to this region. The RPE also accumulates potassium from the sub-retinal space, transports water

from the retinal interstitium toward the choroid, and selectively moves taurine and glutamate from the choroid into the retina to maintain osmolarity and regulate cell volume changes caused by influx and efflux of ions from photoreceptors (Marmor, 1991; Petrosian *et al.*, 2000; Bridges *et al.*, 2001; reviewed in Hamann 2002; Hillenkamp *et al.*, 2004; Mitchell and Reigada, 2008). RPE dysfunction therefore jeopardizes the maintenance of the delicate retinal homeostasis, which typically results in photoreceptor loss.

The RPE secretes several trophic factors that support photoreceptor integrity and viability (Pang *et al.*, 2007). Rods also secrete a protein called: rod-derived cone viability factor, which has been shown to play a role in cone survival (Leveillard *et al.*, 2004); according to these authors, this is one of many other factors potentially secreted by rods that supports cone viability. Rods in the para-foveal region of the macula, where they are at the highest density in the retina (Jonas *et al.*, 1992), are the first retinal cells to die in AMD (Curcio *et al.*, 1996). The RPE-dependent recycling of rod retinoids (referred to as the "visual cycle") may be linked with the fact that rods are lost before cones in AMD, since Müller cells have been shown to posses the ability of recycling cone retinoids (Das *et al.*, 1992).

As the name of the disease implies, the macula in AMD is preferentially affected in terms of functional loss and pathological signs. It is not known for sure why this is the case, but many elements can be taken into consideration when attempting to form a hypothesis. Since age is the main predisposing factor in AMD, one must first consider how aging affects the macula. The high density of

photoreceptors in the macula makes it perhaps the most metabolically active region of the retina. Photoreceptors require extremely high levels of oxygen from passive diffusion through the RPE (reviewed in Stefansson *et al.*, 2010), so one may reason that photoreceptors are very susceptible to DNA damage from the abundant production of reactive oxygen species (ROS). Outer-segment renewal by the RPE minimizes this threat, but with age, mitochondria in the RPE become dysfunctional, impairing the ability of the RPE to phagocytose the outer-segments shed by photoreceptors (Wright *et al.*, 2004; Vives-Bauza *et al.*, 2008). Oxygen consumption also decreases as photoreceptors die, but no feedback mechanism exists to regulate passive oxygen diffusion to the inner retina from the choroid. Therefore, the net level of oxygen in the retina would initially increase without enough cells to metabolize it, exacerbating ROS accumulation (reviewed in Provis *et al.*, 2005); however, Provis *et al.* (2005) also argue that the damage inflicted to this region will eventually hinder oxygen diffusion from the choroid, causing the characteristic retinal ischemia (reviewed in Stefansson *et al.*, 2010) seen during AMD. In addition, light hits the macula at a higher energy than anywhere else in the retina. The macula contains large concentrations of pigments (to be described below) that absorb light energy (Bone *et al.*, 1985; Bone *et al.*, 1988), but concentrations of these pigments are lower in people with AMD (Bone *et al.*, 2001). The vicious cycle of ROS accumulation is then potentiated from increased susceptibility to light damage when the RPE degenerates. For all of these above-mentioned reasons (and more reasons to follow), pathophysiologic changes in the RPE are a key player in AMD formation.

i. Accumulation of Lipofuscin Fluorophores

The retina is constantly under oxidative stress from ROS, primarily: hydrogen peroxide, hydroxyl radicals, superoxide, and peroxynitrite (Brennan and Kantorow, 2009). These compounds are predominantly produced from ultraviolet ray exposure and as a bi-product from the high level of retinal oxygen metabolism (reviewed in Rattner and Nathans, 2006). The RPE contains endogenous antioxidant enzymes and pigments to sequester ROS under normal conditions. Enzymes with antioxidant functions include: ascorbic acid, glutathione peroxidase, catalase, copper / zinc superoxide dismutase, manganese superoxide dismutase, and glutamylcysteine ligase (Neal *et al.*, 1999; Agardh *et al.*, 2006), while lutein and zeaxanthin are highly concentrated macular pigments that block ultraviolet radiation and sequester free radicals, especially peroxynitrite (reviewed in Landrum and Bone, 2001; Santocono *et al.*, 2007). Endogenous antioxidants are saturated from overproduction of A2E free radicals during AMD, so RPE cells become damaged. Similarly, damage may be prevented by antioxidants, such as quercetin, a dietary flavonoid that actively scavenges superoxide and peroxynitrite (Cao *et al.*, 2010); however, without protection, a damaged RPE cell can no longer handle waste products from photoreceptors. Accumulation of waste products, especially oxidized lipoproteins and free radicals, leads to "*para-inflammation"* (Xu *et al.*, 2009). Para-inflammation is described as an intermediate state between basal removal of dead or dying cells by programmed cell death and "frank, overt destructive inflammation" (Xu *et al.*, 2009). This

"intermediate state" can produce enough local cell stress to cause RPE dysfunction and photoreceptor death (reviewed in Holtkamp *et al.*, 2001).

LF, the most common of which is A2E, are bis-retinoid compounds that accumulate with age inside RPE cells (Figure 5; Bindewald-Wittich *et al.*, 2006; Han *et al.*, 2006; Kim *et al.*, 2007; Gutierrez *et al.*, 2010). Light hitting the RPE oxidizes A2E, which will generate ROS that put the RPE under pathological levels of oxidative stress (Broniec *et al.*, 2005). Higher-molecular-weight A2E-like compounds are subsequently formed by spontaneous reaction of A2E oxidation products or by direct reaction of other bis-retinoids with A2E (Murdaugh *et al.*, 2010). Aggregation of these higher-molecular-weight compounds eventually forms granules that can be visualized with fluorescence microscopy. Accumulation of LF may then contribute to atrophic retinal lesions that occur in advanced stages of dry AMD (Schmitz-Valckenberg *et al.*, 2009).

LF that accumulate in RPE cells can be excited with 364nm light and visualized by overlaying a 485nm filter screen. Fundus cameras employ this technology to capture autofluorescent photographs of the retina, but quantification of these photographs is inconsistent (Figure 6; Marmorstein *et al.*, 2002). Computer pixel gray values are compared to the background signal of the same eye, generating a ratio used to measure the amount of autofluorescence (AF); however, gray values are highly variable, even when acquired from the same individual at two closely-spaced intervals (Schmitz-Valckenberg *et al.*, 2009). The relationship between A2E levels in the retina and drusen formation is unclear. Strongest AF signals are usually co-localized with drusen and areas of edema, and

areas of atrophy may extend into previous areas of high AF (Figures 6 and 7); however, areas of high AF can also correspond with locations on color fundus photographs that do not show distinct drusen (Figure 6). Although AF is highly variable and not easily quantified, Schmitz-Valckenberg *et al.* (2009) describe phenotypic patterns of AF expression that could be used as markers to predict AMD progression rate. More research is needed to examine whether AF phenotype is clinically significant for predicting AMD progression.

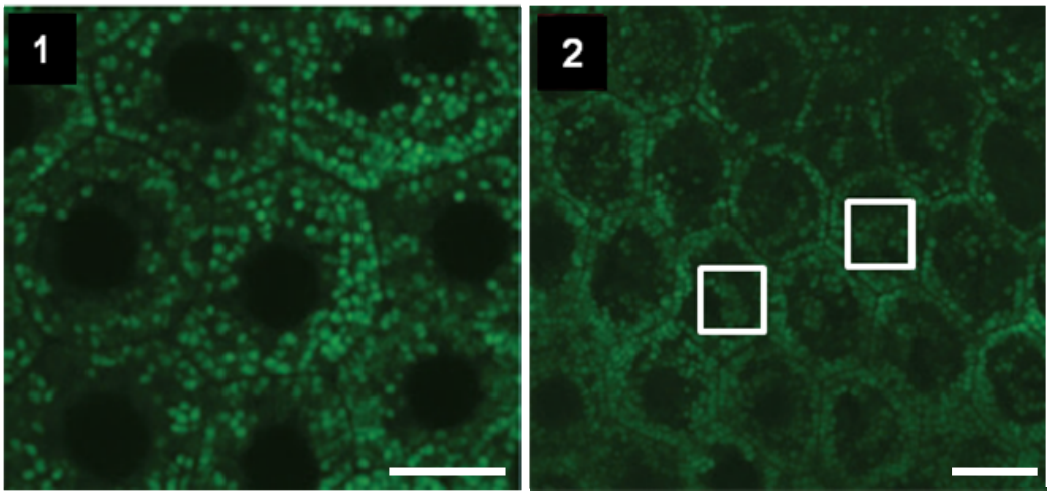

Figure 5: Accumulation of LF granules (green) in RPE cells from human donor eyes: 1) Low density LF granules in periphery of RPE cells from a young adult eye (19 years old, bar = 10μm); 2) High density LF granules in center (white boxes) of RPE cells (along with the low density granules in the cell periphery) from an older adult eye (55 years old, bar = 10μm; adapted from Bindewald-Wittich *et al.*, 2006).

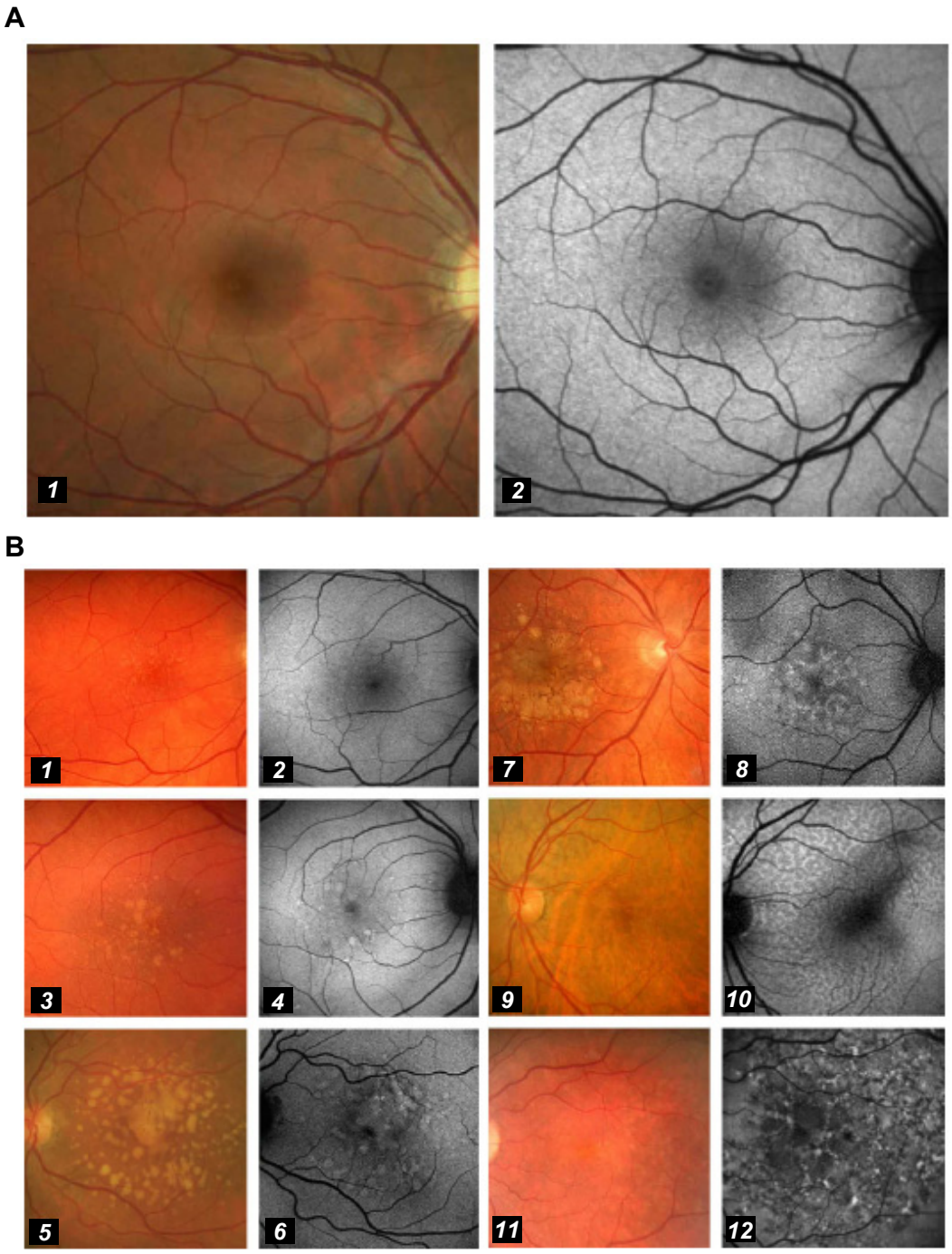

Figure 6: Comparison of fundus AF to color fundus photographs: A) normal fundus (1) and corresponding normal AF photograph (2); B) Fundus photographs of AMD patients showing different drusen formations and pigmentary changes: 2,4,6,8,10, and 12 are AF photographs that correspond to the color photographs of 1,3,5,7,9, and 11, respectively. (adapted from Schmitz-Valckenberg *et al.*, 2009).

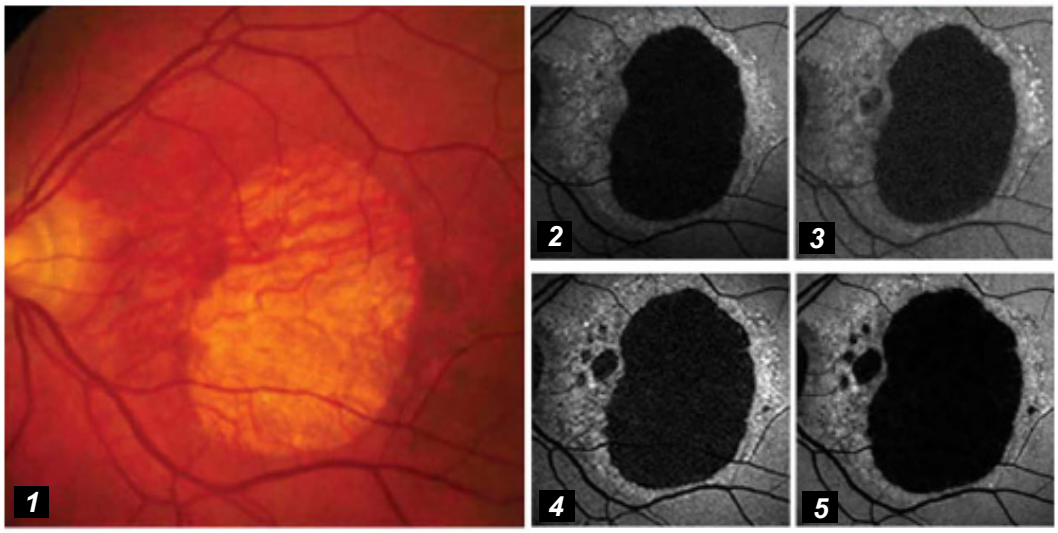

Figure 7: Advanced atrophic AMD: 1) Color fundus photograph; 2-5) corresponding AF measurements taken over the course of disease progression. Note that the area of atrophy extends into previous areas of high AF (adapted from Schmitz-Valckenberg *et al.*, 2009)

ii. Drusen Formation

Waste build-up during photoreceptor degeneration signals a local immune response and pro-apoptotic pathways (Xu *et al.*, 2009). Immune and complement factors elicit drusen formation into the area between the RPE and inner collagenous zone of Bruch's membrane, which can be viewed with optical coherence tomography (OCT; Figure 8; Johnson *et al.*, 2001). Drusen are composed of a wide array of molecules, but major constituents include apolipoproteins: A-I, B-100, C-I, C-II, E, and J (Crabb *et al.*, 2002; Malek *et al.*, 2003; Li *et al.*, 2006), vitronectin (Lommatzsch *et al.*, 2007), esterified and unesterified cholesterol (Li *et al.*, 2007), and complement proteins (reviewed in Rudolf *et al.*, 2008).

Vitronectin is a protective molecule secreted by the RPE that inhibits the membrane attack complex, but is overproduced during wet AMD (Lommatzsch *et*

al., 2008). As described earlier, ApoE protects against nerve fiber loss and the *Y402H* variant of *CFH* reduces inhibition of the alternate complement pathway. In addition, retinal oxidative stress reduces the expression of complement inhibitor proteins on the cell surface of the RPE, leading to unregulated complement activation (Thurman *et al.*, 2009). Although accumulation of vitronectin and ApoE should play a protective role during AMD, the sheer amount of vitronectin and ApoE build-up, and aggregation of complement fragments and other components at the site of inflammation, leads to large deposits that cause pigment epithelial detachments. Pigment epithelial detachments are characterized by a physical elevation of the RPE from Bruch's membrane (Bressler *et al.*, 1994), which increases the rate of progression for developing geographic atrophy and CNV (Cukras *et al.*, 2010). Drusen formation between the RPE and Bruch's membrane may occur by transcytosis (transport of macromolecules inside a cell) of extracellular material from the apical to basolateral side of the RPE (Zhao *et al.*, 2007; Krohne *et al.*, 2010); the extracellular material originally on the apical side of the RPE would displace photoreceptors from the RPE membrane before it migrates to the basolateral side of the RPE. However, in the present model, the RPE reforms contact with photoreceptors once the debris has been transcytosed (Zhao *et al.*, 2007). Since photoreceptors do not appear to be displaced from the RPE due to these sub-RPE deposits in patients, most photoreceptor degeneration could be attributed to the gap between Bruch's membrane and the RPE, which prevents adequate exchange of nutrients between the choroid and RPE. Similarly,

the permeability of Bruch's membrane decreases with age, which also hinders exchange of nutrients between the choroid and RPE (Moore and Clover, 2001).

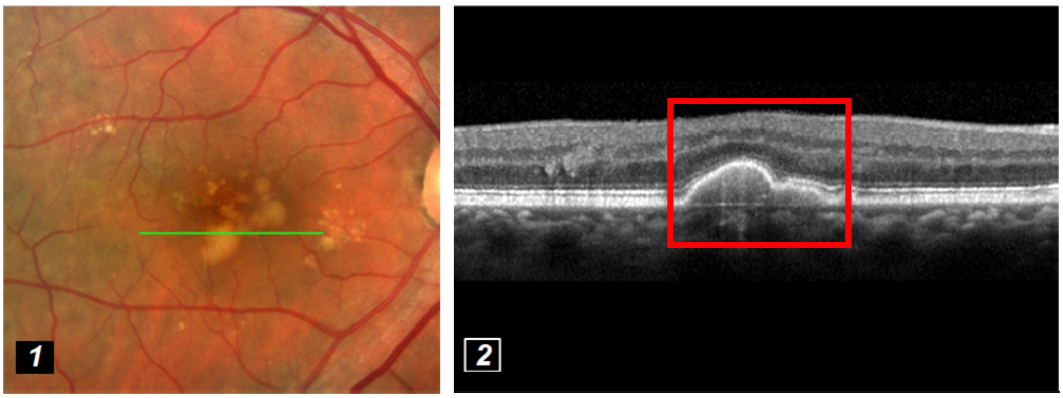

Figure 8: Drusen imaged by vertical slices through the retina, obtained from OCT: 1) Color fundus photograph of dry AMD (horizontal green line corresponds to vertical cutting plane through the retina, for the second picture); 2) Corresponding OCT photograph of a large drusen deposit (red square) under the RPE (adapted from Zweifel *et al.*, 2010). Of note, the location where the OCT plane is placed on the fundus is entirely observer-dependent, which adds some degree of error to the OCT. However, size of particular large drusen may be monitored at subsequent patient visits by obtaining OCT slices through the same plane each time.

iii. Inflammation and Angiogenesis

Increased drusen deposition and subsequent immune signaling results in a self-perpetuating cycle of inflammatory damage that may shift the pathogenesis from dry to wet AMD. ROS recruit inflammatory cells such as neutrophils, mast cells, and microglial macrophages to the RPE (reviewed in Norrby, 2002; Zhou *et al.*, 2005). Inflammatory cells respond to drusen deposits by secreting local, pro-angiogenic factors to cause CNV. Mast cells release pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), fibroblast growth factor-2 (FGF-2), transforming growth factor-beta (TGFβ), tumor necrosis factor-alpha (TNFα), and histamine, to significantly

increase vascular permeability and recruit fibroblasts and macrophages, which secrete VEGF as well (Kvanta *et al.*, 1995; reviewed in Norrby, 2002). Neutrophils also enter the injured site during the initial stages of inflammation to secrete VEGF (Zhou *et al.*, 2005). VEGF is the major contributor to wet AMD pathology, and in addition to being secreted by inflammatory cells recruited to drusen deposits, it is produced in high quantities by RPE cells around sites of neovascularization (Lopez *et al.*, 1996). VEGF induces angiogenesis, stimulates permeability of the microvasculature, enhances endothelial cell survival, inhibits thrombosis, suppresses effects of anti-inflammatory mechanisms, and is a chemotactic factor for recruitment of endothelial cells and monocytes into the site of blood vessel formation (reviewed in Penn *et al.*, 2008). Additional factors also have roles in wet AMD and may act as potential therapeutic targets: Insulin-like growth factor-1 (IGF-1) levels are elevated (Lambooij *et al.*, 2003) and induce VEGF expression locally within the choroid (Sall *et al.*, 2004; Economou *et al.*, 2008) and; pigment epithelial derived factor (PEDF; an anti-angiogenic factor), along with bFGF, are released from the RPE and up-regulated in wet AMD (Rosenthal *et al.*, 2005). Secretions of these factors may suggest that the RPE controls angiogenesis in the later stages of AMD (Martin *et al.*, 2004).

