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Omega-3 Fatty Acids, the Brain and Retina

Editors
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Omega–3 Fatty Acids, the Brain and Retina

Volume Editors

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Preface

Research on omega–3 fatty acids has expanded enormously over the past 10 years. Beginning with the mid 1970s, most of the research focused on the role of omega–3 fatty acids in the secondary prevention of cardiovascular disease. Epidemiological observations, animal studies, clinical intervention studies, and studies at the molecular level firmly established the importance of omega–3 fatty acids, in the prevention and management of cardiovascular disease. Furthermore, studies on the mechanisms and the need to balance the omega–6 to the omega–3 ratio for homeostasis and normal development have been carried out at the molecular level and in transgenic animals using lipidomics and informatics. It is now accepted that docosahexaenoic acid (DHA) and arachidonic acid (AA) are essential for brain development during pregnancy, lactation and throughout the life cycle. Recently, studies on brain and retinal function as well as mental health have dominated the field. That DHA can affect brain function and behavior is no longer controversial. The studies on age-related macular degeneration (AMD) given supplemental DHA have revealed significant interactions between DHA and genetic variants. In animal experiments, deficiencies in DHA show impairments in cognitive development correctable by its repletion. Furthermore, the consumption of DHA or fish oil by humans slows cognitive decline in the aged and in subjects with early Alzheimer's disease (AD) and promotes mental development in infants. Over 60 countries worldwide have supplemented infant formula with DHA and AA, yet the Food and Nutrition Board of the Institute of Medicine has not determined the nutritional requirement of DHA.

There have been a number of volumes in the series of the *World Review of Nutrition and Dietetics* (WRND) on various aspects of omega–6 and omega–3 essential fatty acids (EFA) beginning with Volume 66: Health Effects of Omega–3 Polyunsaturated Fatty Acids in Seafoods, published in 1991, which truly established the field. It was followed by Volume 75: Fatty Acids and Lipids: Biological Aspects, published in 1994.

Volume 83: The Return of Omega-3 Fatty Acids into the Food Supply I. Land-Based Animal Food Products and Their Health Effects, published in 1998. Volume 88: Fatty Acids and Lipids – New Findings, published in 2001. Volume 92: Omega–6/Omega–3 Essential Fatty Acid Ratio: The Scientific Evidence, published in 2003. The present volume 99: Omega–3 Fatty Acids, the Brain and Retina is the sixth in the series, published in 2008.

The volume begins with the paper by Artemis P. Simopoulos on 'Omega–6/Omega–3 Essential Fatty Acids: Biological Effects' which sets the stage for what follows. Dr. Simopoulos emphasizes the changes that have taken place in the food supply that led to high intake of omega–6 and low intake of omega–3 fatty acids, particularly the last 50 years, and the biological effects of the resulting imbalanced omega–6/omega–3 ratio. Major advances have taken place in the concepts of inflammation and proresolution of new lipid mediators, lipoxins, resolvins and protectins discovered by using new approaches mainly lipidomics and informatics. Finally the paper provides an overview of mental illness and eye disease that are presented in detail in the papers that follow.

A number of epidemiological studies across populations have clearly shown an inverse relationship between fish intake and depression, as well as cognitive dysfunction in elderly populations. Animal experiments deficient in DHA have shown deficits in learning and memory. In some studies in Finland and Japan where the fish intake is already high, such inverse relationships have not been seen consistently. But in the majority of studies the data are consistent between low fish intake and prevalence of major depression. For example, prevalence rates of major depression are 50 times higher among countries with little seafood consumption compared to countries with the highest consumption. Furthermore, at autopsy the concentration of DHA is 30% lower in the dorsolateral prefrontal cortex of subjects with major depression. Of interest is the fact that three meta-analyses have reported robust treatment effect sizes for omega–3 long-chain fatty acids that are larger than those reported for most antidepressant pharmaceuticals. Clinical intervention studies have generated data that impulsive violence, post-traumatic stress disorders, personality and substance abuse disorders may be prevented or treated using appropriate amounts of omega–3 fatty acids.

Dr. Hibbeln in his paper 'Depression, Suicide and Deficiencies of Omega–3 Essential Fatty Acids in Modern Diets' reviews the studies of major depression including the ecological and epidemiological aspects, intervention studies, as well as the data on tissue composition. Dr. Hibbeln concludes that a strong and consistent body of data from ecological, epidemiological, case-control tissue compositional studies and randomized placebo-controlled trials indicate that low seafood consumption, omega–3 intake and body compositional status are linked to greater risks for significant depressive symptoms. This body of data is evaluated in light of the Hill Criteria for assessing causality. While substantial work needs to be conducted in the study of omega–3 deficiencies and increased suicide risk, current data suggests an apparent beneficial effect. Religious dietary practices followed for centuries are remarkably consistent with current dietary recommendations issued by international scientific bodies. Modern diets increase the

likelihood of omega–3 deficiencies, perhaps manifested as depressive symptoms and self-destructive behaviors.

In the next paper, 'Application of Serial Structural Magnetic Resonance Imaging Analysis and Proton and 31-Phosphorus Magnetic Resonance Spectroscopy to the Investigation of Cerebral Fatty Acids in Major Depressive Disorder, Huntington's Disease (Chorea), and Myalgic Encephalomyelitis (Chronic Fatigue Syndrome), and in Forensic Patients with Schizophrenia Who Have Seriously and Violently Offended', Dr. Puri et al. describe recent advances in the analysis of serial magnetic resonance structural cerebral scans and in the use of proton neurospectroscopy and 31-phosphorus neurospectroscopy. The authors show how these technologies can be applied to further our understanding of the role played by lipids in the pathophysiology of major neurologic and psychiatric disorders, including major depressive disorder, Huntington's disease (Huntington's chorea), myalgic encephalomyelitis (chronic fatigue syndrome), and schizophrenia and how these techniques may be employed to study the cerebral effects of nutritional supplementation in these and related disorders.

Major discoveries have taken place on the function of neuroprotectin D1 (NPD1) which is produced from DHA. Drs Niemoller et al. present the latest research on the evidence. In their paper, 'Omega–3 Fatty Acid Docosahexaenoic Acid Is the Precursor of Neuroprotectin D1 in the Nervous System', the authors first review the metabolism of DHA in the nervous system. After ingestion, DHA is processed in the liver and transported by the bloodstream to the central nervous system. Once in the retina and brain, it is incorporated into phospholipids in neuronal and photoreceptor membranes where it promotes proper visual and neural activity. DHA is the precursor to NPD1, a potent neuroprotective lipid-signaling molecule. NPD1 is synthesized in response to oxidative stress, ischemia, and certain neurotrophins. NPD1 has been shown to activate protective anti-apoptotic Bcl-2 proteins including Bcl-2, Bcl-xL, and Bfl-1/A1 while inhibiting pro-apoptotic proteins Bad, Bax, Bid, and Bix. NPD1 also actively inhibits interleukin-1β (IL-1β) induction of cyclooxegenase-2 (COX-2) preventing initiation of the inflammatory cascade. Because of its potent protective activity, NPD1 is a promising candidate to support cell survival/repair in retinal degenerative diseases and reduce damage caused by brain inflammation in conditions such as stroke, Alzheimer disease (AD) and epilepsy.

There is a great interest on the mechanisms by which DHA may have a beneficial effect in patients with AD. Dr. Lukiw in his paper 'Docosahexaenoic Acid and Amyloid-β Peptide Signaling in Alzheimer's Disease' discusses the beneficial actions of free DHA and NPD1. Significant molecular, genetic and epidemiological data support the idea that β-amyloid precursor protein (β-APP)-derived peptide and cytokine-induced oxidative stress, and the generation of reactive oxygen species, play important roles in aging and in the development and progression of neurodegenerative disease. Dr. Lukin discusses the role of β-APP, amyloid-β (Aβ) peptides, oxidative stress and apoptosis in AD. While the degree to which brain inflammation plays a causative role in AD is controversial, there is abundant evidence that pathogenic inflammatory signaling con-

tributes significantly to the maintenance and progression of the AD process. DHA reduces both apoptosis and oxidative stress. Dr. Lukiw points out that DHA suppresses the expression of genes involved in inflammation. DHA through the production of NPD1 and other DHA-derived neuroprotectins, provides additional benefits in patients following trauma or stroke. While the interaction of cholesterol and statins with DHA are just beginning to become understood, DHA supplementation in combination with statin therapy demonstrates a significant enhancement in the reduction of serum cholesterol than is observed with statin treatment alone. The fatty acid composition of neuronal membranes, including DHA and cholesterol content, and cholesterol transporters have direct bearing on whether β-APP is processed into neurotrophic or the more neurotoxic species. Dr. Lukiw concludes: 'The chronic nature of AD suggests that neuroprotective and survival factors are progressively lost, switching from an up-regulation in the expression of anti-apoptotic factors to increases in the expression of pro-apoptotic members of the Bcl-2 gene family. Unlike the actions of excessive cholesterol, the neuroprotective DHA and DHA-derived NPD1 decrease the rate of Aβ peptide generation, aggregation, and it's shifting the balance from the expression of pro-apoptotic factors toward the expression of anti-apoptotic, survival-promoting members of the Bcl-2 gene family.'

Administration of the omega–3 fatty acid DHA to humans or experimental animals can improve cognitive performance; the mechanisms underlying this effect remain uncertain. In general, nutrients or drugs that modify brain function or behavior do so by affecting synaptic transmission, changing the quantities of particular neurotransmitters within synaptic clefts or acting directly on neurotransmitter receptors or downstream signal-transduction molecules.

The next paper, 'Administration of Docosahexaenoic Acid, Uridine and Choline Increases Levels of Synaptic Membranes and Dendritic Spines in Rodent Brain', by Dr. Wurtman et al. presents an extensive review of the biochemistry and metabolism of uridine and choline since DHA's effects on synaptic membrane to a great extent depend on its interactions with brain uridine and choline. The authors found that DHA affects synaptic transmission in mammalian brain: gerbils or rats receiving this fatty acid manifest increased levels of phosphatides and specific pre- or post-synaptic proteins per brain cell, and increased numbers of dendritic spines – a precursor of new synapses – on brain neurons. These actions are markedly enhanced in animals which have also received the other two circulating precursors of phosphatidylcholine – uridine (which gives rise to brain UTP and CTP) and choline (which gives rise to phosphocholine). These findings are reproduced also by eicosapentaenoic acid (EPA), but not by the omega–6 fatty acid AA. Administration of the three compounds (DHA, uridine and choline) also increases neurotransmitter release (acetylcholine; dopamine) and affects animal behavior. Conceivably, this treatment might have some use in patients with synaptic loss, e.g. secondary to neurodegenerative disease, or stroke, or brain injury.

Animal experiments and clinical intervention studies have shown that DHA is essential for learning, behavior and memory. How much DHA is needed for normal growth

and development has been estimated from studies with breastfed infants and from clinical intervention studies. Brain development begins shortly after conception. In humans, the brain approaches adult mass within the first 2 years of life during which time the foundation for the neural circuitry of the brain is established. Early access to an adequate supply of substrates for neural development is a key requirement for preventing irreversible cognitive effects.

Hadley et al. in their paper 'An Assessment of Dietary Docosahexaenoic Acid Requirements for Brain Accretion and Turnover during Early Childhood' carried out an extensive review of the data in order to define the DHA requirement during the first 2 years of life by considering data for DHA accretion, concentration and turnover from previously published studies, and prepared a general model for human brain DHA homeostasis. The model is based on physiological demands of the brain rather than on an estimate derived from the caloric contribution of α-linolenic acid (ALA, 18:3n–3). An estimate for the dietary requirement of preformed DHA during early childhood (1–2 years of age) is calculated to be 212 mg/day. This dietary requirement is calculated to be sufficient to support total brain accretion of 3.75 mg/day of DHA.

In the next paper, 'Variation in Lipid-Associated Genes as they Relate to Risk of Advanced Age-Related Macular Degeneration', Dr. SanGiovanni et al. present an extensive review of the functions of both omega–6 and omega–3 long-chain polyunsaturated fatty acids (LCPUFAs), focusing on the role of omega–3 LCPUFAs as factors impacting metabolic processes and environmental exposures implicated in the pathogenesis of AMD. These processes and exposures include neovasucularization, oxidative stress, hemodynamic and hydrodynamic change, and factors affecting cellular survival. Molecules operating with in complex systems to impact AMD pathogenesis include eicosanoids, angiogenic growth factors, matrix metalloproteinases, reactive oxygen species, cyclic nucleotides, neurotransmitters and neuromodulators, pro-inflammatory and immunoregulatory cytokines, and inflammatory phospholipids. The balance and composition of dietary and retinal omega–3 and omega–6 LCPUFAs may affect substrates, availability of biosynthetic enzymes, and gene expression of these molecules; LCPUFAs also act as ligands to a number of transcription factors and serve as precursors to potent cyclooxygenase-, lipoxygenase-, cytochrome P450-derived autocoids that influence vascular sufficiency, cell cycle, and cell survival. In the context of this evidence base, and that on the molecular genetics of AMD, Dr. SanGiovanni and colleagues applied resources from a genome-wide scan to examine the relationship of AMD with polymorphisms in sets of genes encoding enzymes, structural elements, and transcription factors affecting and affected by LCPUFAs and other lipid-based compounds, their precursors, cleavage and biosynthetic enzymes, and metabolites. Their results suggest that variants in genes encoding elements of phosphatidylinositol-based signaling systems are jointly related to a 99-fold increased risk of advanced AMD (odds ratio 99.2, 95% confidence interval 22.3–440.9, $p \leq 1.0 \times 10^{-17}$). This is the first application of joint action models to investigate molecular genetics of AMD related to pathways and gene sets. The pattern of the results suggests that: (1) the phosphatidylinositol signaling sys-

tem may be acting through pleckstrin homology domains to activate a number of protein kinases, G-protein-based signaling cascades, and phospholipases with potential to impact health and disease of the neural and vascular retina; (2) there is a reasonable basis to investigate the relationship of genetic variants in the inositol pathway and the role of Akt/PI3K and dietary LCPUFAs in calcium homeostasis implicated in retinal disease. Deep sequencing of inositol gene sets in clinical populations and mechanistic studies in model systems may yield useful information on strategies for primary and tertiary prevention of sight-threatening AMD.

This volume on Omega–3 Fatty Acids, the Brain and Retina consists of papers written by the scientists that have contributed immensely to the field on the role of omega–3 fatty acids in maintaining homeostasis and in the prevention and management of neurodegenerative diseases due to the aging process or genetic predisposition. The new studies on the DHA as a precursor of resolvins and protectins is a very exciting area that should continue to advance our knowledge of EPA + DHA in the brain and retina. Therefore, this volume should be of interest to psychologists, physiologists, neuroscientists, psychiatrists, ophthalmologists, geneticists, neurologists, pediatricians, obstetricians, geriatricians, and other physicians, as well as nutritionists, dieticians, and policymakers.

Artemis P. Simopoulos, Washington, D.C.

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Omega–6/Omega–3 Essential Fatty Acids: Biological Effects

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The interaction of genetics and environment, nature, and nurture is the foundation for all health and disease. In the last two decades, using the techniques of molecular biology, it has been shown that genetic factors determine susceptibility to disease and environmental factors determine which genetically susceptible individuals will be affected [1–6]. Nutrition is an environmental factor of major importance. Using the tools of molecular biology and genetics, research is defining the mechanisms by which genes influence nutrient absorption, metabolism and excretion, taste perception, and degree of satiation, and the mechanisms by which nutrients influence gene expression. Whereas major changes have taken place in our diet over the past 10,000 years since the beginning of the Agricultural Revolution, our genes have not changed. The spontaneous mutation rate for nuclear DNA is estimated at 0.5% per million years. Therefore, over the past 10,000 years there has been time for very little change in our genes, perhaps 0.005%. In fact, our genes today are very similar to the genes of our ancestors during the Paleolithic period 40,000 years ago, at which time our genetic profile was established [7]. Humans today live in a nutritional environment that differs from that for which our genetic constitution was selected. Studies on the evolutionary aspects of diet indicate that major changes have taken place in our diet, particularly in the type and amount of essential fatty acids (EFA) and in the antioxidant content of foods [7–11] (fig. 1).

Today, industrialized societies are characterized by (1) an increase in energy intake and decrease in energy expenditure; (2) an increase in saturated fat, omega–6 fatty acids and *trans* fatty acids, and a decrease in omega–3 fatty acid intake; (3) a decrease in complex carbohydrates and fiber; (4) an increase in cereal grains and a decrease in fruits and vegetables, and (5) a decrease in protein, antioxidants and calcium intake [7, 9, 12–16] (tables 1, 2). The increase in *trans* fatty acids is detrimental to health as shown in table 3 [17]. In addition, *trans* fatty acids interfere with the desaturation and elongation of both omega–6 and omega–3 fatty acids, thus further decreasing the

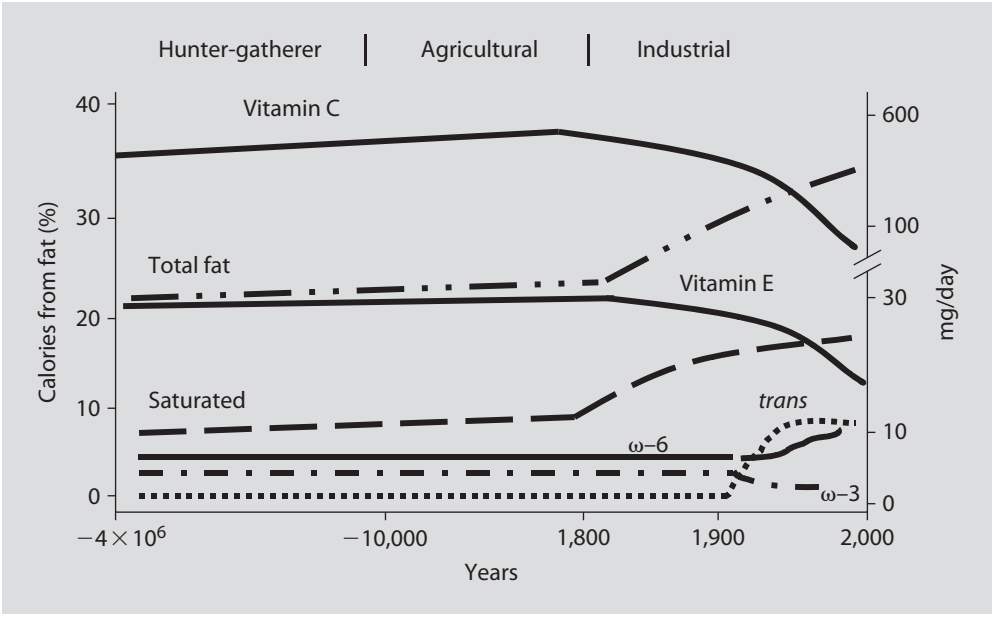

Fig. 1. Hypothetical scheme of fat, fatty acid (ω–6, ω–3, *trans* and total) intake (as percent of calories from fat) and intake of vitamins E and C (mg/day). Data were extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative changes during the preceding 100 years [9].

amount of arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) availability for human metabolism [18].

The beneficial health effects of omega–3 fatty acids, EPA and DHA were described first in the Greenland Eskimos who consumed a high seafood diet and had low rates of coronary heart disease, asthma, type 1 diabetes mellitus, and multiple sclerosis. Since that observation, the beneficial health effects of omega–3 fatty acids have been extended to include benefits related to cancer, inflammatory bowel disease, rheumatoid arthritis, and psoriasis [19], as well as depression and other mental illnesses [20–22]. A balanced intake of omega–6 and omega–3 fatty acids is needed for homeostasis and normal development throughout the life cycle.