Researchers have also found a reduction of endogenous angiogenesis inhibitors (endostatin and thrombospondin-1) in the RPE–Bruch's membrane–choriocapillary complex of AMD patients (Uno *et al.*, 2006; Bhutto *et al.*, 2008). A biochemical barrier in this region may be formed by these inhibitors of angiogenesis to prevent CNV. A Phase I clinical trial for PEDF gene replacement

therapy is underway (ClinicalTrials.gov; NCT00109499), while research into IGF-1 and bFGF inhibition both show repression of CNV, but have not reached clinical testing (Wang *et al.*, 2002; Economou *et al.*, 2008).

Pro-angiogenic factors released during AMD (mainly from inflammation) increase formation of new blood vessels in the choroid. A review by Norrby (2002) simplifies the explanation of angiogenesis into five main steps: 1) Activation of vascular endothelial cells; 2) Degradation of the basal membrane extracellular matrix; 3) Endothelial cell migration and proliferation from the angiogenic stimulus; 4) Formation of a blood vessel lumen by endothelial cells and; 5) Branching of a new blood vessel and network formation. Angiogenesis requires extracellular signaling events, and when these events become uncontrolled, neovascularization from the choroid into the subretinal space leads to retinal detachment, hemorrhages, and edema (Figure 1). Photoreceptors are physically displaced from the RPE due to the invasion of blood vessels, causing RPE dysfunction and photoreceptor death (Lafaut *et al.*, 2001). The leaky vessels from neovascularization also promote Müller cell hypertrophy (due to swelling from increased fluid uptake) and retinal edema, which may dilute ions to slow hyperpolarization / depolarization kinetics involved in phototransduction (Bringmann *et al.*, 2002). AMD therapies are available to target CNV, but irreversible photoreceptor damage has occurred by this point and new blood vessel formation eventually overcomes the capacity of treatment.

The AMD grading scales created by AREDS provide researchers with useful tools to accurately quantify clinical changes attributed to disease progression or

treatment. AMD is a multi-factorial disease with many prospective targets for therapeutic intervention. The accumulation of LF in the RPE contributes to a substantial portion of AMD pathophysiology, since this leads to RPE dysfunction and degeneration, with subsequent photoreceptor death, inflammatory signaling, sub-RPE drusen formation, and CNV. Therefore, reducing accumulation of LF in the retina may provide researchers with an adequate target to delay the onset and progression of AMD.

Chapter 2: Nutritional Supplementation as an Approach to Delay Age-related Macular Degeneration

The environmental and genetic contributions to the accumulation of LF, drusen deposits, retinal inflammation, and angiogenesis, make AMD a challenge to treat. One may first attempt to reduce exposure to the main environmental risk factors, but the natural aging process and genetic contributions to the disease may eventually cause the need for direct therapeutic intervention. No approach has been found to cure AMD: current approaches simply delay its progression to preserve what is left of the retina. According to estimates by Rein *et al*. (2009), access to palliative treatment over the next 40 years may lower visual impairment due to AMD by 34.5%. This chapter will review the current therapies available for AMD and offer insights into delaying the formation and progression of the disease.

A. Current Therapies for Age-related Macular Degeneration

The current standard of care in AMD therapy consists of a three-fold approach: vitamin supplementation, intravitreal anti-VEGF injections, and laser therapy. AREDS, formed by the National Institutes of Health (NIH), provided an intervention aimed to prevent oxidative damage and the subsequent immune response in dry AMD, by supplementing individuals with antioxidant vitamins (15mg β-carotene, 500mg Vitamin C, 400IU Vitamin E per day) and cofactors for antioxidant enzymes (80mg zinc oxide, and 2mg cupric oxide per day; Chan *et al*., 1998). Of note, zinc also has intrinsic antioxidant properties, by competing

with pro-oxidative ions (such as Fe^{2+}) for binding sites on lipids and proteins (Zago *et al*., 2000; Zago and Oteiza, 2001). Fundus examination after supplementation with the original AREDS formula revealed delayed AMD progression and improved odds for not developing a more advanced form (AREDS Research Group, 2001b). AREDS2 was recently developed to test two interventions (Table 2): 1) Omega-3 polyunsaturated fatty acids (PUFAs) and/or macular pigments and; 2) A modified version of the original AREDS antioxidant formula, lowering the amount of zinc to 20mg/day and not supplementing smokers with β-carotene, since high levels of zinc may cause genito-urinary problems and smokers taking β-carotene are at increased risk of developing lung cancer (Evans, 2006). A more detailed explanation of AREDS2 is provided in *Part B* of this Chapter.

Vitamin therapy is ineffective when dry AMD advances to the wet form. Ranibizumab (Lucentis™), bevacizumab (Avastin™) and pegaptanib sodium (Macugen™) are anti-VEGF drugs administered by intravitreal injection to patients with wet AMD. Bevacizumab is a full-length, humanized monoclonal antibody that binds to and inhibits all isoforms of VEGF (Karanjia *et al*., 2008). Ranibizumab is an antibody fragment (derived from the same parent murine antibody as bevacizumab) that binds to and inhibits all subtypes of VEGF-A (Rosenfeld *et al*., 2006). Pegaptanib sodium inhibits $VEGF_{165}$ (a major pathological VEGF isoform), preventing binding to its receptors on endothelial cells (Friberg and Tolentino, 2010). Each drug decreases blood vessel leakage

with subsequent improvement of visual acuity, but only slows down and does not completely prevent the advancement of CNV.

Photodynamic therapy is usually given in conjunction with bevacizumab therapy when anti-VEGF drugs begin to fail. Photodynamic therapy reduces hemorrhaging in the eye through intravenously administering verteporfin (Visudyne^{TM}), followed by activation with a low energy non-thermal laser. Verteporfin in the choroidal vasculature produces short-lived singlet oxygen species upon activation, which ironically is a similar inducer of pathology as the disease. ROS, generated by the laser damage to endothelial cells, promote scar formation that seals the vessels (Silva *et al*., 2008). The multifocal electroretinogram (mfERG) of wet AMD patients is similar before and after the treatment, which suggests that light activation and subsequent signaling in the retina is preserved (Lai *et al*., 2004); however, visual acuity outcomes are not improved (Kurzinger *et al*., 2010; Rudnisky *et al*., 2010). These results suggest that photodynamic therapy does not recover visual function, but only helps to preserve what is left of the retina and delay the onset of future damage.

B. Fish Oil Supplementation

Numerous studies have reported a reduced incidence of developing dry AMD in people who consume high levels of dietary omega-3 PUFAs in the form of docosahexaenoic acid (DHA; 22:6$\Delta^{4,7,10,13,16,19}$) and eicosapentaenoic acid (EPA; 20:5$\Delta^{5,8,11,14,17}$; Figure 9; Mares-Perlman *et al*., 1995; Smith *et al*., 2000; Cho *et al*., 2001; Seddon *et al*., 2001; Chua *et al*., 2006; Seddon *et al*., 2006; Delcourt *et*

al., 2007). More specifically, reports describe people with dry AMD who eat an abundance of oily fish have a 40-50% reduced likelihood of progressing to the wet form (SanGiovanni *et al*., 2004, SanGiovanni and Chew, 2005; SanGiovanni *et al*., 2008).

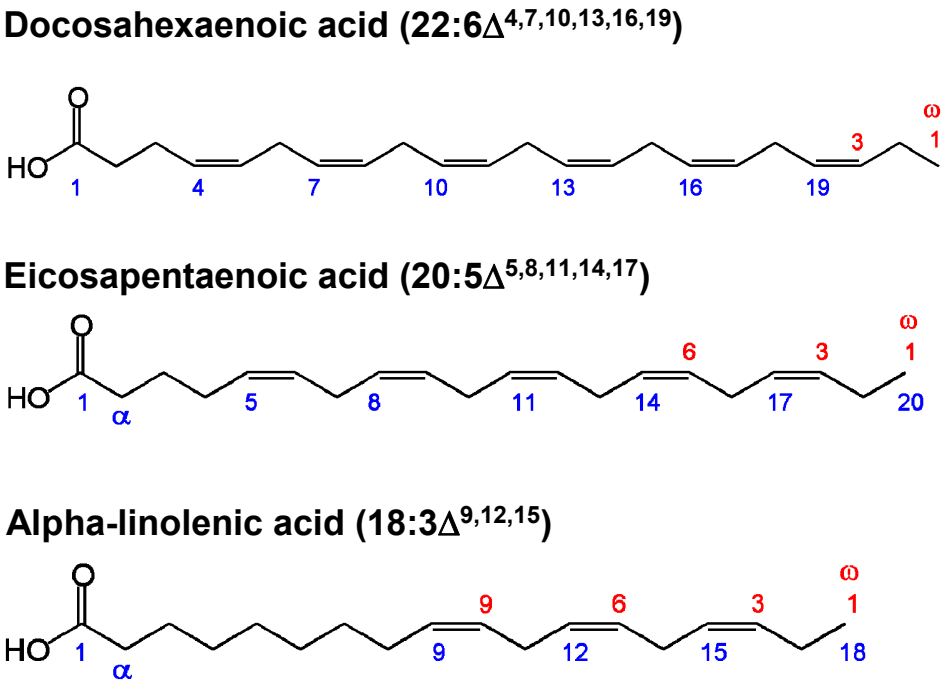

Figure 9: Chemical structures of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and α-linolenic acid (ALA). Red numbers denote carbon numbering from the ω-end and blue numbers denote carbon numbering from the carboxyl end.

The Health Canada (HC) Fish Oil Monograph (HC, 2009) describes common sources of fish oil that contain high amounts of DHA and EPA as: anchovy (Engraulidae); Jack or pompano (any species of Carangidae); herring, shad, sardine, or menhaden (any species of Clupeidae); smelt (any species of Osmeridae); mackerel, tuna, or bonito (any species of Scombridae); sand lance (any species of Ammodytidae) and; salmonids (any species of Salmonidae). Fish

oil is naturally found as a TG, but is commercially concentrated as an ethyl ester (EE). Efficacy of the TG versus EE form of DHA and EPA is unknown. The intestinal absorption is equal for both forms, but the bioavailability of the TG form is substantially higher than the EE form, with significantly less incorporation of the EE form into plasma phospholipids, TGs, and cholesterol esters (Hansen *et al*., 1993; Dyerberg *et al*., 2010). EPA is required for the synthesis of very long chain fatty acids (VLCFAs: over 24 carbons long; Anderson and Ma, 2009; McMahon and Kedzierski, 2009). In the retina, VLCFAs (provided through EPA) and DHA are essential constituents of photoreceptor disc membranes. While the role of VLCFAs remains to be elucidated, numerous studies (to be described in Chapter 3) have shown that VLCFA and DHA deficiencies lead to abnormal retina function in both humans and animals. A key role of DHA is to provide a physicochemical environment that optimizes interactions between several key membrane-bound proteins involved in phototransduction. Although the essential fatty acid, α-linolenic acid (ALA) can be a substrate for the synthesis of DHA, the level of DHA produced from this synthetic pathway is not sufficient to achieve physiological needs in the retina (Sprecher *et al*., 1995; Contreras and Rapoport, 2002). Therefore, intake of preformed DHA from the diet is the most effective means for the body to acquire physiological levels of DHA. AREDS2 is testing whether combined nutritional supplementation with DHA and EPA might slow down the progression of AMD. However, as of yet, no multivariable, highly focused, clinical trials have been created to assess the physiological mechanisms responsible for the potential beneficial effects of fish oil supplementation. By

highly focused, we imply the aspect of experimental design that takes into account the predicted progression of intermediate-stage dry AMD to its wet form: to be described in Chapter 4, our study focuses on patients having intermediate dry AMD in one eye with wet AMD in the other eye, which allows the prediction that the intermediate dry AMD eye is at the most risk to develop wet AMD within 5 years. Our preclinical studies were designed in a similar way, by relying on a monogenic AMD animal model in which the progression of retinal degeneration has been clearly defined.

Our lab assessed the effect of DHA supplementation on *ELOVL4* transgenic mice (Sauvé *et al.*, 2009), after a study by MacDonald *et al*. (2004) showed visual improvement after DHA supplementation in a 15-year-old girl with an Elongation of Very Long-chain Fatty Acid-4 (ELOVL4) mutation. The ELOVL4 protein is found in the endoplasmic reticulum membrane and is thought to catalyze a rate-limiting condensation reaction in fatty acid elongation from C16 to C28-C36 (Jakobsson *et al*., 2006; Molday and Zhang, 2010). Mutant ELOVL4 does not incorporate into the endoplasmic reticulum membrane but instead forms aggresomes in the cytosol and exerts a dominant negative effect on the wild-type ELOVL4 protein (Grayson and Molday, 2005). Although the specific substrate preference of ELOVL4 and the role of VLCFAs in the retina are unclear (Molday and Zhang, 2010), ELOVL4 transgenic mice were used in our experiments as a model of Stargardt-like dystrophy (STGD3), since these mutants have significant central photoreceptor loss, increased accumulation of LF, and a diminished electroretinogram (ERG) response (McMahon *et al*., 2007). Our lab found that

DHA supplementation from one to three months after birth had no effect on the supplemented wild-type (WT+) and ELOVL4 (E4+) mice when compared to the non-supplemented wild-type (WT-) and ELOVL4 (E4-) mice. However, when supplementation was prolonged to six months, RPE function, inferred from the ERG c-wave, was improved in E4+ versus E4-. Kuny *et al.* (2010) has previously described that by 6 months, the outer nuclear layer (ONL) thickness of the ELOVL4 mouse (not supplemented with DHA) retina decreased by 75%, compared to measurements at 1 month of age. We then found that DHA supplementation, starting at 6 months and ending at 12 months, led to maintained a-waves and b-waves and reduced A2E levels in E4+ versus E4- mice. Supplementation from 12 to 18 months of age produced a similar beneficial effect in WT+ versus WT-, but not the E4+ or E4- mice (Sauvé *et al.,* 2009). We hypothesize that too much photoreceptor damage had occurred in the ELOVL4 mice by this stage to be supported by supplementation, based on the findings from Kuny *et al.* (2010) that at 18 months, the ONL is reduced to a single layer of cells and there are no detectable photoreceptor outer segments. The findings from our lab imply that dietary DHA could have a broad therapeutic application, acting on normal age-related ocular processes, which would bypass the primary defect found in the ELOVL4 mouse model of STGD3 (Sauvé *et al.,* 2009).

Apparent contradictory results were published by Li *et al*. (2009) suggesting that DHA supplementation did not prevent the progression of STGD3 pathology in the same mouse model as we used. However, it is important to note that DHA supplementation was obtained by breeding the ELOVL4 transgenic

mouse with fatty acid metabolism-1 (fat-1) mice. Transgenic fat-1 mice express the *fat-1* gene from *Caenorhabditis elegans*, which encodes for an omega-3 fatty acid desaturase (absent in mammals) that can synthesize omega-3 PUFAs from omega-6 PUFAs (Kang *et al*., 2004; Ma *et al*., 2006). The expression of fat-1 was driven by the Chicken β-actin promoter. This promoter was chosen because it yields high levels and ubiquitous expression of the transgene in mice. As such, fat-1 mice accumulate high omega-3 PUFA levels in many organs and tissues (Kang *et al*., 2004). Metabolism of omega-3 PUFAs competes with omega-6 PUFA metabolism, since both pathways utilize the same enzymatic machinery (Contreras and Rapoport, 2002). Our lab has shown that fat-1 mice have very high omega-3 and very low omega-6 PUFA concentrations of fatty acids in the retina (Suh *et al*., 2009). Pathologically high levels of DHA in the retina coincided with an accumulation of the deleterious DHA metabolite, carboxyethylpyrrole (CEP), in abnormally long and tortuous photoreceptor outer-segments (Suh *et al*., 2009). Fat-1 mice also have supra-normal ERG amplitudes (a sign of retina dysfunction) and evidence of retinal stress, such as increased expression of glial fibrillary acidic protein by Müller cells throughout the retina (Suh *et al*., 2009). Considering the pathological state of the fat-1 retina, the experimental design from Li *et al*. (2009) does not provide an appropriate means of supplementing DHA to the ELOVL4 mouse. The opposite situation (high omega-6 and low omega-3) has also been associated with impaired rod photoreceptor renewal and abnormal ERG response in animals and human infants (Neuringer *et al*., 1986; Martinez *et al*., 1988; Pawlosky *et al*., 1997). Therefore,

balance of omega-3 and omega-6 is crucial for the physiological functions in which these fatty acids are involved. For these reasons, results from our nutritional supplementation in ELOVL4 and wild-type mice can serve as a more optimal pre-clinical study on which to expand at the clinical level; however, one could still argue that the ELOVL4 mouse may not be an appropriate model for AMD research, since it uses a phenotype that is expressed at a young age, while we are studying a disease that results from aging. Regardless, patients with AMD have many drusen and increased AF, which supports the notions that LF (A2E being the main constituent) are strongly associated with the etiology of AMD. If DHA slows the progression of A2E accumulation, there could be a subsequent decrease of immune signaling in the retina that results in less deposition of drusen, hence a slower progression from dry to wet AMD.

The results from our mouse studies, which have been submitted for publication, prompted us to create a clinical trial titled: "Omega-3 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) nutritional supplementation to delay the progression of age-related macular degeneration (AMD): The OMEGAlberta Study". Our clinical trial provides a similar intervention as AREDS2 (Table 2), but includes participants *specifically* having wet AMD in one eye, with intermediate dry AMD in the fellow eye (unilateral wet / dry AMD), since these patients are most at risk for developing wet AMD in the fellow eye within five years (Yanoff and Duker, 2009). AREDS2 and the OMEGAlberta Study both grade color fundus photographs with the AREDS 9-step scale, genotype participants, use the Early Treatment of Diabetic Retinopathy

Study (ETDRS) measure of visual acuity, and assess dietary intake of omega-3 PUFAs; however, the OMEGAlberta study also records full-field electroretinograms (ffERGs) and mfERGs, fundus AF, and OCT to quantify the effects of DHA on membrane fluidics, neuroprotection, and disease progression.

Table 2: Comparison of AREDS and AREDS2 experimental arms with our study.

	AREDS	AREDS2 (1st experimental arm)	AREDS2 (2nd experimental arm)	OMEGAlberta Study (MEG-3® 4020EE *plus* Vitalux™ AREDS Formula)
Copper	2mg	2mg	-	2mg
Zinc	80mg	20mg	-	80mg
Vitamin C	500mg	500mg	-	500mg
Vitamin E	400IU	400IU	-	400IU
β-carotene	15mg	15mg (non-smokers only)	-	15mg (non-smokers only)
Lutein	-	-	10mg*	10mg
Zeaxanthin	-	-	2mg*	1mg
DHA	-	-	350mg*	600mg
EPA	-	-	650mg*	1200mg
Placebo	Proprietary	AREDS formula	AREDS formula	None (however, experimental data will be compared against subjects taking AREDS formula)

*In addition to supplementing these together, lutein + zeaxanthin and DHA + EPA are being tested separately without being combined.

According to the HC Fish Oil Monograph (HC, 2009), the maximum combined dose of DHA and EPA (DHA + EPA) should not exceed 1500mg/day for children (1-8y), 2000mg/day for adolescents 9-13y, 2500mg/day for adolescents 14-18y, and 3000mg/day for adults (≥19y). An in-depth summary of fish oil supplementation is provided in the HC Fish Oil Monograph (HC, 2009; Table 3), but note that AMD is not described as an indication for any of the listed clinical trials. The OMEGAlberta Study daily dose of 600mg DHA and 1200mg EPA complies within the safe adult maximum dose of 3000mg DHA + EPA

dictated by HC. Since the daily dose of fish oil complies within HC safe limits, we anticipate no toxicities in our study.

Table 3: Summary of clinical trials that have used fish oil (adapted from HC, 2009).