Imbalance of Omega–6/Omega–3

Food technology and agribusiness provided the economic stimulus that dominated the changes in the food supply [23, 24]. From per capita quantities of foods available for consumption in the US national food supply in 1985, the amount of EPA is reported to be about 50 mg per capita/day and the amount of DHA is 80 mg per capita/day. The two main sources are fish and poultry [25]. It has been estimated that the present Western diet is 'deficient' in omega–3 fatty acids with a ratio of omega–6

Table 1. Estimated omega–3 and omega–6 fatty acid intake in the late Paleolithic period (g/day)[1,2]

Plants	
LA	4.28
ALA	11.40
Animals	
LA	4.56
ALA	1.21
Total	
LA	8.84
ALA	12.60
Animals	
AA (ω–6)	1.81
EPA (ω–3)	0.39
DTA (ω–6)	0.12
DPA (ω–3)	0.42
DHA (ω–3)	0.27
Ratios of ω–6/ω–3	
LA/ALA	0.70
AA+DTA/EPA+DPA+DHA	1.79
Total ω–6/ω–3	0.79[b]

LA = Linoleic acid; ALA = linolenic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid; DTA = docosatetranoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.
[1]Data from Eaton et al. [13].
[2]Assuming an energy intake of 35:65 of animal:plant sources.

Table 2. Late Paleolithic and currently recommended nutrient composition for Americans

	Late Paleolithic	Current recommendations
Total dietary energy, %		
Protein	33	12
Carbohydrate	46	58
Fat	21	30
Alcohol	~0	–
P/S ratio	1.41	1.00
Cholesterol, mg	520	300
Fiber, g	100–150	30–60
Sodium, mg	690	1,100–3,300
Calcium, mg	1,500–2,000	800–1,600
Ascorbic acid, mg	440	60

Modified from Eaton et al. [13]. P/S = Polyunsaturated to saturated fat.

Table 3. Adverse effects of *trans* fatty acids [modified from 17]

Decrease or inhibit
Decrease or inhibit incorporation of other fatty acids into cell membranes
Decrease high-density lipoprotein (HDL)
Inhibit Δ–6 desaturase (interfere with elongation and desaturation of essential fatty acids)
Decrease serum testosterone (in male rats)
Cross the placenta and decrease birth weight (in humans)
Increase
Low-density lipoprotein (LDL)
Platelet aggregation
Lipoprotein (a) [Lp(a)]
Body weight
Cholesterol transfer protein (CTP)
Abnormal morphology of sperm (in male rats)

Table 4. Ratios of dietary omega–6:omega–3 fatty acids in the late Paleolithic period and in current Western diets (USA) (g/day)

	Paleolithic	Western
LA : ALA	0.70	18.75
AA + DTA : EPA + DPA + DHA	1.79	3.33
Total	0.79	16.74

LA = Linoleic acid; ALA = linolenic acid; AA = arachidonic acid; EPA = eicosapentaenoic acid; DTA = docosatetranoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.
Reprinted with permission from reference 15.

to omega–3 of 15–20/1, instead of 1/1 as is the case with wild animals and presumably human beings [7–11, 13, 26–28] (table 4).

An absolute and relative change of omega–6/omega–3 in the food supply of Western societies has occurred over the last 150 years. A balance existed between omega–6 and omega–3 for millions of years during the long evolutionary history of the genus *Homo*, and genetic changes occurred partly in response to these dietary influences. During evolution, omega–3 fatty acids were found in all foods consumed: meat, wild plants, eggs, fish, nuts and berries [29–38]. Studies by Cordain et al. [39] on wild animals confirm the original observations of Crawford [27] and Sinclair et al. [40]. However, rapid dietary changes over short periods of time as have occurred over the past 100–150 years is a totally new phenomenon in human evolution [13, 15, 41–43] (table 5).

Table 5. Omega–6:omega–3 ratios in various populations

Population	ω–6/ω–3	Ref.
Paleolithic	0.79	13
Greece prior to 1960	1.00–2.00	15
Current Japan	4.00	41
Current India, rural	5–6.1	42
Current UK and Northern Europe	15.00	43
Current USA	16.74	13
Current India, urban	38–50	42

Biological Effects and the Omega–6/Omega–3 Ratio

There are two classes of EFA – omega–6 and omega–3. The distinction between omega–6 and omega–3 fatty acids is based on the location of the first double bond, counting from the methyl end of the fatty acid molecule. In the omega–6 fatty acids, the first double bond is between the 6th and 7th carbon atoms and for the omega–3 fatty acids the first double bond is between the 3rd and 4th carbon atoms. Monounsaturates are represented by oleic acid, an omega–9 fatty acid, which can be synthesized by all mammals including humans. Its double bond is between the 9th and 10th carbon atoms.

Omega–6 and omega–3 fatty acids are essential because humans, like all mammals, cannot make them and must obtain them in their diet. Omega–6 fatty acids are represented by linoleic acid (LA; 18:2ω–6) and omega–3 fatty acids by α-linolenic acid (ALA; 18:3ω–3). LA is plentiful in nature and is found in the seeds of most plants except for coconut, cocoa, and palm. ALA on the other hand is found in the chloroplasts of green leafy vegetables, and in the seeds of flax, rape, chia, perilla and in walnuts. Both EFA are metabolized to longer-chain fatty acids of 20 and 22 carbon atoms. LA is metabolized to AA (20:4ω–6), and LNA to EPA (20:5ω–3) and DHA (22:6ω–3), increasing the chain length and degree of unsaturation by adding extra double bonds to the carboxyl end of the fatty acid molecule (fig. 2).

Humans and other mammals, except for carnivores such as lions, can convert LA to AA and ALA to EPA and DHA, but it is slow [44]. This conversion was shown by using deuterated ALA [45]. There is competition between omega–6 and omega–3 fatty acids for the desaturation enzymes. However, both delta–4 and delta–6 desaturases prefer omega–3 to omega–6 fatty acids [44, 46, 47]. But, a high LA intake interferes with the desaturation and elongation of ALA [45, 48]. *Trans* fatty acids interfere with the desaturation and elongation of both LA and ALA. Delta–6 desaturase is the limiting enzyme and there is some evidence that it decreases with age [44]. Premature infants [49], hypertensive individuals [50], and some diabetics [51] are limited in their ability to make EPA and DHA from ALA. These findings are important and need to

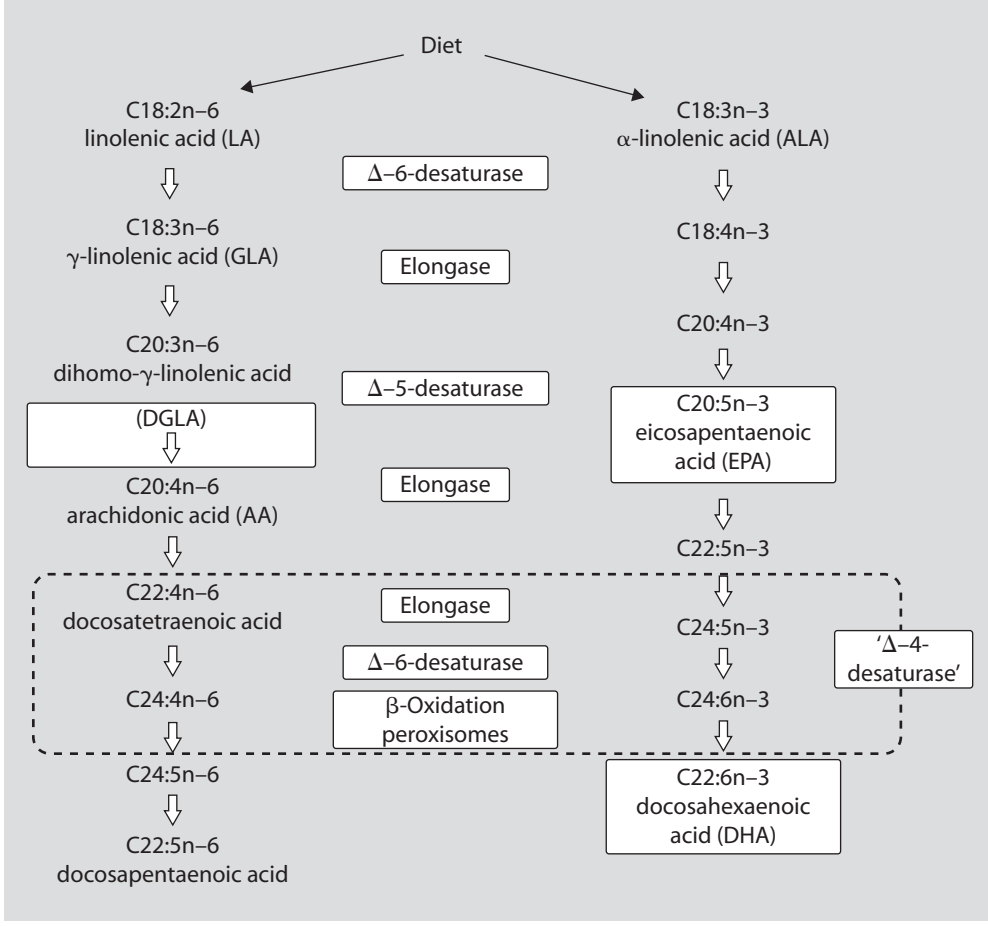

Fig. 2. Elongation and desaturation of omega–6 and omega–3 PUFAs.

be considered when making dietary recommendations. EPA and DHA are found in the oils of fish, particularly fatty fish. AA is found predominantly in the phospholipids of grain-fed animals and eggs.

LA, ALA, and their long-chain derivatives are important components of animal and plant cell membranes. In mammals and birds, the omega-3 fatty acids are distributed selectively among lipid classes. ALA is found in triglycerides, in cholesteryl esters, and in very small amounts in phospholipids. EPA is found in cholesteryl esters, triglycerides, and phospholipids. DHA is found mostly in phospholipids. In mammals, including humans, the cerebral cortex, retina, and testis and sperm are particularly rich in DHA. DHA is one of the most abundant components of the brain's structural lipids. DHA, like EPA, can be derived only from direct ingestion or by synthesis from dietary EPA or ALA.