EPA + DHA	Min. DHA content	Min. EPA content	EPA:DHA ratio	Indication	References
100-3000mg				Maintenance of good overall health	Trumbo *et al*., 2002; Oh, 2005; Simopoulos, 2007
100-3000mg	100mg			Maintenance of cognitive health and brain function	Trumbo *et al*., 2002; Haag, 2003; Fontani *et al*., 2005a; Fontani *et al*., 2005b; Freund-Levi *et al*., 2006; van de Rest *et al*., 2008
150-2000mg	150mg			Development of brain, eyes, and nerves up to 12 years old	Trumbo *et al*., 2002; Haag, 2003; Marszalek and Lodish, 2005; Ryan *et al*., 2010
500-3000mg			0.5:1 to 2:1	Maintenance of cardiovascular health	Kris-Etherton *et al*., 2002; Leaf *et al*., 2003; Oh, 2005; Wang *et al*., 2006
1000-3000mg			0.5:1 to 2:1	Reduce serum TGs / triacylglycerols	Sirtori *et al*., 1998; Nilsen *et al*., 2001; Balk *et al*., 2004; Hooper *et al*., 2004; Oh, 2005
2800-3000mg			0.5:1 to 2:1	Reduce rheumatoid arthritis pain (in conjunction with standard therapy)	Skoldstam *et al*., 1992; Volker *et al*., 2000
1500-3000mg		1000mg	1.75:1 to 2:1	Healthy mood balance	Stoll *et al*., 1999; Peet and Horrobin, 2002; Zanarini and Frankenburg, 2003; Fontani *et al*., 2005a; Fontani *et al*., 2005b; Nemets *et al*., 2006; Sontrop and Campbell, 2006

EPA levels in blood increase rapidly within a week of beginning fish oil supplementation, with DHA levels rising much slower (Metherel *et al*., 2009). EPA and DHA require greater than 4 weeks of intensive supplementation to plateau in the plasma. Plasma half-life of EPA can range from 1-14 days after cessation. Comparatively, cell membranes act as a reservoir for DHA and EPA. DHA and EPA, sequestered in the lipid bilayer, take 1-2 months to return back to baseline after stopping long-term supplementation (Zuijdgeest-van Leeuwen *et al*., 1999; Masson *et al*., 2007). Chapter 3 provides a more detailed explanation of the biochemistry and physiology of DHA and EPA.

Antioxidant vitamin supplementation, intravitreal anti-VEGF injections, and photodynamic therapy are effective for slowing down the progression of AMD, but do not cure the disease. Approaches that delay the onset and progression of AMD are currently the only therapeutic option, and the numerous factors that contribute to AMD pathology put forward a poor prognosis for the development of curative treatments in the near future. The addition of fish oil to antioxidant vitamin supplementation could be more effective at delaying AMD progression than the current standard of care.

Chapter 3: Physiological Implications of Omega-3 Polyunsaturated Fatty Acids in Neural Tissue

Humans do not have desaturases that introduce double bonds beyond C9 in fatty acid chains, so fatty acids having double bonds at C10 or higher are referred to as "essential": they must be taken up by the diet. DHA, EPA, and ALA are the three most abundant essential omega-3 PUFAs (Figure 9). This chapter will review how DHA and EPA (but not ALA) may contribute toward delaying the onset and progression of AMD.

DHA is required for multiple aspects of central nervous system functions. DHA constitutes 40% of the brain PUFAs and can account for up to approximately 95% of the total PUFAs within the mammalian retina (Aveldano and Bazan, 1983; reviewed in Giusto *et al*., 2000; reviewed in Innis, 2003 and 2004, and Singh, 2005), which is an integral part of the central nervous system. DHA is uniquely found as a dipolyene in the retina (mainly in photoreceptor outer-segment discs), incorporated into the *sn*-1 and *sn*-2 positions of phosphatidylethanolamine (PE; 67%), PC (18%), PS (18%), and PI (<5%) by docosahexaenoyl coenzyme A synthases (percentages represent the total amount of the molecular species bound to DHA in the plasma membrane; Salem *et al*., 1980; Aveldano and Bazan, 1983; Reddy *et al*., 1984; Stinson *et al*., 1991; Litman and Mitchell, 1996; Suh *et al*, 1996). The *sn*-1 position of retinal PC is also found with C28-C36 very long chain fatty acids (produced in the retina via ELOVL4), and with DHA in the *sn*-2 position (McMahon and Kedzierski, 2010).

EPA often accompanies DHA in the diet and in nutritional supplements as both play a major role in photoreceptor disc membrane formation (Anderson and Ma, 2009; McMahon and Kedzierski, 2009). EPA levels become elevated up to 60% after ALA supplementation (Mantzioris *et al*., 1994; Burdge *et al*., 2002; Cao *et al*., 2006; Harper *et al*., 2006). High concentrations of EPA are not stored in the retina or brain, but perhaps EPA acts as an intermediate for further metabolic transformation. EPA is a substrate for pathways involved in the formation of VLCFAs. In fact, EPA not DHA, is required for the synthesis of VLCFAs (Suh and Clandinin, 2005). How very long chain fatty acids are acylated only in the *sn*-1 position of PC remains to be elucidated. EPA also exerts a multitude of anti-inflammatory and immunosuppressive effects (reviewed in Chapkin *et al*., 2009). For example, EPA suppresses the generation of ROS in the kidneys of mice (Taneda *et al*., 2010), inhibits inflammatory damage after ischemic brain injury in gerbils (Okabe *et al*., 2010), and reduces inflammation around atherosclerotic plaques (Cawood *et al*., 2010), suggesting that EPA may exert positive immunological effects in the brain and retina through the blood supply to these areas, without being physically incorporated into the membranes. EPA supplementation also reduces ocular inflammation in a mouse model of endotoxin-induced uveitis (Suzuki *et al*., 2010).

ALA supplementation through consumption of flaxseed oil does not significantly alter levels of DHA and it takes roughly two weeks for ALA to return to baseline levels after stopping its intake (Makrides *et al*., 2000; Cao *et al*., 2006). Flaxseed oil supplementation has actually been contraindicated for AMD,

since it contains a high level of omega-6 fatty acids (Cho *et al*., 2001) and leads to a decreased omega-3:omega-6 ratio. This is due to the fact that ALA (omega-3 precursor in the DHA synthetic pathway) and α-linoleic acid (18:2n-6, an omega-6 PUFA) use the same human Δ^6-desaturase, which catalyzes the rate-limiting step of omega-3 and omega-6 PUFA desaturation, leading to the production of EPA and arachidonic acid (AA), respectively (de Antueno *et al*., 2001; Leonard *et al*., 2002). Omega-3 metabolism produces anti-inflammatory eicosanoids while omega-6 metabolism produces pro-inflammatory eicosanoids (explanation to follow), so the omega-3:omega-6 ratio must be fine-tuned to prevent tipping the balance of the enzymatic machinery toward creating inflammatory mediators.

DHA exerts many actions in the central nervous system, including the retina. Arguably, one of the most important attributes of DHA is its contribution to the physicochemical structure of cytoplasmic membranes. The double bonds within the DHA fatty acid molecule greatly increase membrane fluidity of rod outer-segment discs, (Boesze-Battaglia *et al*., 2008) which facilitates the formation of mII from rhodopsin after photoisomerization of 11-*cis*-retinal (Brown, 1994; Litman and Mitchell, 1996; Litman *et al*., 2001). Membrane phospholipids also potentiate the activation of transducin (the G-protein that binds to mII), and subsequent signaling through the phototransduction cascade, by stabilizing the mII-transducin complex (Jastrzebska *et al*., 2009). Furthermore, this mechanism of G-protein coupled receptor signaling in rod outer-segment discs was shown to be less efficient with DHA deficiency in rats (Niu *et al*., 2004). The turnover rate of rhodopsin must also be enhanced to compensate for

the greater amounts being transformed to mII. DHA was shown to co-localize with SNARE proteins of the rod inner segments and promote SNARE pairing in this location (Mazelova *et al*., 2009). SNARE pairing is necessary for the delivery of rhodopsin and plasma membrane constituents back to the rod outer-segments, which turn over following a circadian rhythm. Of note, histological specimens are required to measure the rate of photoreceptor turnover, which does not make this feasible to study in humans. Instead, data from diurnal rodents (ground squirrels and Nile rats) is used as a correlate, and has shown that rod outer-segment shedding takes place within the first three hours after each transition from dark to light adaptation; cone outer-segment shedding is maximal within the first three hours after transition from light to dark adaptation (Anderson *et al*., 1978; Nguyen-Legros and Hicks, 2000).

In addition to providing membrane fluidity, DHA has implications for neural growth and synaptogenesis. DHA deficiency has been linked to the impairment of nerve growth, dendritic arborization, and synaptogenesis (Ahmad *et al*., 2002; Calderon and Kim, 2004; Cao *et al*., 2009). DHA enhances synapse formation by stimulating diacylglycerol kinase activity (Vaidyanathan *et al*., 1994); it can also block delayed rectifier potassium channels (Poling *et al*., 1995). Neural growth depends on DHA-regulated SNARE proteins for fusion of lipid transport vesicles into the plasma membrane (Pongrac *et al*., 2007).

DHA and EPA compete with AA in neural cells for the cyclooxygenase and lipoxygenase enzymes (necessary for the formation of eicosanoids and resolvins), causing an indirect effect of lowering omega-6 PUFA concentration

(reviewed in Calder, 2009). Pro-inflammatory 2-series eicosanoids are derived from AA, whereas less inflammatory 3-series eicosanoids are produced from EPA (Lee *et al*., 1984; Sperling *et al*., 1993; von Schacky *et al*., 1993; reviewed in Calder, 2009); however, the prostaglandins synthesized from AA precursors also regulate brain development, synaptic plasticity, long-term potentiation, and spatial learning (reviewed in Bazan, 2003). Once again, an optimal ratio of AA and DHA in the brain and retina is therefore crucial. Pro-inflammatory cytokines, such as interleukin-6 and TNFα, are released by the RPE in response to fibronectin fragments, which are substrates of the HTRA1 protein implicated in AMD (Austin *et al*., 2009). The RPE also secretes interferon-γ, which signals VEGF expression (Liu *et al*., 2010). DHA and EPA may counteract this pro-inflammatory cytokine expression by inhibiting nuclear factor κ B (NFκB): a transcription factor of inflammatory cytokine gene expression (Lo *et al*., 1999; Novak *et al*., 2003; Chen *et al*., 2005).

EPA produces resolvinE1, which possesses anti-angiogenic properties in the retina (Connor *et al*., 2007); no studies to date have found any harmful metabolites of EPA. DHA was shown to form two metabolites that display strong therapeutic potentials in AMD and retina degeneration in general: neuroprotectin-D1 (NPD-1) and resolvinD1. NPD-1 inhibits oxidative stress, inactivates pro-apoptotic signaling, and reduces inflammation in the RPE (reviewed in Bazan, 2009). NPD1 most likely exerts these effects over specific receptors on RPE cells and human leukocytes, although no specific receptors have been characterized (Bazan *et al.*, 2010). ResolvinD1 exerts an anti-angiogenic effect on retinal blood

vessels (Connor *et al*., 2007). However, DHA, when oxidized can form the deleterious metabolite: CEP, which has been suggested to contribute to the pathophysiology of AMD (Hollyfield *et al*., 2010). CEP is a hapten that promotes a complement-mediated attack on the retina of mice (Hollyfield *et al*., 2010) and has a plasma concentration 1.5 times greater in people with AMD compared to those without (n = 19; Gu *et al*., 2003). It remains to be elucidated whether or not the beneficial properties of DHA supplementation outweigh the potentially harmful effects of complement activation.

Some studies have linked DHA supplementation after birth to improved retinal function (as measured with the ffERG; Uauy *et al*., 1990), increased visual acuity (Birch *et al*., 1998), and enhanced neurological performance (Birch *et al*., 2000; Helland *et al*., 2003), while others have found no beneficial effects of infant DHA supplementation on brain function (Beyerlein *et al*., 2010). DHA may diffuse across the placenta, but a placental fatty acid binding protein also exists to preferentially transport DHA over other fatty acids from the mother to fetus during pregnancy (reviewed in Dutta-Roy, 2000 and Koletzko *et al*., 2007). Even with these mechanisms in place, maternal DHA supplementation does not appear to increase fetal DHA levels over controls (Malcolm *et al*., 2003).

DHA deprivation after birth may affect vision due to its role in synaptogenesis. Synaptogenesis starts at the inner retina before birth and synaptic wiring in the outer retina continues postnatal (reviewed in Sernagor *et al*., 2006). Light-evoked activation of retina cells and intrinsic firing of retinal ganglion cells (RGCs) are responsible for postnatal synaptogenesis (Tootle, 1993; Xu and Tian,

2007; Di Marco *et al*., 2009; Anishchenko *et al*., 2010). The critical period for cortical plasticity is heavily debated in the literature (Daw, 1998; Atkinson, 2000; Fawcett *et al*., 2005; Doshi and Rodriguez, 2007; Birch *et al*., 2009), but the majority of synaptogenesis in the retina (predominantly from dendritic organization of RGCs), lateral geniculate nucleus, and cortex occurs within the first year of birth and is heavily dependent on visual experience (Huttenlocher *et al*., 1982; Garey and de Courten, 1983; Garey, 1984; Xu and Tian, 2007). From 5 months to 5 years of age, visual acuity increases from 6 minutes of arc to 0.75 minutes of arc (Mayer and Dobson, 1982), but cortical remodeling due to visual input may occur up to 8 years of age (von Noorden, 1990). The synaptogenic effects of DHA should then theoretically potentiate retinal development in children before the age of 8. A review by Hoffman *et al*. (2009) describes how many have found an increase in visual acuity (inferred from visual-evoked potential measurements) in infants supplemented with DHA (Birch *et al*., 1998; Hoffman *et al*., 2003 and 2004; Lauritzen *et al*., 2004; Birch *et al*., 2005), while others have found no significant changes (Auestad *et al*., 2001; Jensen *et al*., 2005).

Supplementation of DHA may begin after birth through breast milk. DHA content in breast milk ranges significantly, from less than 0.1% to over 1% of the total human milk fat, depending on the mother's diet (Innis, 2008). As the child gets older, DHA can be obtained through diet or supplementation. Most brain and retinal DHA originates from plasma uptake (reviewed in Innis 2007). DHA levels remain constantly high in the retina, since RPE cells actively recycle DHA from

phagosomal phospholipids and recycle DHA back to photoreceptor cells (Rodriguez de Turco *et al*., 1999); therefore, pathologies affecting the RPE, such as AMD, might preclude such recycling and be associated with lower DHA levels. Rotstein *et al*. (1987) found that DHA levels in the rat retina decrease with age. Omega-6 PUFAs are also taken up by the brain in high quantities, but only DHA will enhance neurite growth (Calderon and Kim, 2004; Cao *et al*., 2005; Kawakita *et al*., 2006). As is a common theme of this chapter, since omega-6 PUFAs compete with the metabolism of omega-3, these must be balanced properly in the diet.

Synaptogenic events that change retinal circuitry may be quantified by the ffERG and mfERG. The ERG is also a widespread diagnostic tool used to measure and track retinal disease states. The ffERG is a non-invasive approach that records a waveform corresponding to the mass potential produced by electrical currents that are related to phototransduction-initiated modulation of neural processing in the retina. The dark-adapted ffERG b-wave reflects pure rod-driven activity (essentially rod bipolar cells) and is obtained using a low intensity flash that is below cone threshold (-2.03 log cd s/m^2; ISCEV; Marmor *et al*., 2009). Diseases that cause a prominent reduction in pure rod b-wave amplitude of this waveform fall into the heterogeneous class of Retinitis Pigmentosa (reviewed in Miyake, 2006; RetNet, 2010). Reduction in pure rod b-wave amplitude and prolongation of implicit times are often the earliest signs of Retinitis Pigmentosa onset (reviewed in Berson, 1987). As the stimulus intensity increases under dark-adapted conditions to exceed the threshold of cone activation

(1.34 log cd s/m^2; ISCEV, Marmor *et al*., 2009), a mixed contribution from rod and cone hyperpolarization produces the mixed a-wave. Diseases that significantly reduce the mixed scotopic a-wave amplitude include: Retinitis Pigmentosa, rod-cone dystrophies, and cone dystrophies (reviewed in Fishman *et al*., 2001). The photopic ffERG a-wave is entirely cone-driven, since the background luminance bleaches rods. Diseases affecting the amplitude of this waveform include maculopathies and cone-rod dystrophies (reviewed in Scullica and Falsini, 2001). The scotopic and photopic ffERG b-wave is produced primarily by on-bipolar cell depolarization, although Müller cell activation might also contribute to some degree (Kellner *et al*., 1998). Complete Congenital Stationary Night Blindness (CSNB Type I) causes an absence of both scotopic and photopic b-waves due to dysfunctional nyctalopin, which is essential for metabotropic glutamate receptor-6 (mGluR6) signaling in on-bipolar cells (reviewed in Miyake, 2006). Incomplete Congenital Stationary Night Blindness (CSNB Type II) is characterized by a mutation in the *CACNA1F* gene, leading to impaired pre-synaptic glutamate release by photoreceptors, which affects the light-driven modulation of both on- (b-wave) and off- (d-wave) bipolar cells and results in a partial reduction of their amplitudes (hence the term: "incomplete"; reviewed in Miyake, 2006). Lastly, oscillatory potentials (OPs), produced under both scotopic and photopic conditions, are related to cells of the inner retina, primarily amacrine cells and RGCs; of note, there are over 40 subtypes of amacrine cells and it is yet unclear as to which one(s) contribute(s) to OP

generation. Diabetic retinopathy and central retinal artery occlusion are examples of inner retina pathology that affect OPs (reviewed in Fishman *et al*., 2001).

Postnatal functional changes can be evaluated with the ffERG (Figure 10; Berezovsky *et al*., 2003). Around the time of birth, the photopic and scotopic a-waves (reflecting cone and mixed rod-cone photoreceptor activity, respectively) are negligible, and are followed by very small b-wave amplitudes (reflecting weak postsynaptic activity from mainly bipolar cells). OPs (reflecting inner retina activity from amacrine cells and RGCs) and flicker amplitudes (reflecting inner retina function related to cone photoreceptor recovery through their visual cycles, likely involving Müller cells) also have small amplitudes in newborns. Within six months of birth, the a-wave, b-wave, and OPs become very distinct, with increased amplitudes and decreased implicit times. The pure photopic cone and mixed scotopic rod-cone a-wave are fully developed by 3 years of age, but the b-wave may still undergo amplitude increments up to ages ranging from 7-14 (Rodriguez-Saez *et al*., 1993). The activity-dependent dendritic organization of RGCs in mice after birth may lead us to believe that most of these ERG changes are due to synaptogenesis; however, there is still the possibility that maturation of synapses and retinal cells, already in place after birth, may contribute to some of these ERG changes as well.

Ante-natal fish oil supplementation (through maternal intake) does not appear to produce substantial ERG findings in newborns, but supplementation after birth may have a greater effect. Birch *et al*. (1992) found DHA supplementation in human infants to significantly increase the rod a-wave

amplitude compared to the non-supplemented group. Similarly, Jeffrey *et al*. (2002) found reduced b-wave implicit times (which indirectly reflect increased kinetics of phototransduction and of synaptic transmission between photoreceptors and bipolar cells) in rhesus monkeys supplemented with DHA, although no significant changes were found in the other 23 ERG parameters that were tested. These results imply that DHA supplementation after birth may potentiate photoreceptor maturation and retinal synaptogenesis; however, more research is needed to substantiate these findings.

All synaptic contacts are formed in the retina, lateral geniculate nucleus, and visual cortex by adulthood. Plasticity driven by visual experience after this point is relatively lower in healthy individuals; however, bordering 60 years of age, Eliaseh *et al*. (2007) describe healthy aging human retinas with extensive dendritic sprouting of horizontal cells and rod- and on-cone-bipolar cells into the ONL; similarly, Cuenca *et al*., 2010 report sprouting of horizontal and bipolar cell processes into the ONL after laser induced intraocular pressure increase in adult albino Swiss mice. Further studies will be required to determine whether such sprouting has analogies with the impact of denervation in the CNS, which induces the sprouting of dendrites in the denervated target and hypersensitivity of the resident neurons (reviewed in Schwarting and Huston, 1996).

Many have found ERG changes as a result of aging in healthy individuals, but the results are somewhat inconsistent (Table 4). These inconsistencies may lead one to ponder whether or not the ERG is a reliable tool for monitoring age-related changes in the eye. One must keep in mind that with the ffERG, light-

driven micro-currents are being measured indirectly as a change in the mass potential (voltage) that these numerous currents contribute to, along a multi-resistor circuit within a live subject. Much room is left for experimental error, especially since heterogeneity between normal aging individuals differs greatly (due to, for example, differences in accumulation of LF or dendritic sprouting) and the effects of aging on the retina might be more subtle than in disease states. Furthermore, regional changes may not contribute significantly to the mass potential and therefore, not alter the ffERG response. One could also argue that participant numbers in some of these aging studies were not high enough to overcome the variable nature of ERG testing on a healthy aging experimental group.

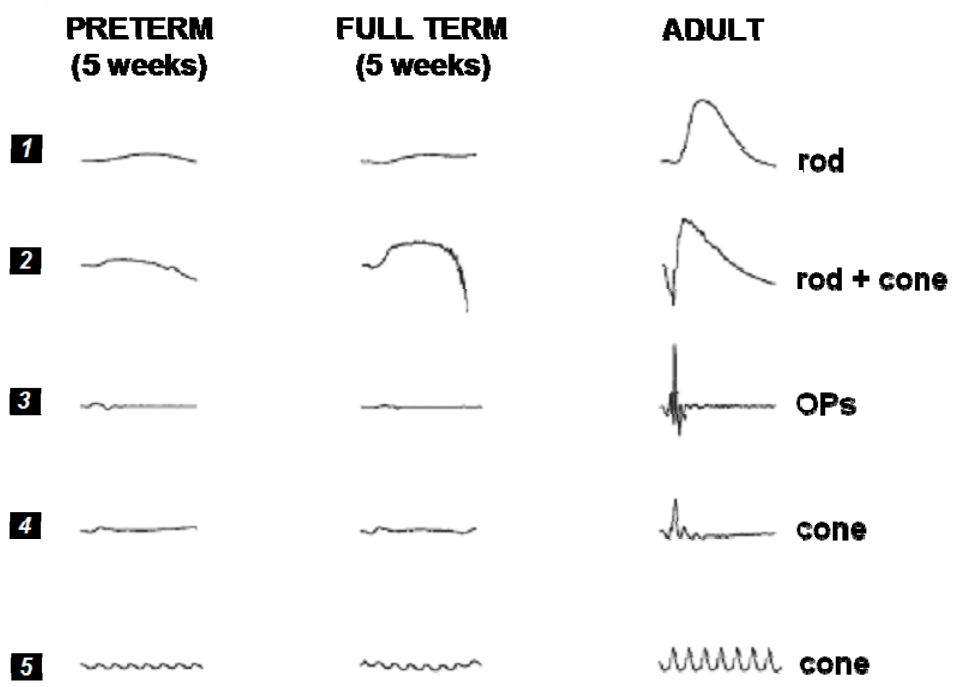

Figure 10: Human ERG changes from birth to adulthood: 1) Scotopic dim-flash, rod driven response; 2) Scotopic bright flash, mixed rod-cone maximal response; 3) Filtered OPs; 4) Photopic bright flash, cone-driven response; 5) 30 Hz flicker, cone-driven response (adapted from Berezovsky *et al*., 2003).

Table 4: Comparison of papers that report senescent-related ERG changes

		A-wave Amplitude		B-wave Amplitude		Sum OP Amplitude	
Paper	**n**	**Mixed Scotopic**	**Photopic**	**Mixed Scotopic**	**Photopic**	**Mixed Scotopic**	**Photopic**
Weleber, 1981	24	↓with age	↓with age	↓with age	↓with age	N/A	N/A
Birch and Anderson, 1992	269	↓with age	↓with age	↓with age	↓with age	N/A	N/A
Kergoat *et al*., 2001	56	↓with age	unchanged	↓with age	↓with age	↓with age	↓with age
Birch *et al*., 2002	100	no change	no change	no change	no change	N/A	N/A
Dornstauder *et al*., 2010	30	N/A	N/A	N/A	N/A	↓with age	↓with age
Freund and Sauve, 2010	63	↓with age	↑with age	no change	no change	N/A	N/A

↓ = decrease; ↑ = increase; n = number of participants

Additional ERG changes have been reported as AMD develops (Feigle *et al*., 2006). The pathophysiological effects of AMD on the retina are much more substantial than in normal aging, so the ERG results as they relate to AMD appear to be more reliable; however, the first clinical sign of dry AMD is a reduction of the ffERG scotopic b-wave amplitude under low intensity stimuli, depicting loss of rod function (Chen *et al*., 2004). Reduced cone response is also seen on the ffERG as decreased photopic a-wave, b-wave, and flicker stimulus amplitudes. Coincidentally, some have found similar effects on the rod and cone pathways with normal aging (Weleber, 1981, Kergoat *et al*., 2001, Birch *et al*., 2002), so separating aging from disease state using the ERG is not realistic at this juncture.

The RPE is substantially affected during AMD so one should expect to see a decrease in ffERG c-wave amplitude before the presence of any changes to photoreceptor function. The ffERG c-wave represents changes in the trans-epithelial potential of the RPE apical membrane, mainly due to the inflow of

potassium that is expelled from photoreceptors during phototransduction. The c-wave magnitude decreases significantly in Best's dystrophy, STGD, cone dystrophies, and with the presence of dominant macular drusen (Rover and Bach, 1985; Rover and Bach, 1987), although none of the literature shows c-wave measurements in AMD patients, *per se* (most likely due to the 10% success rate and unexplained variability in c-wave amplitudes in normal subjects; Marmor, 1991). Rover and Bach (1985) hypothesized that the c-wave is an accurate reflection of RPE function only when it is much more reduced than the b-wave. One must note that the c-wave may be indirectly affected by photoreceptor loss due to a reduction of potassium secretion, simply because less photoreceptors are available to expel it. Theoretically speaking, c-wave measurements in AMD patients would only be useful during early stages of the disease, where RPE changes occur without significant photoreceptor loss. However, due to technical issues (Marmor, 1991), such recordings in humans are simply impractical and unreliable.

The ffERG measurements become increasingly reliable as AMD progresses. The ffERG continually becomes flatter during the progression to wet AMD, and the first negative (N1) and positive (P1) directions of the mfERG waveform significantly decrease compared to age-matched controls (Huang *et al*., 2000). Hood *et al*., 2002 have proposed that N1 and P1 may be generated by the same cells that contribute to the a-wave and b-wave on the ffERG. The mfERG is performed under photopic conditions, so dysfunction of the cone pathway is implicated at the later stages of AMD. Areas of the retina most severely affected

by exudative blood vessels and retinal detachment are shown by discrete areas of low amplitude on the mfERG (Huang *et al*., 2000). Patients with wet AMD have increasingly decreased amplitudes on the *focal* ERG (the focal ERG shines an isolated circle of light over the macula) as visual acuity worsens due to cone photoreceptor loss in the fovea (Nishihara *et al*., 2008). Although sample numbers were low (n=5 per group), our lab also found a decrease in the ffERG scotopic b-wave of wet AMD patients when compared to dry AMD patients (Johnson *et al.*, 2010). We were only able to perform rudimentary non-parametric statistics on these results, so more participants are needed to further clarify the significance of this relationship.

AMD causes severe visual dysfunction, but the retina undergoes compensatory changes similar to those seen in healthy aging individuals. Photoreceptor synapses in patients with AMD retract into the ONL, so bipolar cells grow dendrites into the ONL to reconnect with the photoreceptors (Sullivan *et al*., 2007); however, it remains to be proven whether these synapses are functional and what impact this has on vision. Large-scale cortical remodeling also occurs due to the central scotoma caused by AMD, where part of the visual cortex that normally responds to central stimuli is activated by peripheral stimuli instead (Baker *et al*., 2005). Due to the physicochemical properties of DHA, the plasticity of the retina during AMD should be supported by DHA supplementation, showing preservation of retina function. The additional effects of DHA and EPA on the immune system, inflammation, and angiogenesis should further reduce damage to the retina from AMD risk factors.

Chapter 4: The OMEGAlberta Study

The effects of DHA in relation to phototransduction kinetics, inflammation, neuronal development, and homeostasis, as described in the previous chapters, might correlate with the fact that DHA represents over 50% of all fatty acids in photoreceptor outer-segments. Keeping this in mind, we have constructed a multi-factorial Phase II randomized, controlled clinical trial to analyze the effects of nutritional DHA and EPA supplementation, plus the AREDS Vitalux antioxidant formulation (DHA/EPA+Vitalux), on retina function in people with AMD. Our aim is to delay AMD progression in these people, compared to participants not receiving the intervention. Several additional tests will be performed and correlated with each other to assess the physiological role of DHA/EPA+Vitalux in the retina. If DHA/EPA+Vitalux is proven to delay the progression of intermediate-stage AMD to wet AMD, this knowledge should be implemented by changes in health services and policy relating to the elderly, by providing access to financially affordable sources of DHA/EPA+Vitalux. This chapter is meant to provide an in-depth review and constructive criticism (lengths and limitations) of our proposed study. We have also created a useful manual (Appendix A) for anyone constructing a clinical trial in Canada, especially for those who do not have the support of a clinical research organization (CRO). Please note, the OMEGAlberta Study, as of the date this thesis was submitted, has not been granted approval by Health Canada and the University of Alberta Human Research Ethics Board.

A. Background and Rationale

As we alluded to in Chapter 2, accumulating evidence from AREDS and other trials supports that diets rich in omega-3 PUFAs, such as DHA and EPA, might aid in the prevention of AMD progression (Johnson *et al*., 2008; Chiu *et al*. 2009; Tan *et al*., 2009). Results of AREDS came after measuring the development of CNV and geographic atrophy in supplemented people with bilateral drusen, inferred by annual stereoscopic color fundus photographs over 6 years. Our study is designed to quantify physiological risk factors of AMD and correlate them with disease progression. For the OMEGAlberta Study, we will be supplementing participants with a combination of EPA, DHA, and the AREDS Vitalux formula. This approach to supplementation could have broad therapeutic applications, and perhaps also bypass the multi-factorial primary defects in AMD (Swaroop *et al*., 2009).

B. Study Objectives

We propose to show the protective role of DHA/EPA+Vitalux supplementation on the progression of AMD by performing a highly focused, multivariable, controlled clinical trial on participants with wet AMD in one eye and intermediate-stage dry AMD (determined according to the AREDS clinical scale) in the other eye (fellow eye). The rationale is that in such participants, the fellow eye is at the greatest risk of developing wet AMD within 5 years, upon onset of wet AMD in the other eye (Macular Photocoagulation Study Group, 1997). Therefore, this selection criterion minimizes the intrinsic variability in the

rate of progression of dry AMD between individuals. We hypothesize that: participants taking DHA/EPA supplement pills plus the AREDS Vitalux formula (compared with participants taking the AREDS Vitalux formula alone, which is the standard of care) will have increased plasma DHA and EPA level, and that their intermediate dry AMD eye will have 1) no change in severity (or lower severity) of dry AMD level, compared to previous measurements from the same participant; 2) lower level of LF; 3) higher best-corrected visual acuity; and 4) preserved retina function.

Our ultimate objective is to develop a nutritional manipulation approach to prevent wet AMD. We will test our hypotheses by achieving the following specific aims:

1. Using randomization, half of the participants will be assigned to the DHA/EPA+Vitalux group, with the other half continuing the standard of care with Vitalux supplementation only.
2. We will determine the plasma level of DHA and EPA over total fatty acids just before and during DHA/EPA+Vitalux intake and at 6-month intervals over a period of 5 years. The same DHA and EPA measurements will be performed on the non-treatment arm (Vitalux only).
3. We will genotype each participant for genetic polymorphisms identified as AMD risk factors.
4. We will use several complementary tests to assess retina function and anatomy at baseline, followed by testing at 6 month intervals over a period of 5 years.

5. We will cross correlate the results of DHA and EPA plasma levels, genotyping, and complementary tests of retina function and anatomy.
6. We will identify the incident rate of progression to wet AMD over a period of 5 years.

C. Study Design

i. Participant Recruitment

As mentioned previously, a specific demographic of study participants is to be obtained from retina specialists in Edmonton, Alberta, with unilateral wet/dry AMD. Participants with unilateral wet/dry AMD should be largely motivated to comply with treatment since these participants are at the highest risk to form wet AMD in the fellow eye. For recruiting and ensuring compliance, several key points are to be followed:

1. A poster and brochure suitable for waiting rooms will be displayed to increase awareness and visibility with the clinics' patients and help recruit them.
2. A one-page card stating the inclusion criteria will be distributed with the poster at recruiting doctors' offices.
3. A telephone follow-up every month will be used to check on participants' compliance and general health.

There is a pre-existing relationship with one of the co-investigators: Dr. Tennant, who will be a referring physician. So patients do not feel coerced to participate, Dr. Tennant will only notify the patients of the study's existence, and

if the patient is interested, his office staff will give him/her a brochure and consent form outlining procedures, benefits / risks, timeline, commitment, etc. The patient can then call the research team for additional information and make an informed decision at that point as to whether or not to enroll. Patients will have the consent form obtained from the Doctor's office to sign and give to the research team if they decide to participate. In the event that recruitment levels are too low, we will ask referring physicians to obtain written consent from patients to release their contact information to the research team. We will then contact patients to pursue informed consent.

ii. Inclusion Criteria

Participants must be over 50 years of age and have intermediate-stage dry AMD in one eye (defined as the presence of multiple medium-size drusen or one or more large drusen), with wet AMD in the other eye. Of note, intermediate dry AMD corresponds roughly to grades 3-4 on the AREDS 9-step scale. This group is at the highest risk for developing wet AMD in the fellow eye within 5 years and any effects of DHA/EPA+Vitalux supplementation will be most efficiently identified in these participants. This group will also be the most highly motivated and compliant as a result of their condition. All participants are required to be on the Vitalux AREDS formula since this is the standard of care for people with AMD.

iii. Exclusion Criteria

Any participants that have unstable, serious conditions with a poor five-year survival prognosis will not be included in our study. Since our diet supplementation is not expected to be curative for wet AMD, patients with bilateral wet AMD will also be excluded from our study. The damage in bilateral wet AMD requires a more direct intervention such as anti-VEGF treatment; we are focusing on disease progression of the eye with intermediate dry AMD, using the wet AMD eye as a marker of predictable risk. Other exclusion factors include any one of the following:

1. *Diabetic retinopathy*: the inner retinal neovascularization, macular edema, hemorrhaging and swelling, that are characteristic of diabetic retinopathy (Davis *et al*., 1998), prevent us from accurately attributing retinal changes caused directly by AMD.
2. *Alzheimer disease*: the baseline DHA content of brain phospholipids is significantly lower in people with Alzheimer disease compared to age-matched, healthy controls (Soderberg *et al*., 1991), so we cannot know if DHA levels will attain a high enough therapeutic level in the retinas of these patients.
3. *Anticoagulant use (not including ASA) and/or previous myocardial infarction and/or dialysis*: these patients are at an increased risk for experiencing complications during phlebotomies.
4. *Ocular surgery in the eye with dry AMD (not including cataract intraocular lens surgery):* past surgeries that treated retinal tears or

glaucoma, for example, could possibly alter the progression of AMD in that eye.

5. *Other underlying ocular pathology in the eye with dry AMD (especially glaucoma, dense cataracts, and retinitis pigmentosa):* glaucoma and retinitis pigmentosa will variably increase the rate of retinal degeneration (and profoundly alter retinal physiology), while dense cataracts ultimately prevent us from taking fundus photographs with a clear view of the retina.
6. *Thyroid disease*: ocular manifestations of thyroid disease introduce new variables that we cannot control for, such as changes to ocular surface anatomy, immune system involvement, or intraocular pressure increase (reviewed in Stiebel-Kalish *et al*., 2010).
7. *Uveitis*: causative agents of uveitis (a disease with an extremely broad etiology) can induce pathology in the retina and its vasculature (Sudharshan *et al*., 2010) that could significantly confound our data.
8. *Central geographic atrophy*: drusen disappear as atrophic AMD develops (Sparrow *et al.,* 1997), so people with central geographic atrophy will be excluded from our study; this reduces the variability in the baseline drusen summation, and allows us to see any pigmentary changes in the RPE that may occur.
9. *History of long-term use of fish oil supplements or use of other health products containing omega-3 PUFAs, such as sea algae oils, flax seeds and oil, chia seeds and oil, and / or hemp-derived products*: AMD may not

progress as hypothesized in these participants, due to their initial consumption of omega-3 PUFAs before study enrollment.

10. *Allergies to seafoods (fish and shell fish):* although the supplements we are providing should not contain any seafood protein (the allergen), we will not take a chance that some protein made it into the formulation; therefore, this group of people is at too high of a risk to be included in our study.
11. *Currently using systemic angiogenic therapy*: progression to wet AMD in these participants may be slower than what was hypothesized in our power calculation.
12. *Currently using systemic medications known to be toxic to the retina or optic nerve (Deferoxamine, Chloroquine, Tamoxifen, Chlorpromazine, Phenothiazines, chronic systemic steroid use of more than 10mg per day and Ethambutol):* these medications could alter the ERG recordings, even if no ocular pathology has manifested clinically (reviewed in: Porter and Huehns, 1989; Toler, 2005; Tzekov, 2005; and Li *et al*., 2008).
13. *HIV, any infectious diseases, sexual partners infected with hepatitis or human immunodeficiency virus (HIV)***,** *or donated more than 100 ml of blood in the last month*: complications could arise in these participants as a result of the phlebotomies; in addition, participants with HIV put our research group at risk when drawing blood.
14. *Pregnancy*: changes in serum homeostasis and hormone levels in these participants leave too many variables that could change plasma lipid levels.

iv. Non-Restrictions

The OMEGAlberta Study is a highly focused clinical trial; however, due to our recruitment demographic, there are certain variables that cannot be restricted from our study with respect to obtaining an adequate number of participants. The following variables have been considered and will be noted in participant files, but are not a restriction against participating in the study:

1. S*moking*: cigarette smoke causes oxidative damage, activation of the immune system, and promotes CNV in the retina, thereby accelerating AMD progression (Dhubhghaill *et al*., 2010). People who smoke will be provided with the modified Vitalux AREDS formula for smokers (described in Chapter 2).
2. *Hypertension*: the fragile vasculature of the eye is extremely susceptible to systemic hypertension, which is associated with an increased risk for progression of AMD (DellaCroce and Vitale, 2008).
3. *Rheumatoid arthritis*: inflammatory mediators released by autoimmune diseases, such as rheumatoid arthritis, may confer a greater risk for the development of CNV; however, an epidemiological review by McGeer and Sibley (2005) suggests that rheumatoid arthritis may not be a significant risk factor for the development of AMD.
4. *Hyperlipidemia*: as mentioned in Chapter 1, hyperlipidemia is a major risk factor for AMD, but its prevalence is extremely high in our study demographic, so it would be unrealistic to exclude these people.

5. *Statin use*: the ratios of AA to EPA and AA to DHA are both increased with statin use (Harris *et al*., 2004), which tips the balance towards greater omega-6 PUFA metabolism; however, our supplementation regime should tip the balance back towards a higher ratio of omega-3 PUFA metabolism.
6. *Diabetes*: participants with diabetes, but without any ocular manifestation of diabetes, are to be included; however, DHA and EPA have been shown to increase fasting blood glucose (Woodman *et al*., 2002), so diabetic participants will be informed to strictly monitor their glucose levels.
7. *Mild cataracts*: lens opacities may interfere with the AF signal intensity to some degree.

A full participant history will be documented before any tests are performed. Any previous injuries or pathologies in addition to this list will be noted in the participants' chart and subject to review upon study completion. Baseline DHA and EPA level will be compared with future results since plasma DHA and EPA will be measured at the beginning and every six months thereafter. A baseline LF signal will also be used to compare with future results. Since we will have baseline levels of DHA, EPA, and LF (inferred from AF signal) to compare with our 6-month and 1-year data, we do not foresee a problem having smokers, participants with hypertension, etc. in our study group because all data will be presented as a fluctuation from baseline. Most of the aforementioned non-restrictions should accelerate the disease progression, which actually benefits our study model. Inclusion of these high-risk people will test the limits of DHA on slowing wet AMD progression (we will still be measuring in years 2, 3, and 4

so our data can be compared to the quicker progression rates in the literature if required). Subgroups may also be formed if the data becomes skewed; however, we are aware this will decrease the power of our data.

As described in Chapter 1, quantifying AF photographs can be extremely variable. Bearelly *et al*. (2010) have developed novel semi-automated software to quantify AF changes, but this technology is still proprietary. One may also criticize our supplementation regime since we are providing high concentrations of DHA and EPA to participants, even though DHA is sequestered in plasma membranes for long periods. We are also using plasma DHA levels as a surrogate measure for retinal DHA, since we cannot directly measure DHA concentration in the retina without taking a tissue sample from the eye. Our research group is aware of these potential pitfalls, but since the EE form we are providing has a much lower bioavailability than the TG form, and that we cannot project how each individual sequesters or metabolizes DHA, we require participants to take the supplements everyday to ensure DHA levels remain constantly high in the tissues. The use of plasma DHA as a surrogate measure has been substantiated by Hubbard *et al*. (2006), who found an inverse relationship of STGD3 severity with red blood cell DHA/EPA content; however, we cannot know the duration that the retina has been saturated with these PUFAs. Measuring plasma DHA concentration does, however, assist us for determining patient compliance with taking the supplements. The doses we will provide are within safe limits and no toxicities are expected.