Mammalian cells cannot convert omega–6 to omega–3 fatty acids because they lack the converting enzyme, delta–3 desaturase. LA, the parent omega–6 fatty acid, and

Table 6. Effects of ingestion of EPA and DHA from fish or fish oil

- Decreased production of prostaglandin E_2 (PGE_2) metabolites
- A decrease in thromboxane A_2, a potent platelet aggregator and vasoconstrictor
- A decrease in leukotriene B_4 formation, an inducer of inflammation, and a powerful inducer of leukocyte chemotaxis and adherence
- An increase in thromboxane A_3, a weak platelet aggregator and weak vasoconstrictor
- An increase in prostacyclin PGI_3, leading to an overall increase in total prostacyclin by increasing PGI3 without a decrease in PGI_2, both PGI_2 and PGI_3 are active vasodilators and inhibitors of platelet aggregation
- An increase in leukotriene B_5, a weak inducer of inflammation and a weak chemotactic agent

ALA, the parent omega–3 fatty acid, and their long-chain derivatives are important components of animal and plant cell membranes (fig. 2). These two classes of EFA are not interconvertible, are metabolically and functionally distinct, and often have important opposing physiological functions. When humans ingest fish or fish oil, the EPA and DHA from the diet partially replace the omega–6 fatty acids, especially AA, in the membranes of probably all cells, but especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, and liver cells [reviewed in 8, 52]. Whereas cellular proteins are genetically determined, the polyunsaturated fatty acid (PUFA) composition of cell membranes is to a great extent dependent on the dietary intake. AA and EPA are the parent compounds for eicosanoid production [8] (tables 6, 7; fig. 3).

Because of the increased amounts of omega–6 fatty acids in the Western diet, the eicosanoid metabolic products from AA, specifically prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids, and lipoxins, are formed in larger quantities than those formed from omega–3 fatty acids, specifically EPA [8]. The eicosanoids from AA are biologically active in very small quantities and, if they are formed in large amounts, they contribute to the formation of thrombus and atheromas; to allergic and inflammatory disorders, particularly in susceptible people, and to proliferation of cells. Thus, a diet rich in omega–6 fatty acids shifts the physiological state to one that is prothrombotic and proaggregatory, with increases in blood viscosity, vasospasm, and vasoconstriction and decreases in bleeding time. Bleeding time is decreased in groups of patients with hypercholesterolemia, hyperlipoproteinemia, myocardial infarction, other forms of atherosclerotic disease, and diabetes (obesity and hypertriglyceridemia). Bleeding time is longer in women than in men and longer in young than in old people. There are ethnic differences in bleeding time that appear to be related to diet.

Newly Identified Lipid Mediators: Lipoxins, Resolvins and Protectins

Recent studies have shown that additional lipid mediators are produced from AA, EPA and DHA with potent anti-inflammatory properties [53]. Lipoxins are derived

Table 7. Effects of omega–3 fatty acids on factors involved in the pathophysiology of atherosclerosis and inflammation

Factor	Function	Effect of ω–3 fatty acid
Arachidonic acid	Eicosanoid precursor; aggregates platelets; stimulates white blood cells	↓
Thromboxane A_2	Platelet aggregation; vasoconstriction; increase of intracellular Ca^{2+}	↓
Prostacyclin ($PGI_2/_3$)	Prevent platelet aggregation; vasodilation; increase cAMP	↑
Leukotriene (LTB_4)	Neutrophil chemoattractant; increase of intracellular Ca^{2+}	↓
Fibrinogen	A member of the acute phase response and a blood clotting factor	↓
Tissue plasminogen activator	Increase endogenous fibrinolysis	↑
Platelet-activating factor (PAF)	Activates platelets and white blood cells	↓
Platelet-derived growth factor (PDGF)	Chemoattractant and mitogen for smooth muscles and macrophages	↓
Oxygen free radicals	Cellular damage; enhance LDL uptake via scavenger pathway; stimulate arachidonic acid metabolism	↓
Lipid hydroperoxides	Stimulate eicosanoid formation	↓
Interleukin-1 and tumor necrosis factor	Stimulate neutrophil O_2 free radical formation; stimulate lymphocyte proliferation; stimulate PAF; express intercellular adhesion molecule-1 on endothelial cells; inhibit plasminogen activator, thus, procoagulants	↓
Interleukin-6	Stimulates the synthesis of all acute phase proteins involved in the inflammatory response: C-reactive protein; serum amyloid A; fibrinogen; α1-chymotrypsin, and haptoglobin	↓
C-reactive protein (CRP)	An acute phase reactant and an independent risk factor for cardiovascular disease	↓
Endothelial-derived relaxation factor	Reduces arterial vasoconstrictor response	↑
Insulin function		Increases sensitivity to insulin
VLDL	Related to LDL and HDL level	↓
HDL	Decreases the risk for coronary heart disease	↑
Lp(a)	Lipoprotein(a) is a genetically determined protein that has atherogenic and thrombogenic properties	↓
Triglycerides and chylomicrons	Contribute to postprandial lipemia	↓

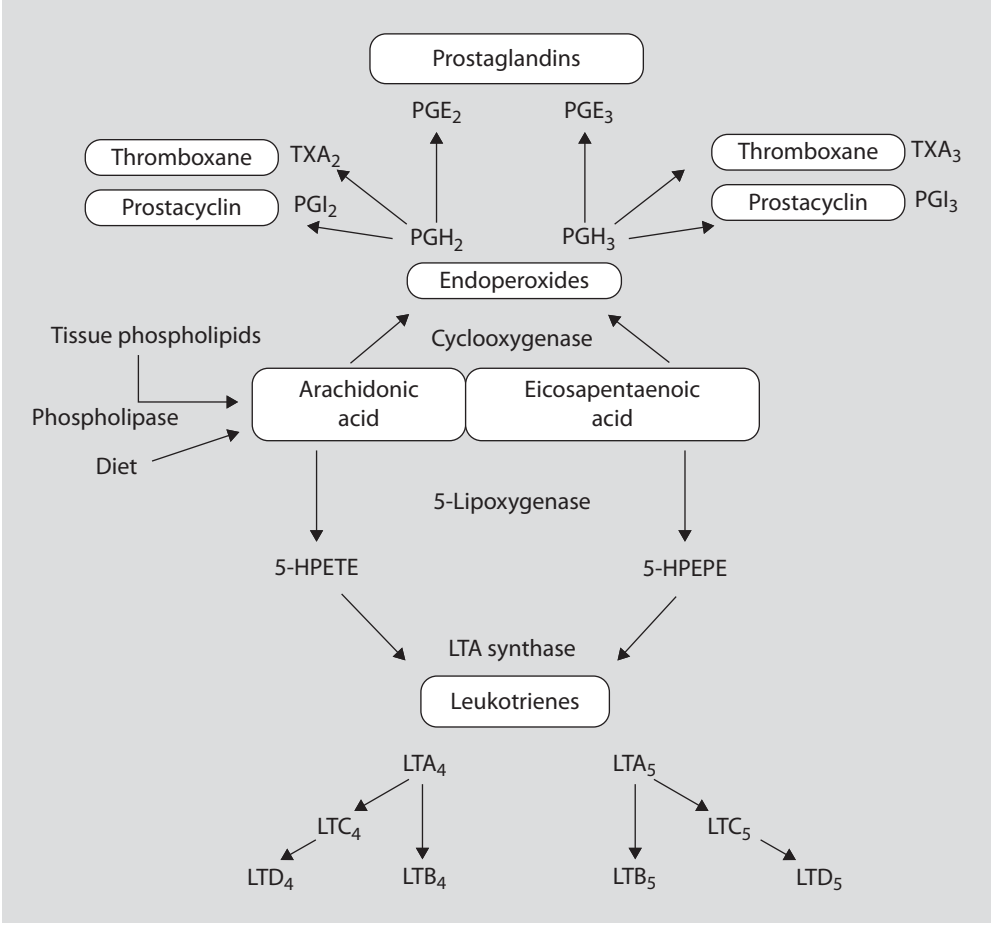

Fig. 3. Oxidative metabolism of AA and EPA by the cyclooxygenase and 5-lipoxygenase pathways. 5-HPETE = 5-Hydroperoxyeicosatetranoic acid, 5-HPEPE = 5-hydroxyeicosapentaenoic acid.

from AA as a result of cell-cell interaction and the sequential transformation by different lipoxygenases. Leukocyte 5-lipoxygenase generates LT4 from AA, which is then transformed to the lipoxin LXA4 in platelets by the oxidase activity of their 12-lipoxygenase. In addition to their anti-inflammatory properties, lipoxins have potent pro-resolution properties, inhibit the formation of inflammatory cytokines, immune cell proliferation and migration. In the presence of aspirin, the acetylation of cyclooxygenase-2 enables it to act as a lipoxygenase forming the lipoxin precursor 15-hydroxyeicosatetraenoic acid from AA, which is then transformed by leukocyte 5-lipoxygenase to 15-epi-LXA4 or 15-epi-LXB4 referred to as aspirin-triggered lipoxins. These aspirin-triggered lipoxins seem to be more potent anti-inflammatory agents than the conventional LX4 [54].

In analogy to the aspirin-triggered lipoxins from AA, bioactive mediators are also produced from EPA + DHA. Serhan and his group [53] used lipidomics and

informatics in studies on EPA and DHA metabolites in the resolution of inflammation which are called resolvins. The resolvin from EPA is RvE1. RvE1 inhibits nuclear factor κB by tumor necrosis factor-α (TNF-α). ChemR23 is the receptor for RvE1 and it is a specific G-protein-coupled receptor. Transcription of ChemR23 is found in cardiovascular, gastrointestinal renal, brain, and myeloid tissue. The ChemR23 shares homology with the receptor identified for the AA-derived aspirin-triggered lipoxins but is molecularly distinct.

Resolvins are derived from EPA and DHA with two chemically unique structural forms, the E-series and the D-series respectively. Resolvin E1 is produced in healthy individuals and is increased in the plasma of individuals taking aspirin and or EPA.

DHA is the substrate for two groups of resolvins produced by different biosynthetic routes, referred to as the 17S- and 17RD-series resolvins during the resolution of inflammatory exudates. D-series resolvins have potent anti-inflammatory actions and are particularly interesting because the brain, synapses and retina are highly enriched in DHA. The D-series resolvins are of interest in the control of inflammation resolution in host defense and in neural tissues.

Another class of lipid mediators produced from DHA are the 10,17S-docosatriene, now known as protectins. When produced by neural tissues is termed neuroprotectin D1 because of its biosynthetic origin. Protectin D1 blocks T-cell migration in vivo, reduces TNF and interferon-γ (IFN-γ) secretion and promotes T-cell apoptosis.