Although the main focus of our study is on the effect of DHA supplementation on the retina, we are still providing EPA in conjunction with DHA. There is a lot of support that suggests DHA and EPA work together, but we have no evidence that increasing VLCFA's in the retina (the main effect of EPA metabolism) will help AMD. However, in the end, we will not know if DHA alone is sufficient. We are looking for a therapy and are not going to risk diminishing the potential benefits to participants by isolating DHA from EPA.

v. Study Arms

This study contains two arms: 1) treatment arm (n=150); 2) non-treatment arm (n=150). Subjects in the treatment arm will take the Vitalux AREDS formula, plus a total of 1200 mg EPA and 600 mg DHA each day (3, 1g capsules of 400mg EPA and 200mg DHA per day; manufactured by Ocean Nutrition Canada Ltd.). Subjects from the non-treatment arm will simply continue taking the Vitalux AREDS formula.

Sample size calculations were performed using the *SAS/STAT Power and Sample Size Calculator* (SAS Institute Inc., Cary, NC, USA) using Fisher's exact test. Of note, these calculations are beyond our expertise, so we collaborated with Dr. Chris Rudnisky, who offered statistical support. Our primary endpoint measures the proportion of patients with an increase in drusen number. Sparrow *et al*. (1997) showed that drusen increases by 30% over seven years, and Abdelsalam (1999) showed that 40% of eyes had an increase in drusen number over only 10 months. Therefore, we hypothesize that 30-40% of the non-

treatment group will have an increase in drusen number over a one-year period. Smith *et al*. (2006) showed that increases in AF intensity significantly co-localizes or surrounds large drusen in the fellow eye of people with early AMD (and wet AMD in the other eye). Although we are aware of the inherent variability in AF testing, we should be able to imply that AF signal intensity is positively correlated with drusen number. If LF contribute to drusen accumulation in the retina, then knocking down A2E levels with DHA should slow down drusen formation. Keeping this in mind, data inferred from our animal models show that DHA should decrease A2E levels in 75% of our participants. We are not sure if an improved phenotype will be seen (i.e. decreased drusen number) at that time, but we expect drusen number to be unchanged in this proportion of people (instead of getting larger). We then conservatively estimate that the addition of DHA and EPA to the AREDS Vitalux formula will decrease the proportion of eyes with an increased number of drusen by 50%. If 30-40% of placebo patients have an increase in drusen number, we should expect approximately 75-80% of the supplemented individuals to have no change (or decrease) in drusen number.

For a comparison of two independent binomial proportions using Fisher's exact test with a two-sided significance level of 0.05, a sample size of 91 eyes per group achieves a power of at least 0.8 when the proportions are 0.2 and 0.4. The actual power is 0.804. This calculation looks at the smallest number of participants necessary to achieve the maximal impact of DHA. The two eyes are not independent since the increase in drusen in one eye adds to the risk of drusen

increase in the other eye. Therefore, all calculations will consider only the eye with dry AMD. To account for the inability to group both eyes together and the fact that our power is based on the *maximal* impact of DHA, we will choose a larger sample size of 150 patients per group (which also accounts for 10% loss to follow-up).

vi. Treatment Arm Nutritional Supplement

1) Formulation

The treatment arm will consume MEG-3® 4020EE capsules manufactured by Ocean Nutrition Canada Ltd. Ocean Nutrition Canada Ltd. refines and concentrates DHA and EPA from oily fish, including: anchovies, sardines, mackerel, and salmon. Each 1g capsule contains on average 370mg EPA (expressed as EE) and 200mg DHA (expressed as EE). MEG-3® 4020EE is marketed as Omega Plus™ and has a Natural Product Number (NPN) under HC: 80000901; however, we will be adding carob and lemon-lime flavoring to mask the fishy aftertaste of the fish oil capsules, so a CTA outlining this modification has been filed under HC. The EE form is being provided, even though the TG form has greater bioavailability. We chose to take this route since the EE form costs less and there is no proof that the TG form is more efficacious. We anticipate that the lower bioavailability should be overcome by the dose concentration and supplementation frequency being provided to participants; however, future studies should examine the efficacy between the EE and TG forms.

2) Contraindications and Anticipated Toxicities

Our daily dose complies within the safe-limits described in the HC Fish Oil Monograph (HC, 2009), so there are no known concomitant medications or health conditions that should affect the safe use of MEG-3® 4020EE. Fish Oil is not normally associated with major or adverse or toxic effects; however, in very rare instances, some people may experience loose stools or abdominal discomfort. Adverse effects that are reported will result in discontinuation of supplementation and natural washout to return omega-3 levels back to baseline. Supplements will be stored in a cool, dry place and kept in air-tight, dark containers to prevent oxidation of the fish oil pills. In addition, supplements will not be used after 3 years of the manufacture date since significant levels of oxidation may have occurred (even in air-tight containers) over this period of time.

3) Safety Variables and Analysis

A Data Safety Monitoring Board (DSMB) will be constructed to review procedures, outcomes, endpoints, and adverse reactions. All results will be presented as a fluctuation from baseline for each tested variable. Since the recruitment period is two years, participants in our study will be at different time points. To accommodate this problem, data will be compiled and reviewed every two months to ensure there are no adverse events that may not be perceived by the participant. This will also allow us to assess the time course of treatment efficacy.

4) Outcome Measures

Supplementing participants with DHA/EPA+Vitalux should decrease accumulation of LF in the RPE and slow down sub-RPE drusen formation

(anatomical improvement), which we hypothesize will delay onset of changes to the ERG and to visual acuity (functional improvement). Since DHA takes approximately 10 weeks to accumulate in the blood following nutritional supplementation (Huang *et al*. 2008), we expect significant DHA plasma elevation after 6 months in the DHA/EPA+Vitalux group when compared to DHA levels measured at time zero. Our *primary endpoint* is to see a reduction (or no change) in the number of soft drusen in the retina after 1 year, without RPE atrophy. Drusen number will also be measured over the next 5 years, since a reduction (or no change) in drusen number may provide the foundation for reduced wet AMD incidence in the fellow eye after 5 years of DHA/EPA+Vitalux supplementation.

vii. Sites and Enrolment Requirements for Participants

When dealing with a novel human intervention, the study group should be composed of experts in each field covered by the research. Our study has brought in world experts in retinal electrophysiology (Dr. Yves Sauvé), ocular genetics (Dr. Ian MacDonald and Dr. Radha Ayyagari), ocular immunology (Dr. Robert Nussenblatt) and omega-3 PUFA nutrition (Dr. M. Tom Clandinin and Dr. Miyoung Suh). A retina specialist from Alberta Retina Consultants, Dr. Matthew Tennant, will grade AMD photographs and Dr. Chris Rudnisky (a General Ophthalmologist with expertise in ocular research) will provide us with statistical support. We have also trained students to perform most of the testing and Jaspreet Garcha, a PhD candidate, will be coordinating the clinical trial. We expect to

save approximately $1000 per participant, per year (Table 5), by utilizing trained and certified volunteers that are part of our lab, and by collaborating with other labs across North America.

Table 5: Tentative cost analysis for the OMEGAlberta Study

2 U of A + 2 RAH Participant Visits per Year:	Approximate Market Value ($)	Charge to Study Group ($)	Money Saved ($)
ETDRS Visual Acuity	38 x 2 = 76	0	76
OCT	87 x 2 = 174	0	174
Fundus Photography + Autofluorescence	112 x 2 = 224	0	224
Parking	27 x 2 = 54	27 x 2 = 54	0
Phlebotomy	10 x 2 = 20	3 (supplies) x 2 = 6	14
ERG	110 x 2 = 220	28 (electrodes) x 2 = 56	164
Plasma DHA analysis	135 x 2 = 270	0	270
SUBTOTALS:	**1038**	**116**	**922**
*Genotyping (not included in cost analysis)	600	0	600
Fish Oil Treatment Arm (per participant cost)	$0.13 per capsule x 3/day x 365 days = 142.35	$0.054 per capsule x 3/day x 365 days = 59.13	83.22
	Treatment Arm Cost / Year / Participant	175.13	1005.22
	Non-treatment Arm Cost / Year / Participant	116	922

All subjects will be recruited in Edmonton, Alberta, from Alberta Retina Consultants and Retina Imaging Centre Ltd., and will be under the continuing care of their ophthalmologists at these locations (regular check-ups every two to three months) for the duration of the study and after it has finished. Participants are required to visit the Royal Alexandra Hospital (RAH) once every six months for AF photographs, OCT, and phlebotomies. Our goal is to see at least 10 participants per trip to the RAH. Each visit should take approximately one hour per participant. Parking for this location will be reimbursed to the participants. Within a few days of the RAH testing, participants are required to visit the Sauvé lab at the University of Alberta (U of A) for ffERGs, mfERGs, ETDRS visual acuity testing, and color fundus photography. Each visit should take approximately 1.5 hours per participant. Parking will be reimbursed for this location as well.

Blood samples obtained from the first visit to the RAH will be centrifuged and sent to the Ayyagari lab at the University of California San Diego for genotyping. Blood samples obtained from each appointment will also be sent to Dr. Miyoung Suh at the University of Manitoba for plasma analysis of DHA and EPA phospholipid content, and to Dr. Robert Nussenblatt at NIH for plasma cytokine analysis.

D. Procedures

i. Phlebotomy

1) Purpose:

The purpose of taking a blood sample is three-fold: analysis of plasma lipids, measurement of plasma cytokine levels, and genotyping of the subject to identify any gene polymorphisms that are candidates for developing AMD. Measurements of plasma lipid levels allow us to evaluate the compliance of the subjects in taking the DHA/EPA supplements and the effectiveness of the supplements in raising DHA levels. Cytokine analysis is a novel approach in AMD research, which could further help elucidate the role of the immune system in AMD. Genotyping will identify any potential genetic risk factors for AMD to help clarify the polygenomic background of AMD. A genetic study questionnaire will be provided to participants beforehand to examine personal and family medical history and history of ocular disease, which can be compared to any target polymorphisms participants may have.

2) Procedure:

Certification of lab members to become phlebotomists required training from a Licensed Medical Laboratory Technologist employed by Alberta Health Services. The blood is drawn from a vein in the arm or on the hand, depending on the ease of identifying a target vein. The site is cleaned with antiseptic and a tourniquet is applied to highlight the vein and facilitate the insertion of a needle. A needle is inserted into the vein and a small amount of blood is collected, after which pressure is applied and the puncture site is dressed. We have obtained

Departmental Approval to draw blood at the RAH and biohazards approval for the Sauvé, MacDonald, and Clandinin labs, to work with the blood. For adverse reactions, the patient is to be slid from chair to floor, carefully protecting his/her head, and a call for medical help is made to the emergency personnel available at the RAH. Blood samples will be stored in Dr. Stephen Harvey's lab (next door to the Sauve lab) which has authorization for working with human blood on the U of A campus. Blood will **only** be used for genotyping, lipid analysis, and measurement of cytokine levels. All materials for phlebotomy will be supplied by our study and the blood will be drawn by our lab members, so there is no burden on Alberta Health Services blood labs and its technicians. Participants will also be provided with a dietary intake questionnaire (DIQ) that will be correlated with the lipid analysis (a nutrition undergraduate student will analyze the data).

3) Analysis:

In summary, blood will be shipped on dry ice to:

1. Dr. Miyoung Suh's lab for the analysis of DHA and EPA plasma content.
2. Dr. Nussenblatt's lab (NIH) for cytokine analysis.
3. Dr. Radha Ayyagari's lab (University of California San Diego) for genotyping to identify candidate AMD gene polymorphisms in: *ABCA4*, *ARMS2/HTRA1, C2/CFB, C3, CFI* and *CFH*. Newly discovered genes will be added to the genetic screening as the study progresses.

ii. Best-corrected Visual Acuity

1) Purpose:

Visual acuity testing has been utilized since the mid 1800s to assess visual function. This is a psychophysical test that requires no physician-patient contact. The Snellen visual acuity chart has been traditionally used, but recently, the ETDRS has designed a visual acuity test for patients with diabetic retinopathy that is now exclusively used for the majority of low vision patients (Figure 11). Visual acuity testing is a quick and efficient way to assess a person's visual function and may also be used to quantify visual improvement after treatment.

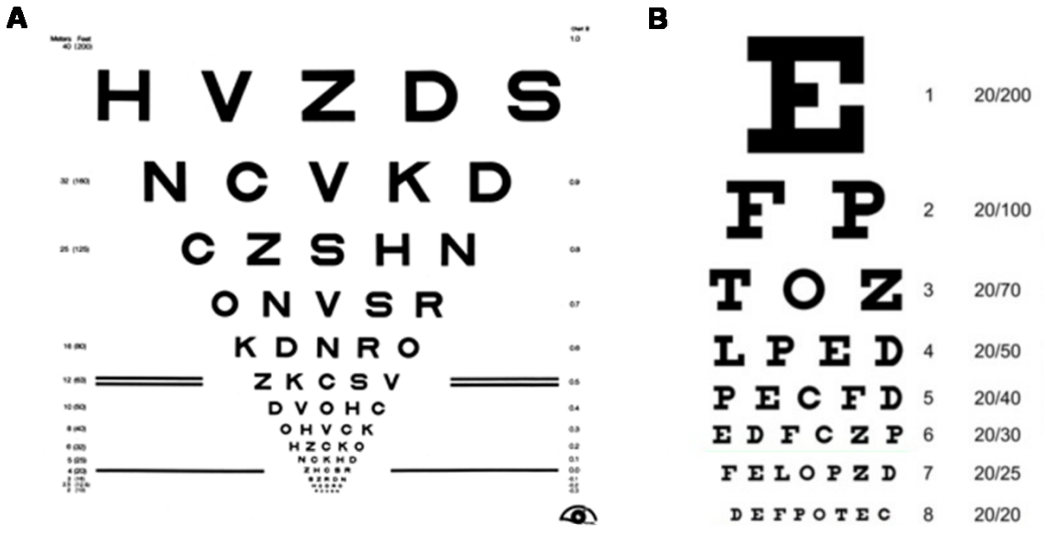

Figure 11: Comparison of two commonly used eye charts for measuring visual acuity: A) Sample ETDRS eye chart. ETDRS eye charts are used as the Gold Standard for visual acuity measurements (adapted from www.Good-Lite.com); B) Sample Snellen Visual Acuity eye chart (adapted from www.apple.com). Arc distance within and between letters on the ETDRS eye chart is highly conserved and the chart itself is placed on a controlled luminance machine to provide uniform light from standardized light emitting diodes. Spacing between Snellen letters is irregular and standard office lighting to illuminate the Snellen chart causes significant glare, which is completely omitted from the ETDRS system (www.Good-Lite.com).

2) Procedure:

The ETDRS visual acuity test is performed with all lights off, except for the illumination light of the ETDRS chart. The participant is first refracted with trial lenses to obtain best-corrected visual acuity (the spherical equivalent is commonly used as a starting point if spectacle correction is known: spherical equivalent = sphere + ½ cylinder). The participant is then seated at the appropriate distance in front of the chart and, with one eye covered, is asked to start at the top and read each line of letters until he/she can read no more. Visual acuity is then recorded with respect to the last complete line he/she got correct plus the number of correct letters from the line below it. A conversion factor for Snellen visual acuity can be calculated with these results.

3) Analysis:

Visual acuity tests will be performed at the Sauvé Lab. The ETDRS scale will be used to measure and compare the best-corrected visual acuity before, during, and after DHA supplementation. Members of our lab have been trained by Opticians at the RAH to administer these tests.

iii. Color Fundus Photography, Autofluorescence, and Optical Coherence Tomography

1) Purpose

Fundus photography is a non-invasive technique with broad applications. By employing different light filters and scanning technology, one can take color fundus photographs, OCT scans, and AF images. Color fundus photographs are graded by retina specialists to determine dry AMD severity. OCT scans generate

a topographical map to detect lesions and elevations of the retina surface, and can take image slices through the retina to detect sub-RPE drusen deposits. AF photography is a non-invasive technique used to detect *in vivo* accumulation of LF. The AF signal is abnormal in people with AMD and can be used to approximate the level of LF in the retina. We can follow the subjects and trace the level of LF in relation to treatment and correlate with other clinical evaluations such as visual acuity. The AF signal can also delineate areas of RPE atrophy to further characterize progression of the disease.

2) Procedure

The procedures for capturing AF, OCT, and color fundus photographs are almost identical to each other. Prior to imaging, dilation of the pupil is required via a mydriatic agent (such as Mydriacil). Multiple exposures are captured to enable researchers to choose the clearest images for analysis.

3) Analysis:

Color fundus photographs will be taken at the Sauvé lab, while AF and OCT will be taken in the photography room at the RAH ophthalmology clinic. Members of our lab were trained to take these pictures by Opticians at the RAH. Dr. Matthew Tennant will grade the color fundus photographs for dry AMD severity using the AREDS 9-step scale, and he will count drusen as a measure of our primary endpoint. Dr. Mahta Rasouli, a third-year Ophthalmology Resident, will grade the signal intensity of AF images to infer the relative amount of LF in the retina. OCT images will be correlated with mfERG recordings to compare lesions with areas of low retina function. We realize that limitations exist for

counting drusen, since drusen may also grow in size to become confluent with adjacent drusen. To overcome this potential setback, we will compare fundus photographs from previous visits to distinguish between drusen growth and increase of drusen number. We are aware of the variability in quantifying AF, but automated grading software may permit more reliable analysis of our photographs in the future (Bearelly *et al*., 2010). For the present time, AF luminance will be quantified by manually measuring the gray-scale values with ImageJ Software (http://rsb.info.nih.gov/ij/; Ayata *et al*., 2008).

iv. Electroretinogram Measurements

1) Purpose

The most sensitive and objective test of retina function is the ERG. Electrodes placed on the inferior scleral surface record electrical fluctuations from the retina upon stimulus to light. Our laboratory has vast experience in the application and interpretation of ERG protocols. One can compare wave amplitudes and implicit times with corresponding baseline data to determine if there is a retinal defect or if a certain treatment improves retina function.

There is a great need for treatments that efficaciously stop or slow down the progression of age-related retina diseases. DHA is hypothesized to slow down the progression of AMD but needs to be proven by accurate, easily accessible and objective diagnostic tools to evaluate retina function in the increasing elderly population. We theorize that standard electrophysiological tests, such as the ERG, can provide an objective diagnostic tool to view the effects of DHA supplementation on retina function in AMD. The overall goal of our research is to

examine how functional parameters of the retina respond to anatomical improvements after DHA supplementation.

2) Procedure

ERG tests are non-invasive and painless. The ffERG measures a mass potential from all cells in the retina upon exposure to a broad flash of light. A reference electrode is placed on the skin just outside the temporal region of the orbit of each eye and microfilament active electrodes are placed on the surface of the sclera within the inferior subconjunctival space (there are no nerves in this area so the patient should not experience pain); the two circuits are grounded by a single electrode placed on the forehead. From our experience, we obtain the most accurate results when the nasal sticker is placed with the wire electrode facing slightly upwards, and the when the reference electrode is placed approximately 1cm temporal to the patient's lateral orbital ridge. A Ganzfield apparatus administers light stimuli and the subsequent retina response is recorded as an ERG waveform on Espion computer software. As described in Chapter 3, the a-wave represents rod and cone hyperpolarization, the b-wave represents on-bipolar cell activation, and OPs are produced by phase-locking of inner retinal cells. Tests performed under different light-adapted states enable the researcher to discriminate between rod-and cone-driven pathways. Low intensity flashes administered under dark-adapted (scotopic) conditions yield a purely rod-driven response because the flash is not strong enough to surpass the threshold of cone activation. High intensity flashes under scotopic conditions yield a mixed rod and cone response because the stimulus surpasses the threshold of activation for both

rods and cones. Light-adapted (photopic) tests yield a purely cone-driven response since rods are saturated from background lighting. The background light does not maximally activate cones so flashes are given at incrementally increasing intensities to recruit larger numbers of cones each time.

The mfERG measures the activity of the central retina. The subconjunctival space is anaesthetized followed by placement of electrodes (shortened by 1 inch) just under the cornea. We obtain best results when the reference electrode and nasal sticker placement are identical to the ffERG procedure, but also ensuring the active electrode sits along the inferior edge of the limbus. A grid of hexagons is mapped onto the retina, with the relative size of each hexagon based on the eccentricity of the region. The mfERG is predominantly performed under light-adaptation since the light is cumulatively shone onto the entire retina using randomized alternating black-to-white flicker stimuli. Computational data obtained from each hexagon is pooled by a computer algorithm to show responses over the entire area of the retina. The mfERG is quantified by amplitudes and implicit times of the N1 and P1 components, which are representative of cone distribution. N1 and P1 amplitudes naturally increase with eccentricity but decrease where there is cone degeneration. Implicit times represent photoreceptor activation kinetics and are computed by percent abnormality compared to control values.