Resolvin E1 and protectin D1 derived from EPA + DHA respectively are potent resolution agonists that activate cell type, neutrophils, macrophages, and epithelial cells to accelerate resolution.

Lipoxins, resolvins and protectins have potent multilevel mechanisms of action in disease models and promote resolution in animal models of oral, lung, ocular, kidney, skin and gastrointestinal inflammation, as well as in ischemia-reperfusion injury and angiogenesis.

Lipoxins, resolvin E1 and protectins act on T cells, dendritic cells and phagocytic cells, therefore they represent a link between the innate and the immune system. Considering that lipoxins, resolvin E1, and protectins are produced from AA, EPA + DHA, it follows that these fatty acids represent a molecular link between the two systems.

Inflammation is at the base of many chronic diseases such as cardiovascular disease, obesity, diabetes, arthritis, mental illnesses and cancer, as well as many autoimmune diseases. These diseases are characterized by increased amounts of IL-1 and IL-6. Increased dietary intake of omega–6 fatty acids is associated with higher levels of TXA_2 and LTB_4. LTB_4 is a proinflammatory AA metabolite that along with IL-1 and IL-6 contributes to inflammation. The discovery of the newly identified mediators lipoxins, resolvins, protectins and neuroprotectins form AA (lipoxins) EPA (resolvins) and DHA (protectins and neuroprotectins) indicate that the resolution of inflammation is not just a passive termination of inflammation, but rather an active biochemical and metabolic process. These families of endogenous pro-resolution molecules

are not immunosuppressive but instead function in the resolution of inflammation by activating specific mechanisms to promote homeostasis [54].

The Balance of Omega–6/Omega–3 Fatty Acids Is Important for Health: Evidence from Gene Transfer Studies

Further support for the need to balance the omega–6/omega–3 EFA comes from the studies of Kang et al. [55, 56], which clearly show the ability of both normal rat cardiomyocytes and human breast cancer cells in culture to form all the omega–3s from omega–6 fatty acids when fed the cDNA encoding omega–3 fatty acid desaturase obtained from the roundworm *Caenorhabditis elegans*. The omega–3 desaturase efficiently and quickly converted the omega–6 fatty acids that were fed to the cardiomyocytes in culture to the corresponding omega–3 fatty acids. Thus, omega–6 LA was converted to omega–3 ALA and AA was converted to EPA, so that at equilibrium, the ratio of omega–6 to omega–3 PUFA was close to 1/1. Further studies demonstrated that the cancer cells expressing the omega–3 desaturase underwent apoptotic death whereas the control cancer cells with a high omega–6/omega–3 ratio continued to proliferate [57]. More recently, Kang et al. [58–60] showed that transgenic mice and pigs expressing the *C. elegans fat-1* gene encoding an omega–3 fatty acid desaturase are capable of producing omega–3 from omega–6 fatty acids, leading to enrichment of omega–3 fatty acids with reduced levels of omega–6 fatty acids in almost all organs and tissues, including muscles and milk, with no need of dietary omega–3 fatty acid supply. This discovery provides a unique tool and new opportunities for omega–3 research, and raises the potential of production of *fat-1* transgenic livestock as a new and ideal source of omega–3 fatty acids to meet the human nutritional needs. Furthermore, the transgenic mouse model is being used widely by scientists for the study of chronic diseases and for the study of mechanisms of the beneficial effects of omega–3 fatty acids [61]. The fat-1 transgenic mice produce and store higher levels of EPA + DHA in their tissues than wild-type mice, and as a result generate increased levels of resolvins and protectins.

Omega–3 Fatty Acids and Gene Expression

Previous studies have shown that fatty acids released from membrane phospholipids by cellular phospholipases, or made available to the cell from the diet or other aspects of the extracellular environment, are important cell signaling molecules. They can act as second messengers or substitute for the classical second messengers of the inositide phospholipid and the cyclic AMP signal transduction pathways. They can also act as modulator molecules mediating responses of the cell to extracellular signals. Recently it has been shown that fatty acids rapidly and directly alter the transcription

of specific genes [62]. In the case of genes involved in inflammation, such as IL-1β, EPA and DHA suppress IL-1β mRNA whereas AA does not, and the same effect appears in studies on growth-related early response gene expression and growth factor [62]. In the case of vascular cell adhesion molecule (VCAM), AA has a modest suppressing effect relative to DHA. The latter situation may explain the protective effect of fish oil toward colonic carcinogenesis, since EPA and DHA did not stimulate protein kinase C. PUFA regulation of gene expression extends beyond the liver and includes genes such as adipocyte glucose transporter-4, lymphocyte stearoyl-CoA desaturase-2 in the brain, peripheral monocytes (IL-1β and VCAM-1) and platelets [platelet-derived growth factor (PDGF)]. Whereas some of the transcriptional effects of PUFA appear to be mediated by eicosanoids, the PUFA suppression of lipogenic and glycolytic genes is independent of eicosanoid synthesis, and appears to involve a nuclear mechanism directly modified by PUFA.

Mental Health and Eye Disease

Psychologic stress in humans induces the production of proinflammatory cytokines such as IFN-γ, TNF-α, IL-6 and IL-1. An imbalance of omega–6 and omega–3 PUFA in the peripheral blood causes an overproduction of proinflammatory cytokines. There is evidence that changes in fatty acid composition are involved in the pathophysiology of major depression [63]. Changes in serotonin (5-HT) receptor number and function caused by changes in PUFA provide the theoretical rationale connecting fatty acids with the current receptor and neurotransmitter theories of depression [64–68]. The increased C20:4ω–6/C20:5ω–3 ratio and the imbalance in the omega–6/omega–3 PUFA ratio in major depression may be related to the increased production of proinflammatory cytokines and eicosanoids in that illness [66]. There are a number of studies evaluating the therapeutic effect of EPA and DHA in major depression. Stoll and colleagues [69, 70] have shown that EPA and DHA prolong remission, that is, reduce the risk of relapse in patients with bipolar disorder.

Kiecolt-Glaser et al. [71] studied depressive symptoms, omega–6/omega–3 fatty acid ratio and inflammation in older adults. As the dietary ratio of omega–6/omega–3 increased, the depressive symptoms, TNF-α, IL-6, and IL-6 soluble receptor (sIL-6r) increased. The authors concluded that diets with a high omega–6/omega–3 ratio may enhance the risk for both depression and inflammatory diseases.

Dry eye syndrome (DES) is one of the most prevalent conditions. Inflammation of the lacrimal gland, the meibomian gland, and the ocular surface plays a significant role in DES [72, 73]. An increased concentration of inflammatory cytokines, such as IL-1, IL-6, and TNF-α, has been found in tear film in patients with DES [74]. Miljanovic et al. [75] investigated the relation of dietary intake of omega–3 fatty acids and the ratio of omega–6 to omega–3 with DES incidence in a large population of women participating in the Women's Health Study. A higher ratio of omega–6/

omega–3 consumption was associated with a significantly increased risk of DES (OR 2.51; 95% CI 1.13, 5.58) for >15:1 vs. <4.1 (p for trend = 0.01). These results suggest that a higher dietary intake of omega–3 fatty acids is associated with a decreased incidence of DES in women and a high omega–6/omega–3 ratio is associated with a greater risk.

Age-related macular degeneration (AMD) is the leading cause of vision loss among people 65 and older. Both AMD and cardiovascular disease share similar modifiable factors [76–80]. Fish intake has been reported to have protective properties in lowering the risk of AMD [81, 85], especially when LA intake was low [81, 82]. In a study involving twins, Seddon et al. [86] showed that fish consumption and omega–3 fatty acid intake reduce the risk of AMD whereas cigarette smoking increases the risk for AMD.

Conclusions and Recommendations

Western diets are characterized by high omega–6 and low omega–3 fatty acid intake, whereas during the Paleolithic period when human's genetic profile was established, there was a balance between omega–6 and omega–3 fatty acids. Therefore, humans today live in a nutritional environment that differs from that for which our genetic constitution was selected.

Both omega–6 and omega–3 fatty acids influence gene expression. The balance of omega–6/omega–3 fatty acids is an important determinant in maintaining homeostasis, normal development, and mental health throughout the life cycle.

The new lipid mediators from AA, EPA + DHA such as lipoxins, resolvins, and protectins are potent pro-resolution and anti-inflammatory agents and appear to be the molecular link between the innate and adaptive immune systems, indicating the importance of nutrition in maintaining homeostasis and the need to decrease omega–6 fatty acid intake while increasing omega–3 fatty acid intake.

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Depression, Suicide and Deficiencies of Omega–3 Essential Fatty Acids in Modern Diets

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Background

Optimal brain function requires the ready availability of brain critical nutrients, especially during periods of extreme stress. Deficiencies of brain-specific nutrients, in addition to inadequate calories, may not only substantially contribute to suboptimal cognition and alertness, but may manifest as severe psychiatric disorders including adjustment disorders, major depression, impulsive violence and suicide. The mammalian brain is primarily composed of fats and lipids, usually 60% wet weight. Approximately 30% of fatty acid pool cannot be made de novo and must be obtained through the diet and are thus termed essential fatty acids. Seafood, fish oils and fortified foods are rich sources of the long-chain ω–3 fatty acids (ω–3 LCFAs: eicosapentaenoic (EPA), docosapentaenoic (DPAω–3), and docosahexaenoic acids (DHA)). DHA is selectively concentrated in neuronal membranes comprising 14% of total fatty acids. Plausible biological mechanisms linking dietary deficiencies of ω–3 LCFAs with psychiatric illness include: depletion of serotonin and dopamine levels by 50% in animal models [1], impaired neuronal migration, connectivity, timed apoptosis, and dendritic arborization, such that there is an irreversible disruption in the neuronal pathways that regulate behavior [2] neuroinflammatory processes and dysregulation of the hypothalamic pituitary adrenal axis [3].

This work does not represent any policy or position of the National Institutes of Health and is solely the scientific opinion of the author.

Defining Deficiency

The absence of signs of deficiency related to critical target tissues has been considered in formulation of required dietary intakes. DHA is selectively concentrated in synaptic neuronal membranes and, comprises nearly 14% of all brain fatty acids and is necessary for optimal neurological function [4]. Thus, deficit intakes of DHA or ω–3 LCFAs supporting DHA are likely to manifest as signs or symptoms of neural dysfunction including neurodevelopmental and neuropsychiatric impairments. Neurodevelopmental impairments are identified as signs and symptoms of deficiency in setting the dietary reference intakes (DRIs) [5] for biotin, folate, iodine and iron. Psychiatric and neurocognitive impairments are also identified as signs and symptoms of deficiency in setting the DRIs [5] for vitamin B_6; depression and confusion, vitamin B_{12}; mood changes, confusion, insomnia and cognitive impairments, biotin; depression, lethargy and hallucinations, folate; irritability and difficulty concentrating, niacin; depression and apathy, pantothenic acid; irritability, restlessness, apathy and malaise, thiamin; apathy, irritability, confusion decreased short-term memory, iodine; hypothyroidism and learning impairments and iron; impaired cognition and decreased work capacity. Severe symptoms of major depression may be used as a categorical diagnosis and can potentially be used to calculate an estimated average requirement. Major depression is commonly a chronic degenerative disease. Neurodevelopmental and neuropsychiatric impairments are clearly identified by the Food and Nutrition Board as signs and symptoms of deficiencies for several nutrients [5], thus setting a precedent potentially applicable to ω–3 LCFA's deficiencies in similar outcome parameters.

The viewpoints of Sir Austin Bradford Hill [6] are routinely evaluated in assessing the strength of the body of epidemiological and other evidence for possible relationships to causality. We posit that DHA and ω–3 LCFA deficiencies are causally related to an increase risk of psychiatric disorders, specifically major depression. Evidence linking deficiencies in ω–3 LCFAs in psychiatric disorders has recently been reviewed in a UK Parliamentary Inquiry Report on Nutrients and Mental Health [7] and by the American Psychiatric Association in the treatment recommendations issued for ω–3 LCFAs in psychiatric patients [8]. Both find consistent data across ecological cross-national studies, epidemiological studies, case-control comparisons of blood and brain tissues, in double-blind randomized placebo-controlled trials and meta-analyses of these trials, that low fish consumption or low ω–3 body compositional status increases risk of depression and other affective illnesses. Here we assess in detail ecological, epidemiological dietary intake data, blood and tissue composition data and randomized clinical intervention trials for evidence of causal links between low intakes of ω–3 fatty acid and risk of significant depressive symptoms. Data linking low intakes of ω–3 LCFAs to risk of suicide or suicidal ideation is beginning to emerge and shows great promise.

Hill 'Criteria'

Strength

The ecological and epidemiological associations between low seafood and ω–3 LCFA intake are strong. Ecological studies indicate that in comparison to countries with the highest consumption, low seafood consumption is associated with a 65-fold higher risk for lifetime prevalence of major depression ($r = -0.84$, $p < 0.0001$) [9], a 50-fold higher risk for postnatal depression ($r = -0.81$, $p < 0.0001$) [10], a 30-fold higher risk for bipolar spectrum disorder ($r = -0.80$, $p < 0.0003$) [11], and a 10-fold higher risk of death from homicide mortality ($r = -0.63$, $p < 0.0006$) [12]. Correlation coefficients in these ranges are considered to be strong.

Consistency

'Consistent findings observed by different persons in different places with different samples strengthen the likelihood of an effect' [6]. We find a relationship of low ω–3 LCFA status and greater risk of affective illnesses to be consistent across ecological studies, epidemiological studies, case-control studies and biological tissue sample studies. In particular, epidemiological studies have reported strong associations between low seafood intake and greater risk of depression with a high degree of consistency. Among 1,767 subjects in Northern Finland, Tanskanen et al. [13] found that both the risk of being depressed (odds ratio (OR) 0.63; 95% confidence interval (CI) 0.43–0.94; $p = 0.02$) and the risk of having suicidal ideation (OR 0.57; 95% CI 0.35–0.95; $p = 0.03$) were significantly lower among frequent lake-fish consumers compared with more infrequent consumers. In a birth cohort of 5,689 Finnish subjects, the risk of depression was 2.6-fold (95% CI 1.4–5.1) greater and risk of suicidal thinking was 1.5-fold (95% CI 1.0–3.0) greater comparing females with rare fish consumption to regular consumers [14]. Finnish fishermen ($n = 6,410$) consume twice as much fish but have a lower risk of mortality from alcohol-related diseases (OR 0.59; 95% CI 0.41–0.82) and suicides (OR 0.61; 95% CI 0.39–0.91) compared to the general population, after adjustment [15]. In contrast, Hakkarainen et al. [16] found no associations between the dietary intake of ω–3 fatty acids or fish consumption and self-report of depressed mood, hospitalization for a major depressive episode, or suicide among 29,133 Finnish men. However, there was a high covariance with fish and ω–6 linoleate consumption, which was 20-fold higher than ω–3 LCFA from fish [17]. Thus, it is difficult to determine which factor was specifically associated with an increased risk of depression. Among 21,835 Norwegians, users of cod liver oil were significantly less likely to have depressive symptoms than non-users after adjusting for multiple possible confounding factors (OR 0.71; 95% CI 0.52–0.97) [18]. In a longitudinal follow-up study of 13,017 French subjects, subjects consuming fatty fish, or having an ω–3 LCFA intake >1 en%, had significantly reduced risk of single or recurrent depressive episodes [19]. In the Zutphen Study of the Elderly, high intakes of ω–3 LCFAs (mean 407 mg/day) were associated with lower risk of

depressive symptoms (OR 0.46; 95% CI 0.22–0.95) compared with low intakes (21 mg/day) [20]. Among 7,903 Spanish subjects, moderate consumption of fish had a relative risk reduction of >30% [21]. Among 10,602 men from Northern Ireland and France, the greater depressed mood is associated with lower fish intake in a non-linear relationship [22]. A similar non-linear relationship between greater depression and lower fish intake was described among a UK population (n = 2,982) [23]. Jacka et al. [24] also found no association between fish consumption and depression defined by DSM-III criteria in a New Zealand population (n = 755). Murakami et al. [25] found no association between fish intake and lower risk of depressive symptoms among 618 adults. However, the mean ω–3 LCFA intake was approximately 0.37 en% far above the putative estimated average requirements and recommended dietary allowances presented here, thus the majority of the population may have adequate intakes. We conclude that epidemiological studies based on reports of dietary intakes have found an association between low ω–3 LCFA intake and significant depressive symptoms with good consistency.

Specificity

Tissue compositional studies have fairly consistently reported a lower ω–3 LCFA status and/or a higher ω–6 LCFA status among depressed subjects. The most specific evidence of tissue compositional deficits is the finding that DHA was 22% lower in the postmortem orbitofrontal cortex of patients with a major depressive disorder [26]. A similar deficit of DHA was found in the orbitofrontal cortex of patients with a bipolar disorder [27]. The pathophysiology of depressive disorders is thought to involve deficits in orbitofrontal cortex function [34]. These specific deficits in brain composition, combined with the epidemiologically based tissue compositional studies, indicate that deficits of ω–3 LCFA, in particular EPA and DHA, are associated with depressive illnesses. Adams et al. [28] found a significant positive correlation between the severity of depression and both the erythrocyte phospholipid arachidonic acid (AA) and EPA ratio and erythrocyte EPA alone. Maes et al. [29] found lower ω–3 LCFAs in serum phospholipids and cholesteryl esters of depressed patients compared to controls. Edwards et al. [30] reported lower EPA and DHA concentrations in the erythrocytes of depressed compared to control subjects. They also noted a biological gradient in which lower erythrocyte DHA correlated with greater severity of symptoms ($r = 0.80$, $p < 0.01$). Peet et al. [31] also reported a nearly 50% reduction in DHA in the erythrocytes of depressed subjects. Among a community sample of the elderly in Bordeaux, plasma EPA alone was inversely associated with severity of depressive symptoms [32]. Among adolescents in Crete (n = 90), depressive symptoms were negatively associated with EPA and positively associated with the ω–6 fatty acid dihomo-γ-linolenic acid in adipose tissue [33]. Among 247 healthy males in Crete, mildly depressed subjects had significantly reduced (–34.6%) adipose tissue DHA levels compared to non-depressed subjects. Multiple linear regression analysis indicated that depression related negatively to adipose

tissue DHA levels [34]. Among 3,884 elderly subjects in Rotterdam, ω–3 LCFAs were significantly lower (5.2 vs. 5.9%, p = 0.02) and ratios of ω–6 to ω–3 LCFAs were higher (7.2 vs. 6.6, p = 0.01) comparing subjects with depressive disorders to controls [35]. Among a US community sample (n = 207), higher plasma AA and lower EPA concentrations were associated with greater depression and neuroticism [36] and erythrocyte ω–3 LCFAs are decreased in patients with social anxiety disorder [37]. EPA levels in erythrocytes were significantly lower in suicide attempters than those of the control subjects [38]. When the highest and lowest quartiles of EPA in RBCs were compared, the ORs of suicide attempt was 0.12 in the highest quartile (95% CI 0.04–0.36, p for trend = 0.0001) after adjustment for possible confounding factors [38]. A bias against publications failing to find tissue compositional differences may exist, however most published studies indicate a lower ω–3 LCFA body composition status among depressed subjects.

Depression Associated with Other Medical Disorders

Compared to controls, depressed patients with acute coronary syndromes had significantly lower concentrations of total ω–3 and DHA, and higher ratios of AA/DHA and AA/EPA [39]. A second study of patients with acute coronary syndromes reported higher depression severity scores which were significantly associated with lower DHA levels, with similar but non-significant trends observed for EPA and total ω–3 LCFA levels [40]. Consistent with these reports, Schins et al. [41] found higher plasma AA/EPA ratios comparing depressed to non-depressed subjects among 50 postmyocardial infarction patients. Kobayakawa et al. [42] found no differences comparing depressed and non-depressed lung cancer patients, but used a very low cut point to define depression. No differences in ω–3 LCFA compositions were found comparing depressed and non-depressed patients with multiple sclerosis [43].

Temporality

Deficits in ω–3 LCFA tissue status can be caused by either low intakes of ω–3 LCFA and/or excessive intakes of competing ω–6 fatty acids, for example greater dietary intakes of ω–6 linoleic acid, ranging from 1 to 8 en%, results in 10-fold lower tissue concentrations of ω–3 LCFA [44]. Strong temporal relationships have been reported between increasing intakes of the ω–6 fatty acid, linoleic acid and greater prevalence rates of major depression [45] and homicide mortality in five different countries between 1960 and 2000 (20-fold higher risk, r = 0.94, p < 0.0001) [46].