3) Analysis:

ERG recordings will take place in Dr. Yves Sauvé's lab at the University of Alberta and be performed by trained members of our lab. We will measure and

quantify the mfERG (x2 to reduce variability) and ffERG (x1) to compare with previously obtained normalized data from age-matched healthy individuals. Parts of the ffERG to be quantified (under scotopic and photopic adaptation) are: 1) a-wave amplitude and implicit time; 2) b-wave amplitude and implicit time; 3) oscillatory potentials and; 4) adaptation after a 20 minute transition from light to dark, with probing flashes every 2 minutes (shows progression from pure rod-, to mixed-, to pure cone-driven response). Parts of the mfERG to be quantified (under light-adaptation) are: 1) N1 / P1 amplitudes and implicit times and; 2) 3-dimensional map superimposed onto fundus photographs. After six months of supplementation, we expect to see a functional preservation (or possibly an improvement) of implicit times and a- and b-wave amplitudes, especially under dark-adaptation, since DHA should preserve phototransduction kinetics and rod function (because rods are the first to be affected in AMD). As the study progresses, we hypothesize preservation of mfERG amplitudes that would suggest decreased cone degeneration and delayed progression to wet AMD.

v. Dietary Intake Questionnaire

1) Purpose:

By simply recording the amount and type of food consumption over two weekdays and one weekend day, one can asses a representative dietary intake of a person's vitamins, minerals, fatty acids, and other nutrients. Data obtained from the DIQ can be correlated with the incidence and progression of AMD.

2) Procedure:

Participants must fill out the DIQ, provided from our study group, by being as specific as they can: listing ingredients, weighing the portions, describing the method of preparation, and including brand names wherever possible. A record of food consumption must be filled out separately for two weekdays and one weekend day. Of note, the DIQ is a more accurate reflection of dietary intake than the Food Frequency Questionnaire used by AREDS, which only asks participants about the quantity and frequency of food items they consume that fall under a pre-determined list (and participants must recall what they ate, instead of recording right after eating, as with the DIQ).

3) Analysis:

A Nutrition undergraduate student will analyze the DIQs and compare results with baseline omega-3 levels from our blood samples. Since some evidence has shown that omega-3 fatty acids consumed with a low intake of α-linoleic acid may benefit people with AMD (Seddon *et al*., 2001; Tan *et al*., 2009), we will also estimate the intake of α-linoleic acid from the DIQ. All data obtained from the DIQ will be coded and kept confidential so there is no risk of data infringement. These forms will be put on the Secure Diagnostic Imaging (SDI) SecureServer (described in *Part C* of Appendix) for remote access by our research group.

E. Data Analysis, Privacy and Confidentiality

i. Randomization

Participants are to be assessed a randomized number generated from Microsoft Excel upon enrolment in the study. This number corresponds to their name and contact information, which will be entered into a password-protected file. The researchers deal only with the numbers, but a third party (an individual separate from our study group) has the password to the name file if an emergency situation occurs.

ii. Analysis Parameters

A teleophthalmology account will be set up on the SDI SecureServer to analyze fundus photographs and DIQs. Subjects will be randomized to a number code, so the analysis will be done blind. When analyzing the data, only one eye of a patient is used for the study. Data will be compared using parametric *t*-tests if the data follow normal distributions and Wilcoxon Rank Sum if the data fail to follow normal distributions. All parameters assessed from this study are to be cross-correlated. For instance: 1) we will examine whether DHA and EPA levels are inversely proportional to the severity of dry AMD, whether certain genotypes respond better to DHA/EPA+Vitalux treatment, or whether drusen size is proportional level of LF (inferred from AF) and; 2) we will compare specific ERG parameters against age-matched data we have acquired over the past two years in our lab using novel ERG tests. Implementation of these ERG parameters might provide novel insights into retinal dysfunctions that occur in AMD.

iii. Analysis Pipeline

Preliminary data will be taken from the first visit, six months after baseline measurements, and analyzed within the year. The power of this data will be greatly reduced since not all participants will have been recruited; however, the data will be submitted to the DSMB for review of safety and efficacy (described in *Part C* of the Appendix). Upon review of the previous version of our protocol, the Northern Alberta Clinical Trials and Research Centre (NACTRC) has determined this study to be low risk, so it only requires a one-time Data Safety and Monitoring Plan and an annual progress report that is to be completed at the end of the first year. The one year endpoint will be analyzed two years after study start. Full recruitment is expected by this point so our power requirements will be fulfilled. The 5-year endpoint will be analyzed seven years after study start. Anticipated publications are at the 2nd and 7th years of the study. We look forward to submitting many papers since a large number of variables are to be measured. If our treatment proves to be effective, our goal is to educate the public and lobby for adding DHA/EPA supplementation to the standard of care for AMD treatment.

iv. Confidentiality

Personal health records relating to this study will be kept confidential. Any research data collected about participants during this study will not identify them by name, only by their initials and a coded number. Participants' names will not be disclosed outside the research clinic. Any report published as a result of this study will not identify participants by name. For this study, the Medical Monitor may need to access participants' personal health records for health

information such as past medical history and test results. He may also need to contact participants' family physicians and other health care providers to obtain additional medical information. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study.

The personal health information collected in this study will need to be checked from time to time against participants' medical records by representatives from the DSMB assigned to this study, the Human Research Ethics Board (HREB) at the U of A, and the Health Products and Food Branch of HC, to monitor the research and verify the accuracy of study data. In Canada, study information is required to be kept for 25 years. Even if a participant withdraws from the study, the medical information which is obtained for study purposes will not be destroyed. Participants have a right to check their health records and request changes if personal information is incorrect.

F. Participant Safety

i. Risk / Benefit Assessment

There will be a qualified investigator who is responsible for the conduct of the clinical trial at each site. The qualified investigator for the RAH and U of A is Ian MacDonald, M.D. The DSMB, consisting of members with no affiliation to the study, will be put in place once the clinical trial application has been addressed by HC. We will comply with the guidelines of HC and NACTRC after

a formal risk assessment of this study has been conducted, as to who will be on this board. The following outlines the anticipated risks and benefits of our study:

1. The Ocean Nutrition MEG3® 4020EE supplement does not have a NPN or Drug Identification Number (DIN) under the United States Food and Drug Administration or HC. According to the position of the American Dietetic Association and the Dietitians of Canada, there is no perceived risk associated with a normal diet of 500mg DHA and EPA per day (Kris-Etherton *et al*., 2007). The dose we are providing is above this amount, but our dosage regimen complies with HC safety standards of fish oil consumption. All participants will also be on AREDS vitamins, the current standard of care for AMD treatment.
2. The duration of the assessment and tests may be slightly mentally fatiguing to individuals due to their inherent length (one and a half hours for U of A visit and one half hour for the RAH visit). To compensate, the visits will be broken up into parts, so that the participants have short rest periods in between tests.
3. Blood samples allow for monitoring of DHA and EPA levels since they are being manipulated in the study. If participants take extra DHA/EPA supplements beyond what we provide, or if they do not take them as outlined, the blood samples will reveal this. Drawing blood is a routine procedure and the risks are minimal. Risks include bleeding, fainting or light-headedness, bruising, or infection. Blood will be drawn infrequently

(at 6 month intervals) and in small amounts (~10ml per visit) to further minimize these risks.

4. The ERG provides insight into AMD pathology and the potential therapeutic targets or actions of DHA/EPA+Vitalux in the retina. The pupil-dilating agent required for fundus photography and ERG recordings may cause discomfort for 45 seconds but will not cause any damage to the eye. The bright flashes associated with the ERG may be slightly uncomfortable for the subjects, but will not cause damage to the retina. The cornea may get a superficial scratch from the electrode; however, this risk is extremely minimal. Participants will be warned that they cannot drive with pupils dilated.
5. ETDRS visual acuity testing is consistently reliable and accurate with the benefit of being entirely non-invasive, so there is no risk of harm to the individual.
6. The subjects of the study will be under the continuing care of ophthalmologists who can assess them during their regular check-ups. If adverse events are viewed by the Medical Monitor or DSMB, the information will be sent to the participant's ophthalmologist, who will deem whether or not it is necessary for the participant to be seen outside of regularly scheduled checkups.

ii. Voluntary Participation / Premature Withdrawal / Discontinuation Criteria

Participants are free to withdraw from the research study at any time, and continuing medical care will not be affected in any way. If the study is not undertaken or if it is discontinued at any time, the quality of participants' medical care will not be affected. If any knowledge gained from this or any other study becomes available which could influence a participant's decision to continue in the study, he/she will be promptly informed.

Participants are to be monitored by their ophthalmologist at regularly scheduled checkups (once every 2-3 months). Any adverse reaction to the treatment is grounds to stop the study or change the administered dosage of DHA/EPA+Vitalux. In case of a need to re-adjust the dosage, participants will be instructed to reduce the dosage to one capsule per day, followed by an increase of one capsule per day at one week increments, until the participant is back to taking the full dosage of three capsules per day. The subject will be promptly removed from the study if he/she experiences any severe symptoms that cannot be attributed to an underlying pathology or illness known before the trial. Also, all serious adverse events will result in withdrawal from the trial, which will be reported to the HC Natural Health Products Directorate in an expedited manner.

Participants who no longer satisfy the inclusion or exclusion criteria during the trial will also be withdrawn. Although the levels of serum DHA/EPA will be used to measure compliance, participants will not be excluded from the study due to non-compliance. Rather, the participants will be encouraged to

follow their dosing regimens in order for them to experience optimal benefits. Should a participant choose to withdraw at any time from the study, that participant's data will be included in the analysis only if he or she consents to the use of his/her data upon withdrawal. Participants may contact the Patient Relations Office of Alberta Health Services at: 780-342-8080, or the U of A HREB at: 780-492-2615 if they have concerns about rights as a study participant. These offices have no affiliation with the study investigators. If participants become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost. By signing the consent form, participants are not releasing the investigators, institutions and/or sponsors from their legal and professional responsibilities.

Genotyping, ETDRS visual acuity measurements, color fundus photograph grading, and assessment of dietary intake are measures already being performed in AREDS2, and are to be used for the OMEGAlberta, to track the clinical progression of AMD after DHA/EPA+Vitalux supplementation, in order to elucidate the role of genetic predisposition and diet on AMD formation in our study population. The OMEGAlberta Study further aims to compare the physiological effects of DHA/EPA+Vitalux supplementation on the retina, in relation to the clinical progression and epidemiology of AMD, by tracking the level of LF and employing novel ERG tests. Our experimental design should enable us to discern whether or not DHA decreases the accumulation of LF in the RPE, which we hypothesize, will prevent photoreceptor dysfunction and loss, to ultimately slow down drusen formation and delay the progression of AMD.

Concluding Remarks

The multi-factorial etiology of AMD makes the primary defect(s) of this disease a challenge to quantify. Many have found treatments that target individual risk factors for AMD, but the self-perpetuating cycles of drusen deposition, inflammation, and oxidative damage become exponentially devastating to the retina as the disease progresses. We have hypothesized that RPE dysfunction could contribute to a significant portion of AMD pathology, but the factors that contribute to this dysfunction are highly variable between individuals. Supplementation with fish oil and antioxidants should delay the actions of many of the agents that cause RPE damage and the subsequent pathological response, but this therapy alone will never treat or cure AMD. More research is needed to find therapies that specifically abolish the primary causative agent(s) of AMD, or that restore the retina to proper functioning after damage from the disease has already occurred. In the meantime, we must educate the public on ways to minimize exposure to AMD risk factors, in the hopes of delaying onset or progression of the disease.

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Appendix A: Constructing a Clinical Trial in Canada

Clinical Trials originating from Canada require registration at ClinicalTrials.gov and approvals from an institutionally-governed Human Ethics Research Board (HREB) and Health Canada (HC; federally governed). Many research groups employ Clinical Research Organizations (CROs) to submit these applications. The application/approval process is not well-defined in the literature for investigators who want to save money and learn how to submit applications independently. This lack of information caused several barriers and delays for our research group during the application process, so the following sections provide a step-wise guide for submitting concrete applications in a timely fashion, based on our experiences.

A. Study Protocol

The application process for new clinical trials must always start with a comprehensive protocol. One should complete the protocol before applying to any of the committees, thus preventing unforeseen circumstances that could severely delay approval. Outlined below are sections we created in our protocol to satisfy the various submission requirements stipulated by the HREB Human Ethics Research Online (HERO) application, HC Clinical Trial Application (CTA), and ClinicalTrials.gov Registered Clinical Trial (RCT) application. Only simple explanations are provided for the following sections of the protocol, since the remainder of this thesis will analyze questions asked by each committee in

more detail. Following a close reading of the HERO, CTA, and RCT application sections, the protocol sections (outlined below) can be completed. Writing the different applications serves as a secondary review of the protocol; weaker elements of the protocol may be brought to light. Investigators should be aware of this and must be prepared and willing to make changes to the proposed protocol throughout the application process. Each application is different and will inspire special changes to the protocol; however, the investigator should try submitting the same protocol to HREB and HC.

i. Investigators and Collaborators

Clinical trial investigators must have a thorough knowledge of the disease and intervention under study. The study group should seek help from experts in the field to add credibility to the project by including investigators from different disciplines to ensure all aspects are covered. Graduate students and undergraduate volunteers are also important to propagate interest in the field and help with procedures.

ii. Background

Background information of the clinical question under investigation is meant to be informative and succinct, but should draw the interest of the reader. This section should briefly review the subject, as opposed to a lengthy examination of the literature. The review should focus on material that leads into the study rationale.

iii. Rationale

The issue's importance must be addressed after reviewing the background information. The investigator must provide a convincing, affirmative answer to the reviewers' inevitable question, "Why is conducting this study important?" Information from similar studies should be incorporated into this section to justify reasons for creating a new study. If human studies are unavailable, the research group should use animal data; if animal studies have not been performed, the study will most likely be deemed unethical and approval will not be granted.

iv. Research Objectives and Study Design

The research group should outline questions to be answered during the study, and how the proposed tests will provide these answers. Hypotheses of end results should be supported by previous animal data and physiology of the intervention. Creation of a descriptive experimental platform is crucial for a study to run smoothly. The study design should include descriptions of timelines and procedures for patient recruitment, eligibility criteria (inclusion / exclusion / non-restriction criteria), study arms, sites, and outcome measures. One should always collaborate with a physician practicing in the field to create and justify inclusion / exclusion criteria. Collaboration with a statistician is also useful for computing power calculations, which determine the minimum number of participants necessary to yield significant results that will support or reject the hypothesis. Necessary participant numbers are easiest to obtain when recruitment occurs over a range of clinical trial sites; however, study logistics may become complicated with equipment availability and cost. The research group must divide participants

into study arms (experimental and control) to determine efficacy of the intervention. Pre-determined outcome measures should be listed to dictate which study procedures and tests must be used to determine efficacy or provide insight about *how* the intervention works.

v. Methods/Procedures

Medical equipment in Alberta is challenging to access due to cost, number of machines, and long clinic wait times; therefore, strong justification must be provided for performing each procedure. At this point, a dilemma may occur, since the research group must budget cost with equipment accessibility. Money was saved for our clinical trial by training lab members and volunteers on each piece of equipment and gaining access to hospital equipment after clinic hours, bypassing payment of clinic staff salaries. One should keep in mind that any test with the possibility of causing an adverse event, not performed by a registered physician, should be conducted at a hospital, where support staff is available. To ensure consistent collection of data, we then created standard operating procedures and patient screening forms for each site, which also provide us with the materials to train new lab members as the study progresses.

vi. Data Analysis, Privacy and Confidentiality

Research ethics boards dictate that data must be stored in a safe place, such as a locked cabinet, password-protected computer, or secure server. We created data safety monitoring procedures for each site to protect participant privacy, confidentiality, and study results.

vii. Participant Safety

Participant safety is the chief concern of every clinical trial. A Data Safety Monitoring Board (DSMB), which is an independent panel of physicians and academics, is constructed to overlook participant safety. A detailed benefit/risk assessment and early withdrawal procedures must be included in all consent documents and HREB / HC safety assessments.

Information required for *Parts B, C, and D* of this Chapter should be included in the protocol, following the previously described template.

B. ClinicalTrials.gov Registered Clinical Trial Application

All clinical trials in North America must be registered at ClinicalTrials.gov. To obtain a username and password, contact the relevant organization's Protocol Registration System (PRS) administrator by clicking on the "Request contact information" link at http://prsinfo.clinicaltrials.gov/gettingOrgAccount.html. This website is slightly difficult to navigate, so when registering or updating the clinical trial after obtaining the username and password, type in the website: https://register.clinicaltrials.gov/. ClinicalTrials.gov allows researchers to quickly search for any clinical trial being conducted in North America. This website states whether a clinical trial is active, recruiting, completed, or withdrawn. Investigators are required to update the status of their RCT every six months.

The research group should include only a minimal amount of information on the ClinicalTrials.gov application to protect against others copying the clinical trial. We briefly described the purpose, condition affecting participants, and intervention. ClinicalTrials.gov provides straightforward questions about study type and design, primary and secondary outcome measures, study arms, participant numbers, and eligibility criteria. Once again, only a minimum amount of information is required, since ClinicalTrials.gov is purely a registration system; it has no governance over ethical approval for the clinical trial. Once the application is submitted and approved, ClinicalTrials.gov issues the Principal Investigator an identifier in the form of: NCT########. This number can be added to the protocol, but note it is not the same protocol number required for the HERO application.

C. Health Ethics Research Online Application

The University of Alberta (U of A) requires a HERO application to be submitted before commencing any clinical trials at the institution. The U of A HREB (2010) has produced a document titled: "HERO HELP – New Study Human Ethics Application" (*HERO HELP*). This document provides guidance to Principal Investigators setting up studies at the U of A. We have described below each section our study group was required to fill out in the HERO application. Please note that other specific studies may require supplementary sections in HERO that we will not describe; however, these forms are straightforward and guidance can be obtained using *HERO HELP*. The following explanation of the

HERO application parallels and expands on *HERO HELP* and important sections of the online HERO Application, but is intended to focus specifically on constructing an investigator-initiated clinical trial in Canada:

1 – Study Staff, Funding, Location

1.1 Study Identification

The study should be identified by both a short and long title (Subsections 1.0 and 2.0, respectively). Large clinical trials generally use an acronym for the short title (for example: AREDS, The OMEGAlberta Study), which provides others with a quick identifier to perform internet searches about the study. The long title should be more descriptive by identifying the intervention and experimental group. Subsection 3.0 asks to select the appropriate research ethics board (REB). There are currently five REBs to chose from at the U of A: 1) Arts, Science, Law; 2) Education, Extension, Augustana, Campus Saint Jean; 3) HREB Biomedical Panel; 4) HREB Health Panel (also known as HREB Panel B) and; 5) Physical Education & Recreation, Agriculture, Life & Environmental Sciences and Native Studies. HREB governs all clinical trials conducted from the U of A Health Sciences Faculties (Medicine and Dentistry, Nursing, Pharmacy and Pharmaceutical Sciences, Physical Education, and Rehabilitation Medicine), Alberta Health Services, or Covenant Health. The research group must apply to the biomedical panel if the study deals with invasive interventions or measures to human subjects, regardless of the severity level. If interventions or measures are non-invasive, one should apply to Panel B. The U of A Research Services Office is notified by HERO after the study has been approved, to release funds for

investigator-initiated trials originating from the U of A (Subsection 4.0). Trials originating from Alberta Health Services, which go through the Northern Alberta Clinical Trials and Research Centre (NACTRC), and Covenant Health (including Institute for Reconstructive Sciences in Medicine), also require notification of HREB approval to release funds. NACTRC must be notified about all sponsor-initiated trials, even though these types of studies are privately funded.

The Principal Investigator is responsible for the entire study and answers directly to the HREB. Principal Investigators that are graduate students or trainees are not accepted in an ethics application, although the type of project may be classified as graduate student research. Study coordinators and co-investigators do not have to hold the status of "supervisor", but must be employed by the department conducting the research. People added as study coordinators (Subsection 8.0) or co-investigators (Subsection 9.0) become part of the "authorized list", enabling them to review and make changes to the ethics application. Anyone else who is part of the study can be added to Subsection 10.0: Study Team. Study Individuals cannot view or make changes to the application but will become official team members of the study group once ethics approval is obtained.

1.2 Additional Approval

HREB is not the only entity required to approve a study. Each department within the Faculties of Health Science has its own review board, and internal review may be required within the department. One should consult the department Chair for advice regarding the process of internal review.