Biological Gradient

A biological gradient in the reduction of risk of depression is evident in ecological studies and epidemiological studies. Progressively greater exposure to ω–3 LCFA from fish consumption generally leads to a progressively lower incidence of

psychiatric symptoms and illness as reviewed above. Direct compositional analyses of tissue composition usually report a similar biological gradient, as reviewed above. In ecological and epidemiological studies, negative exponential equations consistently best describe the biological gradient relationships [44].

Plausibility

DHA is a nutrient required for neurological development and cannot be substituted by any other molecule [4, 47]. Studies of the multiple interacting mechanisms linking ω–3 LCFA and depressive symptoms have recently been reviewed [2, 48]. Plausible biological mechanisms linking dietary deficiencies of ω–3 LCFAs with psychiatric illness include: depletion of serotonin and dopamine levels by 50% in animal models, impaired neuronal migration, connectivity, timed apoptosis, and dendritic arborization, such that there is an irreversible disruption in the neuronal pathways that regulate behavior [2], neuroinflammatory processes [29, 49] and dysregulation of the hypothalamic pituitary adrenal axis [3]. ω–3 LCFAs may prevent vascular contributions to depression [50]. Inadequate serotonergic and dopaminergic function has long been recognized in the pathophysiology of depression and is the target of most pharmaceutical treatments. Concentrations of serotonin and dopamine were nearly doubled in the frontal cortex of piglets among piglets fed infant formula supplemented with DHA and AA for 18 days [1]. Unconditioned mild stress induced a significant decrease in the tissue levels of serotonin in the frontal cortex, striatum and hippocampus in the range of 40–65%. Interestingly, the ω–3 LCFA supplementation reversed this stress-induced reduction in 5-HT levels and decreased aggressive behavior [51]. One generation of ω–3 LCFA deficiency markedly increased depressive and aggressive behaviors in rats [52]. Consistent with these animal studies, Hibbeln et al. [53] found the lower plasma DHA concentrations were correlated with lower concentrations of the metabolites of both serotonin and dopamine in cerebrospinal fluid among healthy controls. Low cerebrospinal fluid concentrations of these metabolites have been repeatedly reported among suicidal and impulsive patients. Chronic alcohol use, in the context of a low ω–3 diet, depleted DHA levels by 50% in rhesus frontal cortex (from 14 to 7%), suggesting that depression and impulsive behaviors associated with alcohol may be attributable, in part, to depletion of ω–3 LCFAs [54]. Alcohol-induced depletion of neural tissues may contribute to the high rates of violence and depression among alcoholics [45]. We conclude that many known biological mechanisms plausibly link ω–3 LCFA deficiencies to depressive and aggressive pathologies.

Coherence

'Coherence between epidemiological and laboratory findings increases the likelihood of an effect' [6]. One example of the coherence is the role of ω–3 LCFA in relationship to depressive symptoms in pregnancy. We posited that since maternal DHA is selectively transported to the fetus, mothers without sufficient dietary intakes may become depleted, leaving them more vulnerable to depression symptoms during or after

pregnancy [10]. A cross-national ecological study supported this proposition linking both low maternal milk DHA composition and low seafood consumption with higher rates of postnatal depression [10]. In a series of animal experiments, Levant et al. [55–57] have demonstrated depletion of regionally specific brain DHA in a single reproductive cycle, with multiple parity and alterations of dopamine and dopamine-related behaviors. Decreased brain DHA was associated with decreased hippocampal brain-derived neurotrophic factor, increased corticosterone responses to stress and increased immobility on the forced swim test [58]. Otto et al. [59] found that postpartum depression symptoms were associated with a slower recovery of DHA plasma status. A recent randomized controlled trial among pregnant women reported significantly lower depression scores and higher rates of clinical response (62%) to 3.5 g/day of ω–3 fatty acids compared to placebo (27%) [60]. Another randomized controlled trial reported a trend towards efficacy, but was described by the authors as underpowered [61]. Recent non-blinded trials of EPA and DHA supplementation have also reported a reduction of depressive symptoms related to pregnancy of 50% [62, 63]. Two prior epidemiological studies (from New Zealand and Japan) reported no association between seafood consumption and pregnancy-related depressive symptoms. The New Zealand study included only 80 women [64] and consequently did not have sufficient power. Although the Japanese study had much larger numbers (n = 865) it used a cut point of 9+ (rather than 13+) on the Edinburgh Postnatal Depression Scale and almost all the subjects had a relatively high intake of oily fish with very few subjects consuming zero ω–3 from seafood [65]. In contrast, Sontrop and Campbell [66] found low intakes of ω–3 LCFAs from seafood among smoking and single women associated depressive symptoms in pregnancy when more clinically significant cut points were used. In summary, the status of studies in pregnancy-related depression is coherent and promising: animal studies, adequately powered intervention and epidemiological studies do support the proposition that ω–3 LCFAs may have therapeutic benefit.

Experiment

The specificity and efficacy of ω–3 LCFAs in reducing significant depressive symptoms has been assessed in the meta-analyses of randomized placebo-controlled trials. Three recent meta-analyses of up to 11 randomized placebo-controlled trials of ω–3 fatty acids have each reported large treatment effect sizes of ω–3 LCFA in reducing significant depressive symptoms [63, 67, 68]. In 2006 the accumulation of data was sufficient enough for the American Psychiatric Association to issue treatment recommendation for ω–3 LCFAs [63]. Since that time, several confirmatory studies have also been published. Jazayeri et al. [69] reported similar response rates (defined as a 50% reduction in depressive symptoms) comparing patients receiving 1 g of EPA alone (50%) to fluoxetine (56%) but significantly better rates (81%) when patients received both EPA and fluoxetine in combination. Antypa et al. [70] found a reduction of depression-related cognitive symptoms even among healthy controls. Mischoulon et al. [71] reported antidepressant efficacy of DHA 1 g/day, but not at higher doses. Dinan et al. [72] reported

that ω–3 LCFA levels and ratios of EPA to AA predicted clinical responses to antidepressants. Freund-Levi et al. [73] found that reduction of aggressive and depressive symptoms in Alzheimer's patients to ω–3 LCFAs appeared to depend upon their APOEω4 genotype. Some randomized controlled trials have failed to find treatment effects [74–77]. However, Elkin et al. [78] have established that if clinical trials include subjects without a sufficiently high initial severity of depression, antidepressants were unlikely to demonstrate treatment effects because of inadequate power and floor effects inherent in the clinical study of depression [78]. A common feature of the clinical trials that have failed to find antidepressive effects of ω–3 LCFAs has been the enrollment of patients below the cut points of depressive symptom severity established by Elkin et al. [78] with one exception [75]. Thus, insufficiently powered trials should not be considered as definitively negative trials. The demonstration of efficacy in randomized trials appears to depend upon adequate symptom severity, baseline ω–3 LCFA status, perhaps the relative amounts of EPA and DHA, and perhaps allelic variance. The evidence from the currently published placebo-controlled randomized trials and meta-analyses of these trials indicate that ω–3 LCFAs are effective in treating severe depression.

Analogy

Nutritional deficiencies in vitamin B_6, vitamin B_{12}, niacin, folate, pantothenic acid iodine and iron results in reversible neuropsychiatric symptoms. Deficiency symptoms for these nutrients are rarely confined to one organ system. Deficiencies in ω–3 LCFAs may similarly increase risk of several chronic diseases. We have previously proposed that an increased risk of cardiovascular disease and an increased risk of affective disorders are two different manifestations of a common deficiency of ω–3 LCFAs [45].

Suicide Risk, Prevention and Treatment

Epidemiologic data indicate that low fish consumption is a risk factor, but certainly not a sole determinant for suicide mortality. In a 17-year follow-up of 256,118 Japanese subjects [79], those who ate fish daily had a lower risk of death from suicide (OR 0.81; 95% CI 0.27–0.91) compared to subjects eating fish less than daily, but that result was not adjusted for confounding variables. We examined 1,767 subjects in northern Finland and reported that frequent fish consumption (twice per week or more) significantly reduced the risk of reporting depressive symptoms (OR 0.63, $p < 0.03$) and of reporting suicidal thinking (OR 0.57, $p < 0.04$) after adjustment for confounding variables [80]. De Vriese et al. [81] reported that the seasonal variation in ω–3 plasma status closely correlated with the seasonal variation in suicide rates in Belgium.

These epidemiological observations are consistent with the assessment of ω–3 LCFA body compositions directly among patients. Among suicide attempters without depression as a primary diagnosis, low concentrations of plasma EPA alone were robustly

correlated with greater psychopathology on rating scales of impulsivity, guilt, future suicide risk, and most subscales of the Comprehensive Psychopathological Rating Scale [82]. In a case-control study of 200 subjects in Dalian, China, Huan et al. [38] found 30% lower RBC concentrations of EPA among suicide attempters and a dose response association of low EPA status and greater risk. Low DHA status predicted greater risk of a new suicide attempt in a follow-up study of more than 800 days, 5% of subjects above the median split had new attempts compared to 50% having new attempts among those below the median split [83]. These subjects also had resting PET scans quantifying regional glucose uptake. Future suicide attempters had greater activity in the anterior cingulate and limbic forebrain, consistent with the suspected pathophysiology of severe depression and posttraumatic stress disorder (PTSD). Low DHA in plasma phospholipid robustly predicted this regional hyperactivity ($r = -0.86$, $p < 0.0001$) indicating that low DHA status may increase limbic system-mediated anxiety [83]. In a randomized blinded placebo-controlled trial, we have reported a 45% reduction in suicidal thinking and a 30% reduction in depression among patients with recurrent self-harm recruited from an emergency room 84]. This intervention used 2 g/day of ω–3 LCFAs in a 12-week trial of 49 subjects. Subjects also reported a reduced perception of daily stresses and anxiety which is likely relevant to reduction of risk of the development of PTSD. While substantial work needs to be conducted in the study of ω–3 deficiencies and increased suicide risk, current data suggest an apparent beneficial effect.