1.3 Study Funding Information

Every clinical trial requires a source of funding, whether by government grant, institutional funds, or contract with a private sponsor. Obtaining adequate funding is the single-most challenging hurdle to overcome when setting up a clinical trial. Many researchers at the U of A use start-up funds and operational grants offered by the university, but these do not amount to a lot of money. The current state of government funding for investigator-initiated Health Sciences research in Canada is grim. Last year, the Canadian Institutes of Health Research (CIHR) funded only 18% of all Canada-wide applications (http://www.cihr-irsc.gc.ca/e/193.html). In Alberta, the current loss of the Alberta Heritage Foundation for Medical Research (AHFMR), with as of yet unspecified funding allocated to Health Sciences from the novel agency: "Alberta Innovates", has further hindered the progress of Health Sciences research; however, new mandates of this restricted agency should hopefully include provisions to fund clinical trials. In relation to vision research, very few Principal Investigators get funded from charities such as the Foundation Fighting Blindness and the Canadian National Institute for the Blind, so researchers may outsource to private sponsors. For-profit funding by private sponsors is a lucrative avenue to follow, but research may lose credibility due to product endorsements and vested interests. Private sponsors may also elect to only provide funding if the contract states that the company can retain the research group's intellectual property.

1.4 Conflict of Interest

Section 1.4 directs ethical questions to investigators, especially those receiving funding from private sponsors, to assess conflicts of interest. If any of the investigators (or their immediate family) receive personal remuneration that is not budgeted, have proprietary or executive interests in the product under study or the study sponsor, or can be compensated on the basis of study outcome, a Conflict of Interest Declaration must be submitted to the HREB. Based on this declaration, the HREB can deem whether or not the study meets ethical standards to produce unbiased, balanced results without the possibility of data falsification for personal gain.

1.5 Study Locations and Sites

Any site where research will be conducted, and the procedures performed at each site, must be described in Section 1.5. We recommend obtaining site approvals at this point to ensure that each location is receptive to the proposed project. People in charge of clinical trial sites will want to look at the study protocol to ensure that standard operating procedures are functionally feasible using the available equipment, space, and time. Meetings with site advisors provide opportunities to fix possible problems with the protocol, preventing time-consuming addenda from being filed once ethics approval is granted. We also ensured that collaborators from external institutions have obtained, or at least will obtain, the necessary ethics approval to participate in the clinical trial.

2 – Study Summary

2.1 Study Objectives and Design

Subsections 1.0-3.0 ask for start and end dates of the proposed study. These dates do not have to be fixed, but should represent a realistic timeline to complete the endpoint measurements outlined in the protocol, taking into account time for participant recruitment. Note that the end date does not apply to the period required for data analysis after human participation in the study has finished.

Subsection 4.0 asks for an abstract or lay summary of the proposed research. Unlike a scientific abstract, the lay public must be able to comprehend the summary. The short abstract should include a brief background of the proposed intervention, rationale behind the proposed intervention, a simple summary of procedures being performed, and a short explanation of the study endpoints. A more detailed description of the proposed research can then be added in Subsection 5.0, which should include background, treatment range, hypotheses, methods/procedures, and endpoints. An expanded explanation of methods/procedures can be provided in Subsection 6.0, if necessary; we recommend using Subsection 6.0 to provide a detailed explanation as to *why* each test is being performed. For each procedure, one should outline the historical background, relevance of being included in the study, detailed method of performing the test, and how the results will be analyzed. The study group should also mention in Subsections 7.0 and 8.0 whether the research proposal has received independent reviews (i.e. granting agency, scientific peer review, etc.) or

is building upon a previously approved study separate from HERO. According to *HERO HELP*, HREB will look at these other applications to decide whether the study warrants review for scientific or academic merit to be continued.

3 – Risks and Benefits Assessments

3.1 Risk Assessment

Ethical policies for the conduct of research involving humans in Canada have been designated by a group called the Tri-Council. The Tri-Council is a collaboration of CIHR, Natural Sciences and Engineering Research Council of Canada (NSERC), and Social Sciences and Humanities Research Council of Canada (SSHRC). In Subsection 1.0, the risk to participants must be classified as "minimal" or "greater than minimal". The Tri-council Policy Statement (*TCPS*) definition of the standard of minimal risk states: "if potential participants can reasonably be expected to regard the probability and magnitude of possible harms implied by participation in the research to be no greater than those encountered by the subject in those aspects of his or her everyday life that relate to the research, then the research can be regarded as within the range of minimal risk. Above the threshold of minimal risk, the research warrants a higher degree of scrutiny and greater provision for the protection of the interests of prospective participants." In order to determine the level of risk a participant may encounter, HERO provides a rating scale for general physical and psychological risks.

General physical and psychological risks and discomforts are rated on a scale of 0 to 10 in Subsection 2.0 (0 = no likelihood, 5 = moderate likelihood, and 10 = extreme likelihood of the participant being harmed or distressed), followed

by detailed explanations of short- and long-term risks in Subsection 3.0. Our study group brainstormed risks for Subsection 3.0 before providing the general ratings in Subsection 2.0. Ideally, the study protocol should be created to "do no harm". All clinical trials entail some degree of risk, so constructing a plan to manage each risk and discomfort while mitigating harm is key (Subsection 4.0). One should always remember to put the participants' safety and ease before the goals of the study, even if this necessitates compromises to the protocol. Participants are more likely to be compliant for the study's duration if risks and discomforts are minimized and managed efficiently. Conversely, one cannot alter the protocol so much that it makes the results meaningless. Therefore, a compromise must be made to weigh risks against benefits and maximize participant compliance, realizing that not all subjects will last the entire duration of the study.

Every clinical trial requires a Medical Monitor. The Medical Monitor must have a medical degree and is responsible for the care of each participant during the study; however, to maximize care, subjects should also be given access to an independent health-care provider that can follow participants between study visits. Independent health-care providers include family physicians and specialists who have amassed a great deal of knowledge about their patients through following each person over a lifetime. These physicians may use their relationship with patients to more accurately assess changes to physical and mental health that the study group might not perceive. Participants can also be

referred directly to their primary health-care providers if investigators notice adverse events during the study (Subsection 5.0).

3.2 Benefits Analysis

In constructing a good protocol, one's aim is to have the benefits outweigh the risks, but this does not guarantee that the study is ethical. For example, a study could look at the success rate of a new treatment for terminal cancer: all patients have a month to live without the possibility of benefiting from any other treatment, animal studies show efficacy in half of the models studied, but the treatment is fatal in the other half. One could say that the patient is going to die soon and therefore has nothing to lose with the treatment. However, one could also say that the patients' last month of life is sacred and should not be put to chance with the treatment. Of course, there are many other arguments to be made on this issue, but the ethical dilemma remains the same: should a patient take the chance of living a longer life when there is a possibility of immediate death? The reward seems to outweigh risk in this scenario, since the patient could live for a few extra years instead of dying in a month; however, the patient may experience excruciating pain and die sooner than without treatment. Although the clinical trial we constructed does not have to deal with this extreme, we still ensured that the benefits greatly outweighed the risks, and mitigated harm wherever possible.

The best-case scenario of any clinical trial has everyone benefit from participation. Unfortunately we cannot prove exactly how the intervention acts without using controls to compare outcomes. The use of controls only allows half the participants to receive maximum treatment. Control participants must receive

pre-existing precursor treatments or "standard of care" as a placebo comparator; however, if the new intervention has no precursor drug or treatment, the control group must receive an inactive placebo. Fortunately for our trial, all participants enrolled in the OMEGAlberta Study are taking anti-oxidant vitamins (Vitalux AREDS formula), which is the standard of care for age-related macular degeneration (AMD).

The benefit-to-risk ratio for novel research must favour the wellbeing of participants, while still advancing knowledge in the field (Subsection 4.0). *HERO HELP* provides an adept explanation of the ethical dilemma involved with conducting potentially harmful clinical research: "Modern research ethics...require a favourable harms-benefits balance—that is, that the foreseeable harms should not outweigh anticipated benefits. Harms-benefits analysis thus affects the welfare and rights of research subjects, the informed assumption of harms and benefits, and the ethical justifications for competing research paths. Because research involves advancing the frontiers of knowledge, its undertaking often involves uncertainty about the precise magnitude and kind of benefits or harms that attend proposed research. These realities, as well as the principle of respect for human dignity, impose ethical obligations on the prerequisites, scientific validity, design and conduct of research." Therefore, researchers must explain how their study advances science for the betterment of people afflicted with the condition (Subsection 2.0) and how society benefits as a whole (Subsection 3.0). The clinical trial that is being constructed should focus on the improvement of public

health through knowledge transfer, public education, advancing the standard of care, or providing patients with access to treatment.

4 – Participant Information, Recruitment, and Informed Consent

4.1 Participant Information

Properly justified inclusion (Subsection 1.0) and exclusion (Subsection 2.0) criteria ensure the study is well-controlled. These criteria are also made to accommodate the minimum number of participants required to yield significant data. Achieving a balance between keeping a study well-controlled and recruiting the required number of participants is challenging, since one must consider accessibility of required participant numbers that fit the inclusion criteria. Necessary compromises are therefore made for participant recruitment to be feasible. For example, our study requires participants to be older than 50 years of age and have wet AMD in one eye with intermediate dry AMD in the fellow eye. People in this group are at highest risk of developing wet AMD in the fellow eye within 5 years, so our intervention period will be shortened and required participant numbers will be reduced, while keeping the study power at an appropriate level to yield significant results. However, we cannot realistically expect to recruit enough participants by excluding people with hypertension, rheumatoid arthritis, hyperlipidemia, or diabetes (without retinopathy) since the majority of people over age 50 are affected by at least one of these conditions. Our study will be less controlled by including these people, so more participants must be recruited to reduce variability in the data. The exact number of

participants required, taking these factors into account, is provided by performing a detailed power calculation based on statistics presented in the literature.

Power calculations are used as the gold standard for sample-size justification (Subsections 4.0 and 5.0). Values for these calculations are found through an in-depth review of disease incidence, prevalence, and progression amongst the study population, with estimates of how these numbers will change after therapeutic intervention. Estimates should foremost be based on previous human trials related to the intervention, but if no such trials exist, one must resort to animal data. Clinical values do not completely correlate with corresponding animal data, so one should make conservative estimates when putting these values into the power calculation. Different statistical tests can yield a power calculation of the required number of participants. We used a Fisher's exact test from the *SAS/STAT Power and Sample-size Calculator* (SAS Institute Inc., Cary, NC, USA), to compute our power data. Fisher's exact test allows input of "yes" or "no" variables into a Chi-square-like table. For example, we hypothesize that drusen number *will* (yes) decrease over 1 year of fish oil supplementation (primary endpoint) and wet AMD *will not* (no) develop over 5 years of fish oil supplementation. Of note, the absolute value obtained from the sum of drusen for each eye in the experimental and control arms may also be compared between groups. This measurement requires completely different statistical measures that will be performed after experimentation, but these calculations are unnecessary for our current power analysis. Unlike the Chi-square test, Fisher's exact test will not underestimate the probability of the expected observed results, which means

there is a smaller chance the study will be too underpowered to yield reliable data (Dr. Chris Rudnisky: OMEGAlberta Study Statistical Advisor).

Studies of long duration, such as our own, should have primary and secondary endpoints to avoid "putting all the eggs in one basket". Primary outcome measures are created to obtain data after a short period of time (i.e. one year) and assess whether the treatment is working. Early data analysis allows researchers to publish pilot results that are useful when applying for grant renewals, since committees are most likely to extend funding if early results show efficacy. Likewise, if treatment looks to be ineffective, the study can be terminated early enough to prevent the granting agency from wasting future funds. Secondary outcome measures provide a broader evaluation of the intervention and are the focus of the study. For example, our study measures changes in drusen number as a primary endpoint, but will ultimately measure prevalence of wet AMD after 5 years (secondary endpoint), which is in direct correlation to drusen number.

Subsection 6.0 asks for the expected start and end dates of the recruitment/enrolment period. At this point, we recommend creating a recruitment and enrolment timeline in the protocol to facilitate patient recruitment soon after administrative approval is granted. Patient-visit schedules should outline the number of patients seen per week, with anticipated visit times, at each location. Site supervisors also need patient-visit timelines to ensure that access can be granted without disrupting current operations. Timelines must account for the anticipated number of participants getting through the trial in a reasonable and

realistic amount of time, bearing in mind the amount of funding and labour force available to the study group.

4.2 Recruit Potential Participants

Undue pressure may be put on study participants if relationships exist with any of the researchers. Participants could be family members, employees, or students of members of the research team, but in most cases when dealing with clinical trials, participants are patients of physicians on the study team (pre-existing relationships must be identified in Subsection 1.0). Ethical concerns are raised with pre-existing physician/patient relationships, since obtaining participants is simpler when recruiting from the practice of a physician involved in the study. One should use an *intermediary* to prevent participants from being manipulated, coerced, or pressured into joining the study.

4.3 Recruitment Contact Methods

Subsections 1.0 to 4.0 require a description of the proposed process for making contact with potential participants. Intermediaries are separate from researchers who may or may not have pre-existing relationships with potential participants. Intermediaries may be advertisements or people that provide information about the study to potential participants. Based on the information obtained from, for example, a poster, brochure, or recruiter, the subject can then decide whether to contact the research team for more information. Any recruitment material used as an intermediary (including an oral description of the study given by the person acting as an intermediary) must be described in Section

4.2 (Subsection 2.0) and attached in the documentation section of HERO for review.

For our study, we chose to have ophthalmologists inform their patients about the study (all physician offices and recruitment locations, for example, where posters will be displayed, must be listed in Subsection 4.0). The ophthalmologists who are part of the research team are instructed not to explain the study to their patients, but instead provide a consent form which explains the study in detail. If the patient reads the form and would like to know more, he/she can then contact the research team's intermediary directly, without going through the ophthalmologist. The study group should also add a clause to the HERO application that states if recruitment is low, referring physicians can obtain consent from patients to release their contact information to the research group; an underpowered study does not ethically benefit participants already enrolled since data will be deemed insignificant.

4.4 Informed Consent Determination

Per *TCPS* guidelines, informed consent is obtained when "the potential subject chooses freely, and with full information…to participate in the research." For our clinical trial, all participants must be capable of giving informed consent, so exploring the avenues of obtaining third-party consent was unnecessary. Signed, informed consent (including third-party consent) is a requirement of clinical trials registered under HC (Sedgeworth and Derewiany, 2006). HERO provides a consent form template titled: "*EEASJ Information & Consent Letter*", which is found under the Forms and Templates tab. HREB prefers that study

groups use this template. From our experience, this template is confusing and poorly constructed, so we arranged our consent form differently. Regardless of format, a consent form consists of an information sheet and consent checklist. The information sheet and checklist must be at a reading level appropriate to the audience. HERO asks for the specific reading level, which is represented as the Flesch-Kincaid Grade in the Microsoft Word spell-check summary. A consent form has no page limit, but aims to be short and concise and target a Grade 8 reading level. The information sheet should be divided into: 1) study overview; 2) purpose; 3) timeline and locations; 4) procedures; 5) benefits/risks; 6) confidentiality; 7) voluntary participation; 8) expense reimbursement; 9) compensation for injury and; 10) contact names and telephone numbers. The consent checklist must include relevant material for biohazards procedures (i.e. phlebotomies) and ask questions about the participant`s understanding of the implications of being involved in the study.

Subsection 2.0 provides additional options for indicating consent (explicit oral, implied by overt action, implied by inaction/non-objection). The gold standard for any study is a signed consent form, firstly because it indicates the subject has read and fully understands the study information, and secondly because it provides documented proof if liability problems arise. Subsection 3.0 raises the issue of people with special needs who need assistance to give informed consent. Of note, special needs may include non-English speaking or visual / physical impairment, but *does not* include mental retardation. People who fall under “special needs” are competent to give informed consent, but cannot access

the consent material. These people may be accommodated, for example, by providing translated consent documents (Subsection 3.0).

A double-blind study should be created whenever possible, so that neither the researchers nor the participants know which people are receiving the intervention. Double-blind studies are created by coding participants and using a third-party to administer the intervention. The third-party also has access to original participant files in the case of adverse events or early withdrawals. In studies with multiple endpoints, some of the experimental data obtained from the participant may still be useful for analysis. Therefore, if an early withdrawal occurs, the third-party can inform the participant of the group he/she was enrolled in, without compromising the data obtained (Subsections 4.0 and 5.0).

Clinical trial sites must respect participant privacy since human intervention is involved (Subsection 6.0), so the interaction of study participants with non-participants should be limited (i.e. do not provide a survey to participants in a classroom where some people are not involved in the research). In addition to privacy, participants should be comfortable and motivated to come to each site. Incentives and/or reimbursements that make study participation enjoyable must be described in Subsection 7.0 (i.e. reimbursement for parking, selecting a study group that will benefit the most from treatment). One should keep in mind that incentives and/or reimbursements cannot include profitable remuneration to participants, as this could constitute bribery; however, these incentives may still be used to avoid deterrence from participation (due to, for example, inconvenience or discomfort).

4.8 Study Population Categories

As stated in *HERO HELP*, "Section 4.8 is intended ONLY to capture general information for administrative reporting on areas of research. It does NOT speak to inclusion/exclusion criteria or recruitment methodology." Therefore, simply place a check beside anything that applies to the research group's study demographic.

5 – Research Methods and Procedures

5.1 Research Methods and Procedures

Items selected in Subsection 1.0 will prompt additional pages of questions. For our study, we were required to obtain biohazards approval for blood collection. Every study is different, but as a general rule it is important to select everything that applies, so that all pertinent documents are attached and questions answered when the application is submitted.

Subsection 2.0 asks if the study involves a clinical trial. *HERO HELP* defines a clinical trial as "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes...Health related interventions include any intervention to modify a biomedical or health related outcome (i.e. drugs, surgical procedures, devices, behavioural treatments, diet, or process-of-care changes). Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events." Section 7 of the *TCPS* further explains that clinical trials undertaken in biomedical or health research "may include questions that are not directly related

to therapy, in addition to those that directly affect the treatment of the subjects." For example, the primary objective of our study is to prevent AMD progression (proven by visual acuity testing, fundus autofluorescence (AF), color fundus photography, and multifocal electroretinogram; mfERG), but we are performing additional tests (full-field electroretinogram; ffERG, genotyping, lipid analysis, optical coherence tomography; OCT, and dietary intake questionnaires; DIQs) to gain insight into disease pathology and the physiology of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), plus Vitalux AREDS formula supplementation.

All clinical trials that involve a drug, device, biologic, vaccine or natural health product that is not marketed in Canada or will be used outside the parameters of its officially approved use by HC, require registration and approval by submitting a CTA to HC (explained in *Part D* of this Appendix). Subsection 3.0 asks for a registry and registration number, if available, for registered clinical trial(s). The registry and registration number are contained in a No Objection Letter (NOL) from HC that says they have reviewed the CTA and have no objections to starting the clinical trial; however, to obtain a NOL, ethics approval must be granted by the institutional REB. *Part D* of this Appendix details how to overcome the flaw in the system, but at this point, simply submit the HERO application without a NOL. Through the application process, the study group will become acquainted with many people at HREB who, upon request, will quickly send the proper ethics documents for CTA approval. The turnaround time at HC

is much slower than HREB, so conditional HERO approval should be obtained (contingent only on receiving the NOL) before submitting to HC.

All tests described previously in Section 2.1 must be added to Subsection 5.0, listing the people who will be administering the tests and their qualifications. Test administration offers an effective avenue to save money for investigator-initiated clinical trials. We suggest that the study group make training available for undergraduate volunteers and lab employees to become certified to administer tests. Members of the study group may be qualified to offer certain training, but some procedures, such as phlebotomy, require training and approval by a Licensed Medical Laboratory Technologist. Based on the procedures being performed in our study, we expect to save approximately $1000 per patient, per year, by administering the tests ourselves without hiring hospital staff or university technicians. Of note, many of these test results could be interpreted diagnostically, but are being administered by technicians, not medical doctors. Therefore, the technician cannot report test results to participants, but can send a letter to the participant's physician instead, who will then disclose the results and interpretation at his/her own discretion (Subsection 6.0).

5.2 Clinical Trial

Subsection 1.0 requires the research group to create a number for the protocol, which is commonly the Principal Investigator's initials, followed by the year. The date the protocol was last modified is put into Subsection 1.2, and the protocol number and date should also be placed in the footer of the protocol (of note, any protocol modifications require changing the protocol date). Subsection

2.0 asks whether the study is an investigator-initiated clinical trial. Our study is an investigator-initiated clinical trial since it is authored, initiated and conducted by a member of a university or provincially regulated health service provider who is not funded by a commercial sponsor such as a pharmaceutical company or CRO. Clinical trial phase must then be selected in Subsection 4.0, keeping in the mind the following *TCPS* definitions for each phase:

Phase I clinical trials examine acute, dose-related toxicities of new drugs, normally using healthy patients, and may be combined with Phase II clinical trials to include patients with terminal disease when all other treatment has failed. Phase I clinical trials may also involve patients who already have the disease, if the intervention will significantly alter gene expression or protein levels in the organ of a healthy individual. For example, gene therapy for Leber Congenital Amaurosis (ClinicalTrials.gov: NCT00481546) causes upregulation of the human *RPE65* gene that could produce unnecessary, harmful amounts of protein if upregulated in a healthy individual; however, testing in this Phase I clinical trial is still strictly for safety, not for efficacy.

Ethical concerns are raised in Phase I clinical trials when pharmaceutical companies pay healthy participants to take new drugs for testing, which compromises the process of free and informed consent. According to the *TCPS*, Phase I and Phase I/II trials require additional vigilant monitoring from the academic sector for adverse events. Phase II clinical trials, such as the OMEGAlberta Study, examine short-term drug toxicities and efficacy of new drugs in a small sample-size of people having the target disease / condition.

Phase III clinical trials are performed in a much larger sample-size with the disease / condition and are meant to examine efficacy of improving survival or quality of life. Phase II and III clinical trials use a placebo control or comparator to examine toxicities and efficacy of the experimental therapy. For example, our clinical trial uses the Vitalux AREDS formula that the patients are already taking, as a comparator to measure efficacy of the new intervention.

Phase IV clinical trials continue after a drug is marketed to examine *long-term* efficacy and toxicities. Pharmaceutical companies often pay private physicians to market a drug in his/her practice, while examining long-term side-effects. The physicians' professional integrity could be compromised in this situation. Phase III and IV clinical trials often utilize multiple centres to increase participant numbers or accommodate other disciplines into the research. The involvement of high numbers of participants warrants greater vigilance by the study team and organized communication between sites to ensure proper monitoring of participant safety. Each centre requires individual REB approval in addition to overall study approval. Once study data are collected, sponsors may hold legal property over the initial analysis and interpretation of the results. Investigators in many sponsor-initiated clinical trials sign non-disclosure agreements, legally preventing them from reporting results without consent of the sponsor. The Government of Canada Interagency Advisory Panel and Secretariat on Research Ethics is working to legally address ethical non-disclosure issues such as adverse event reporting and dissemination of research results (PRE, 2008), but the process has been going on for the past two years and no changes

have been made to *TCPS*. The *TCPS* does, however, state that it is the researcher's duty to have final analysis of results and that he/she must disseminate this knowledge, preferably through peer-reviewed journals. If a clinical trial does not obtain the preferred results and is unpublished, the researcher is still equally responsible to release results to the public domain. Our clinical trial is investigator-initiated, so it does not have an industrial sponsor. Ocean Nutrition Canada Ltd. has graciously sold us supplements at cost, while allowing us to retain academic property through signing of Confidential Disclosure Agreements.

Subsection 5.0 asks to state provisions made to break the code of a double-blind study in an emergency situation. Contacting a third-party that is always available but is not part of the study is an efficient way to access coded patient files. The third-party can release decoded participant information to the necessary health-care providers if an emergency situation occurs. If at any time there are concerns of safety or toxicity to an individual, one must promptly remove the participant from the study and evaluate whether the treatment or an underlying pathology is causing the problem (Subsection 7.0). HREB and HC must be notified if the treatment causes safety concerns.

5.4 Data Safety and Monitoring for Clinical Trials

Clinical trials that test a therapy outside its officially approved use require a DSMB or study monitor that is not associated with the study, in addition to safety monitoring by the investigators (Subsection 1.0). A DSMB is created after a formal risk assessment by HC and NACTRC, usually after CTA and HERO approval. Members on the DSMB are selected on the basis of guidelines

pertaining to the formal risk assessment and have no affiliation with the study (Subsection 2.0). The DSMB has grounds to stop the study or call for protocol modifications if safety concerns are raised (Subsection 3.0).

5.6 Sound or Image (other than audio- or video-recorded interviews) or Material Created by Participants and; 5.7 Interviews, Focus Groups, Surveys and Questionnaires

When obtaining private material from participants, such as photographs, audio recordings, or surveys, the material must be coded and stored on secure servers to protect the participants dignity and privacy, since this material may be disseminated to the public as results of the study.

5.9 Investigational Drugs, Devices, Biologics, Vaccines or Natural Health Products

Information about the health device (name, manufacturer, type, HC approval status, and NOL control number with date) must be added to Subsection 1.0. For our trial, we put "NOL Pending" as the HC approval status for reasons we described earlier.

5.10 Food, Nutrition, and Nutraceuticals Information

Dietary levels of a nutrient may coalesce with levels achieved by the interventional supplement and become toxic. Dietary products must come from a trusted source and be produced within acceptable standards for food safety. A dietician should be made available by the study team to educate participants about healthy food consumption while enrolled in the trial.

5.11 Health and Biological Specimen Collection

If biological specimens (i.e. blood tissues or fluids) are being collected, additional biohazards approval is required for each site. In Subsections 3.0 to 5.0, explain how the specimen will be collected and stored, including all intended uses. Section 5.13 provides contact information for the Biosafety Officer at the U of A, to whom the study group must submit a cover letter with the Principal Investigator's name and department, project title, and grant information. The research group is also required to attach an experimental plan of the procedures involving biohazards. The Biosafety Officer will work with the research group to obtain biohazards certification for each site that deals with biological specimens.

5.12 Registries and Databases (including Biobanks)

Clinical trial data must be stored in a secure place to respect the safety and privacy of study participants (i.e. store digital files in a password-protected file). Our study conveniently has access to the Secure Diagnostic Imaging (SDI) teleophthalmology server, enabling researchers secure remote access to participant data. The SDI SecureServer was created to provide an acceptable standard of care and diagnostic evaluation to patients through the use of high resolution, stereoscopic digital photographs, and visual acuity and intraocular pressure measurements (www.teleophthalmology.com). Physicians with remote access to this information can screen patients without unnecessary referrals, reducing the number of patient visits, and can also follow patients after treatment to determine future testing. Pictures are simply loaded into the password-protected SDI ImagePackager software and uploaded onto the 128-bit encrypted,

password-protected SDI SecureServer (compliant with the Health Information Act).

Paper files must be stored in a locked cabinet within a secure location. One should keep records of individuals who are granted access to patient files and "de-link" information wherever possible. Participant information is "de-linked" when it is not directly linked to personal identifiers submitted to the database. As described earlier, identifiable participant information should be separated from study data and put in care of the third-party.

6 – Data Privacy and Confidentiality

6.1 Data Collection

We have alluded to data privacy and confidentiality in many of the earlier sections. Subsections 1.0 to 5.0 are redundant, asking how participant anonymity will be respected.

6.2 Data Identifiers

Subsection 1.0 asks to select items that will be used as personal identifiers. For safety purposes, one should be sure to include the participant's full name, address, telephone number, and full date of birth. The participant's healthcare number should also be collected, since this would prove useful in the event of an adverse reaction. There is no need to include other identifiers (i.e. social insurance number, full-face photograph, etc.) if not relevant to data collection. HC mandates that all data and master lists be kept for a minimum of 25 years after study completion (Subsection 4.0).

6.3 Data Confidentiality and Privacy; and 6.4 Data Storage, Retention, and Disposal

Sections 6.3 and 6.4 are redundant to many previous sections that describe data privacy and confidentiality in HERO. Any lab members who have never dealt with human data should be trained to do so, and collaborators should be legally bound to privacy by signing confidentiality agreements (confidentiality agreements are drafted by NACTRC).

7 – Documentation

7.1 Documentation

Once a document has been attached to Section 7.1, it cannot be removed. Type "(REMOVE)" in the file name if a document needs to be discarded. Recruitment posters and brochures, consent forms, questionnaires, and a final version of the study protocol should be attached to this section

D. Health Canada Clinical Trial Application

All clinical trials performed in Northern Alberta must go through NACTRC to obtain Administrative Approval. More specifically, this service acts as a necessary intermediate between the U of A and HC. Documents and approvals are funneled through NACTRC, so that both institutions can easily communicate with each other. Cases occur in which the University HREB requires HC approval, but at the same time, HC requires HREB approval. NACTRC works between the two institutions to prevent deadlocks from slowing the application process. NACTRC aids research coordinators and Principal

Investigators of investigator-initiated clinical trials free of charge, but sponsor-initiated clinical trials are charged by NACTRC for services rendered. In any case, we recommend corresponding with NACTRC at the very beginning of the study application process to gain insight into the necessary avenues to be followed. Every clinical trial is different, requiring certain application forms that may not be necessary for other applications. After reading the study protocol, NACTRC puts together a package specific to the study, so that all required forms are sent to HC. For example, our study proposed to use a Natural Health Product (NHP) that has not been given a Natural Product Number (NPN) by HC, so our study requires different forms than an investigational new drug application. NACTRC is very convenient, but not all sites have a service like this. The remainder of this section is meant to review the CTA for a NHP that has not been approved by HC, or is being administered outside safe limits. As mentioned earlier, applications for other kinds of interventions require different forms, but these are simply obtained from NACTRC.

HC requires submission of a CTA in hard copy and on compact disc. The hard copy must follow the format mandated by HC, and digital files put in properly named folders (Figure 12).

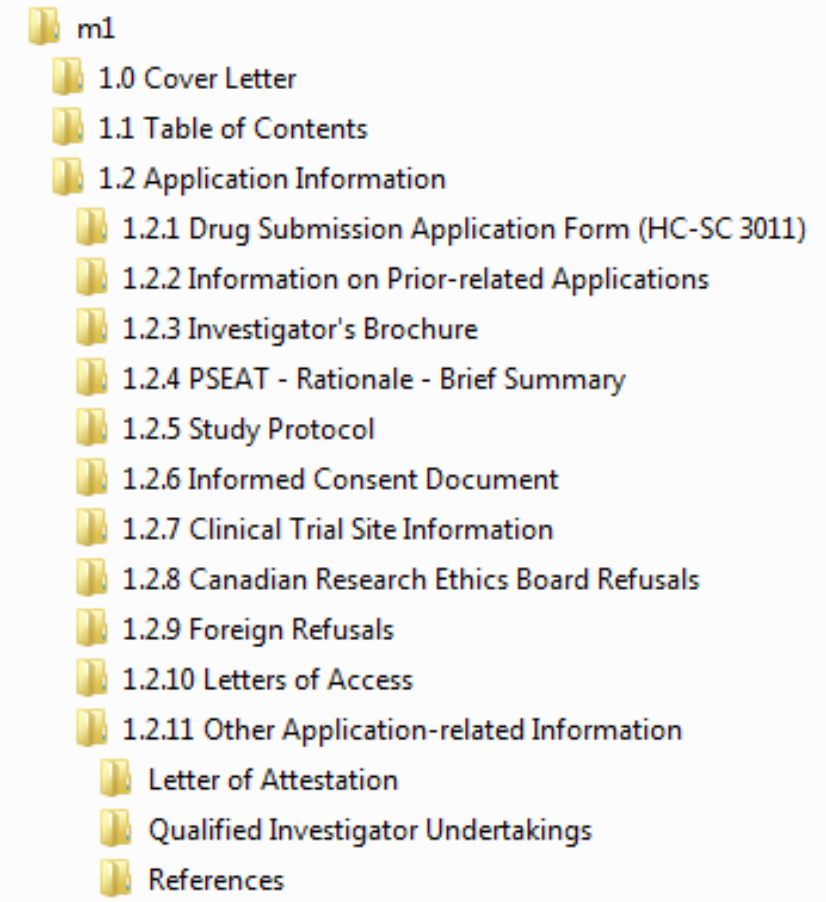

Figure 12: CTA folder organization for digital copy (m1 → 1.0 Cover Letter; 1.1 Table of Contents; 1.2 Application Information)

For our study, the CTA began with two cover letters, one from the Principal Investigator and the other written by Alberta Health Services. The cover letter must contain the study title in bold, with a brief explanation of the purpose, describing the proposed intervention and main outcome measures. Lastly, the Principal Investigator (for investigator-initiated clinical trials, this will be the person signing the cover letter and submitting the application) and co-Principal Investigators must be identified, along with these individuals' credentials. The rest of the application must be formatted on the compact disc as follows:

1.1 Table of Contents

One should note that section titles in the Table of Contents are slightly different than what must be submitted on the compact disc (Figure 12).

1.2 Application Information

Section 1.2 must contain the following subsections:

1.2.1 Clinical Trial Application and Attestation Form

Part 1: Applicant and Contact Information

Subsection A asks for sponsor information. Investigator-initiated clinical trials are generally sponsored by the Governors of the University. The contact for investigator-initiated applications is always the Principal Investigator (Subsection B).

Part 2: Research Ethics Board(s)

Part 2 asks whether REBs have approved or refused the protocol, which seems like a situation where HREB wants HC approval at the same time HC wants HREB approval. To overcome this dilemma, NACTRC collaborates with HREB to send a conditional letter of approval (described in Section 1.2.8) if the ethics application has been submitted without the NOL from HC. Any Canadian REB refusal letters must be attached to Section 1.2.8, and foreign refusals to Section 1.2.9.

Part 3: Clinical Trial Application Information

The trial phase, protocol number, and title must be entered into Subsection A (refer to *Section iii* of this Chapter for an explanation of clinical trial phases). Subsection B asks if other submissions contain evidence to support safety,

efficacy and/or quality of the NHP. If so, add the Master File Number (MF#) for the application and enclose a letter of access to the Master File (Master Files are described in Section 1.2.2 of the CTA). Subsection C asks what documents are being included in the application, so one should fill in this section at the very end to check the correct boxes that coincide with what is enclosed.

Part 4: Clinical Trial Application – Amendment

This section is required only if changes are being made to an application that has already been approved. All changes need to be submitted as an addendum to HREB as well.

Part 5: Clinical Trial Site Information

The Clinical Trial Site Information Forms (Subsection A) are submitted once REB and HC approval have been obtained. A letter is added to Section 1.2.7 of the CTA that states Clinical Trial Site Information forms will be submitted after REB and HC approval. The Qualified Investigator Undertaking forms (Subsection B) for each site are submitted with the original application. These are added to Section 1.2.11 of the CTA. There must be a Qualified Investigator for each site, who is a physician or dentist, responsible for the medical care and medical decisions conducted at the site. The Qualified Investigator can be the same person for each site and may also be a co-Principal Investigator.

Part 6: Study Product Information

HC has strict guidelines about the study product information presented in the CTA. In fact, HC sent this section back to us for many minor revisions. The

information required to complete *Part 6* can be found in a product monograph or finished product specification sheet obtained from the manufacturer (Table 6).

Table 6: Summary and explanation of questions asked about medicinal and non-medicinal ingredients of the NHP and placebo.

QUESTION	EXPLANATION
A. Standard or Grade	Refers to the compound purity; sometimes this is not included in the finished product specification sheet.
B. Scientific Monograph	Review of research results pertaining to the medicinal ingredient, usually outlining the biochemistry, metabolism, indications, pharmacology and toxicology of the compound. HC has generic monographs for NHPs, but a monograph for the specific product can be obtained from the manufacturer if it has one.
C. Proper Name	Name of the medicinal compound as it is seen on the monograph.
D. Common Name	Refers to the lay name of the product.
E. Quantity per dosage unit	Refers to the amount of medicinal compound per pill, capsule, etc. Quantity per dosage unit does not refer to the amount of each individual medicinal constituent forming the compound.
F. Synthetic	State "yes" if the medicinal compound has been chemically produced. For example, MARTEK purifies DHA from genetically modified algae (synthetic), whereas Ocean Nutrition concentrates DHA from fish oil (natural).
G. Animal Tissue	State "yes" or "no" to whether animal tissue is used, regardless of the stage at which it was used in the preparation.
H. Potency	The amount of each constituent of the medicinal ingredient must be represented as mass *and* percent.
I. Source	If ingredient is from animal tissue, be sure to include the taxonomic family of the animal. Identify the type of tissue that was used under: "Material".
J. Extract	Usually only pertains to plant-derived ingredients.
K. Method of preparation	May pertain to plant-derived ingredients or animal tissue. For example, flaxseed can be crushed, milled, or cold-pressed; animal tissue can be fixed, frozen, homogenized, extracted, etc.

1.2.2 Information on Prior-Related Applications

Two documents must be added to Section 1.2.2: "Ongoing Trials" and "Prior Related Applications". Ongoing trials are referenced in the manufacturer's Master File for the product under investigation. HC has copies of all Master Files

for marketed compounds, but needs permission from the manufacturer to access it for each new clinical trial. Getting permission from the manufacturer to allow HC access to the Master File can be tedious. HC must be provided with a Letter of Access from the manufacturer (Section 1.2.10); however, some manufacturers will not provide the study group with a Letter of Access, since confidentiality could be breached. In this situation, one should ask the manufacturer to provide HC with access upon direct request by HC during the review period. HC can be simply provided with contact information of the manufacturer's person/department in charge of handling these requests. In addition to the Ongoing Trials letter, a review of Prior-Related Applications must be attached. The review should list all peer-reviewed papers that report studies of the product and its actions, stating dosage, outcomes, and adverse reactions.

1.2.3 Investigator's Brochure

Investigator's brochures are not always available for the product. Our group was unable to obtain investigator's brochures for the study products, so we tried attaching product monographs of similar compounds; however, these were rejected. If no investigator's brochure is available, one should simply state this in Section 1.2.3 and provide HC with an informative review of the literature in Section 1.2.2 of the CTA, describing previous trials that used the product.

1.2.4 Protocol Safety and Efficacy Assessment Template

A Protocol Safety and Efficacy Assessment Template (PSEAT) must be submitted in addition to the study protocol, even though the study protocol contains everything mentioned in the PSEAT. The PSEAT is used by HC as a

summary of the safety and efficacy of the investigational compound. The introduction lists product information (dosage form, strength, route of administration, proposed indication, etc.) and contact information of the Principal Investigator. A lengthy summary of the protocol must be attached to cover background / rationale, objectives, study design and duration, site information, list of investigators, sample-size, patient population, inclusion/exclusion criteria, drug formulation, dosage regimen, washout period, pre-study screening and baseline, treatment/assessment visits, concomitant medication, rescue medication and risk management, premature withdrawal/discontinuation criteria, efficacy variables and analysis, safety variables and analysis, and statistical analysis. HC provides guidance notes for the PSEAT protocol summary about information required for each section; however, answering this part of the PSEAT should simply require a summary of what is already stated in the protocol.

1.2.5 Study Protocol

The protocol should be complete by this stage. As stated earlier, sections can be created in the protocol that do not follow the PSEAT to the letter, but the study group must ensure all information from the PSEAT is stated in the protocol.

1.2.6 Informed Consent Document(s)

These documents should already be reviewed, modified, and passed by HREB, so one can simply attach the consent document(s) to this section.

1.2.7 Clinical Trials Site Information

Refer to Part 5 of Section 1.2.1 of the CTA.

1.2.8 Canadian Research Ethics Board Refusals / REB Supporting Documents

Refer to Part 2 of Section 1.2.1 of the CTA. If the clinical trial has passed all Canadian REB applications, a letter is required that says no refusals have been issued. A letter from HREB must also be obtained, stating the institution has reviewed the ethics application and has decided to grant approval, contingent upon HC having no objections. NACTRC will add three more letters to show that the U of A HREB complies with requirements of the HC Food and Drug Regulations.

1.2.9 Foreign Refusals

Refer to Part 2 of Section 1.2.1 of the CTA. One must write a letter to say that no refusals have been issued if the trial has passed all foreign ethics board applications.

1.2.10 Letter(s) of Access

Refer to Section 1.2.2.

1.2.11 Other Application-Related Information

Section 1.2.11 must include a signed Letter of Attestation from the Principal Investigator, stating that all electronically submitted information matches the material submitted in hard copy. Included in this section are the Qualified Investigator Undertaking forms, as well as references that aid in the approval process. Instead of providing citations, the research group must attach PDF files and complete printed copies of the full documents being referenced. NACTRC also provides two documents for the Principal Investigator to sign. The first document confirms the trial is “investigator-initiated”, and the second is a

Principal Investigator-created risk assessment of the study. These two documents are independently reviewed by NACTRC in addition to the evaluation from HC.

1.3 Electronic Review Documents

Recent changes to HC policy requires all CTAs to be submitted in hard copy and on compact disc. HC documents are found on the HC website, but can be more conveniently obtained from NACTRC. All HC template files are locked, which is extremely inconvenient when trying to "track changes" after the first review. To track changes, we revised the document, printed it off, then crossed out corrections and highlighted additions by hand.

E. Operational and Administrative Approval

Once HC has approved the CTA and sent the study group a NOL, HREB will grant approval to the HERO application. Operational Approvals must then be obtained for each health authority listed on the NACTRC website. Applications for Operational Approvals are performed online at http://www.clinicaltrials.ualberta.ca/op_app.php. Operational Approval is not the same as Site Approval; however, site approval is often necessary to obtain operational approval. For example, the Royal Alexandra Hospital (RAH) is a health authority with many departments: site approval for the Department of Ophthalmology must be obtained before RAH Operational Approval is granted.

NACTRC compiles and reviews the HREB approval, funding information, and Operational Approvals to decide whether to grant Administrative Approval.

Once administrative approval is obtained, patient recruitment and testing can officially begin. As per Post-Authorization Requirements, any modifications to the clinical trial, premature withdrawals, or adverse drug reactions must be reported to the HREB and the corresponding Directorate at HC.