Cost Savings to Healthcare Systems

Because of the well-established benefits to cardiovascular health, cost-efficacy analyses have been conducted for use of ω–3 LCFAs for secondary prevention for cardiovascular disease. The Lewin Group (2006) [89] determined that supplementation with 1,800 mg/day of ω–3 long-chain fatty acids was estimated to prevent 384,303,000 hospitalizations due to cardiovascular disease and save USD 3.1 billion over 5 years in the USA, using Congressional Budget office methodology. Similar reductions in misery and economic costs could be expected for the reduction in burden of neuropsychiatric illnesses. For example, depressive disorders appear to respond to ω–3 LCFAs more rapidly (within 2–4 weeks) and have a larger treatment effect size, in comparison to the well-documented effects in the reduction in cardiovascular risk [44]. The use of ω–3 LCFAs in primary prevention is likely to be much more cost-effective than providing treatment of established neuropsychaitric illness. A RAND study indicated that evidence-based care using psychotherapy techniques, for PTSD and major depression, could save as much as USD 1.7 billion, or USD 1,063 per returning veteran; the savings come from increases in productivity, as well as from reductions in the expected number of suicides [85]. The use of ω–3 LCFAs in primary prevention of cardiovascular disease and psychiatric illness could potentially result in much greater savings in cost and human misery.

Cultural Symbolism

It may not be too broad a leap to begin to question if our societal happiness or sense of collective meaning be impaired by deficiencies in ω–3 fatty acids? One approach to examining that question has been an evaluation of the meaning of fish as a cultural symbol [86]. The data from randomized studies in major depression, suicide and aggression indicate that fish is a food with psychotropic properties because it is rich in long-chain ω–3 fatty acids that improve mental wellbeing, i.e. change emotional states. Central to the neuroscience of the assignment of meaning to a visual object is the pairing of that object to an emotional state. Symbols of foods or other substances with psychotropic properties may become paired to the emotional states the substances induce. We posited that traditional cultural medical practices and religious symbolism reflect the ability of long-chain ω–3 fatty acids in fish and seafood to reduce depressive or dysphoric states [86]. Symbols of fish may have become consciously and unconsciously associated with the healing of mental illness and the optimization of emotional wellbeing sacred to both religion and healing. Throughout time, religious and spiritual practitioners have altered their dietary practices, observed their altered internal states, and linked dietary practices to spiritual beliefs using religious and cultural symbols. In traditional Chinese medicine, seafood is used to calm excessive aggression. In Hinduism, Buddhism, Shinto, Islam, ancient Middle Eastern religions, Judaism, and Christianity, fish is symbolically associated with central tenets of faith and healing. For at least six millennia among independent cultures, fish has nearly universally been symbolically associated with sacred symbols of peace and religion. A recent study of a religious group that strictly adheres to the Christian Orthodox Church is remarkably consistent with this proposition [87]. As proscribed by the Church, adherents followed dietary laws for abstinence from meat, eggs, and dairy products for most Wednesdays, Fridays and other periods such as 40 days before Christmas and 40 days before Easter, as well as August 1–15 and other shorter periods, adding up to about 180 days per year [88]. Seafood is not only allowed but is also expected to be eaten and resulted in higher levels of adipose DHA in comparison to controls [87]. These higher adipose DHA levels were also correlated with lower levels of depressive symptoms. Thus, the culturally encoded dietary laws which affirm their religious identity also changed their body compositions of DHA and improved mental wellbeing. The treatment recommendations of the American Heart Association and American Psychiatric Association to consume seafood 2–3 times per week are remarkably similar to the dietary practices of the Christian Orthodox Church.

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Serial Structural MRI Analysis and Proton and ^{31}PMR Spectroscopy in the Investigation of Cerebral Fatty Acids in Major Depressive Disorder, Huntington's Disease, Myalgic Encephalomyelitis and in Forensic Schizophrenic Patients

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Magnetic Resonance Scanning

Spectroscopy revolutionized the study of organic chemistry and biochemistry during the last century. While X-ray crystallography, mass spectrometry and infrared spectroscopy cannot be used non-invasively in the study of fatty acid metabolism in adult human brains, fortunately nuclear magnetic resonance spectroscopy can be so used. In the context of human in vivo studies, nuclear magnetic resonance spectroscopy is more commonly referred to as magnetic resonance spectroscopy, partly to avoid the potentially pejorative and upsetting word 'nuclear' when talking to human volunteers and patients, and partly because the technique involves the use of the same magnetic resonance imaging (MRI) scanners as are employed to carry out structural MRI.

The technique requires a strong magnetic field, preferably at least 1.5 T when applied to the adult human brain. Certain atomic nuclei in the brain interact with this strong static magnetic field. These include protons and the 13-carbon and 31-phosphorus isotopes. In lay terms, each of these nuclei can be considered to possess more than one possible energy level in the magnetic field. Upon exposure of the brain to a short pulse of radiofrequency energy in a magnetic resonance scanner, some of these nuclei absorb the radiofrequency energy and enter a higher quantum energy state. Recovery of the previous, lower, quantum energy state is associated with the reverse process of the release of energy. The latter is measured as an amplified signal by the head coil receiver.

With all such measurements, there is the phenomenon of 'noise'. For example, when a normal radio receiver is not tuned correctly to a radio station, then one hears a lot of static – noise. (Actually, some of this noise may derive from electromagnetic radiation which had its origin in the big bang.) Then, as one tunes in to a correct station frequency (a resonance frequency), the signal-to-noise ratio becomes high enough to allow the station to be heard clearly. The individual nuclei also have particular resonance frequencies. In order to improve the signal-to-noise ratio, multiple readings are taken. Since the noise is assumed to be random, successive additions of the readings lead to relative enhancement of the true signals against a diminishing background of noise signals which tend to cancel each other out. Furthermore, in a given molecule, all the protons do not usually resonate at the same frequency, and all the 31-phosphorus nuclei do not usually resonate at another single frequency (different from that for the protons). This is because the resonance frequencies for given nuclei are partly determined by the electron structure of the molecule. (In classical electromagnetic physics, one can consider that the motion of these charged electrons in a magnetic field gives rise to an electric current which in turn gives rise to an associated and opposing magnetic field, in accordance with Maxwell's equations; this causes varying levels of shielding of the nuclei from the applied external magnetic field.) The difference between the resonance frequencies of a given nucleus and of a reference nucleus is calculated and the chemical shift of the given nucleus is then defined in terms of the ratio of this difference to the resonance frequency of the reference nucleus. The chemical shift is usually expressed in parts per million (ppm). The signals from the brain are plotted as peaks against the chemical shift, the latter constituting the abscissa of the graph. The area under each peak is directly proportional to the concentration of the corresponding nucleus in the sample (for instance, a brain voxel) under study. Moreover, the shape of the peak(s) yields information about the electrochemical environment of the nucleus in the molecule. Further details of this technique are beyond the scope of this article but may be found in the paper by Cox and Puri [1].

Magnetic resonance scanning also offers a non-invasive method of studying brain structure which, as with magnetic resonance spectroscopy, does not involve the use of ionizing radiation. Serially acquired high-resolution structural MRI scans of the brain can now be registered, that is to say, they can be positionally matched, with great accuracy. Upon successful anatomical registration, the scan first acquired chronologically may be electronically subtracted from the second scan. The resulting subtraction image highlights any regions of anatomical change in the brain, to the resolution of the technique used.

The registration technique used by our group in studying cerebral structural changes in relation to fatty acids, as described in this article, is a rigid-body subvoxel technique whose development was pioneered by Hajnal and Bydder [2]. In this technique, the brain is considered to be a rigid body whose spatial coordinates have changed between scans, in respect to the scanner, owing to spatial and rotational transformations. In mathematical terms, the technique assumes 6 rigid-body degrees

of freedom: 3 spatial and 3 rotational. The data matrix corresponding to the second (and subsequent) scan is spatially transformed in each of three mutually orthogonal directions and rotated about three mutually orthogonal axes and the resulting matrix compared with the matrix corresponding to the first, baseline, scan. The differences between the two matrices are then evaluated, and a chi-squared statistical index of goodness-of-fit is calculated. Based on these results, the whole process is repeated, in order to try to minimize the overall difference between the two sets of data. By means of such an iterative process, an optimum positional match between the two datasets is achieved. It turns out that this method can detect differences in position which are far smaller than the dimensions of a voxel; that is, the procedure can detect subvoxel shifts [3]. Such subvoxel shifts are particularly detectable at steep signal intensity boundaries, such as those which occur between cerebral tissue and cerebrospinal fluid, such as the boundary of the lateral ventricles. In a further development of this technique, Nadeem Saeed and Basant Puri developed a semi-automated method for the quantification of ventricular volumes. Further mathematical details of both techniques are beyond the scope of this article but may be found in a paper by Puri [4].

Depression

While working with ultra-pure eicosapentaenoic acid in patients with schizophrenia, schizoaffective disorder, and dyslexia, including a large double-blind, placebo-controlled trial in schizophrenia, the first author became convinced that eicosapentaenoic acid appeared to have antidepressant properties. This hypothesis found support from epidemiological and biochemical evidence, which are now briefly outlined.

In his epidemiological study of the annual prevalence of major depression versus apparent fish consumption (fish catch plus fish imports minus fish exports) in nine countries, Hibbeln [5] found a significant negative correlation between these two variables.

Maes et al. [6] reported a seasonal variation in the severity of depression in 104 consecutively admitted depressed patients between November 1983 and April 1985, with peaks in Zung Self-Rating Depression Scale scores being found in April-May, with lows occurring in August-September. They also reported a significant seasonality for suicide but not homicide from an analysis of the data on suicide, violent suicide, non-violent suicide, and homicide (categorized according to ICD-9) for all of Belgium for the period 1979–1987 [7]. Seasonality was present in violent but not in non-violent suicide. The number of violent suicides increased with age and was more prominent in men. The violent suicide spectral chronograms of younger and elderly persons were distinct in the occurrence of peaks in March-April and August, and lows in December-January. There was no significant relationship between violent suicide and homicide. The same group subsequently took monthly blood samples

from 23 healthy volunteers from Belgium, during the period from December 1991 to December 1992, to test for polyunsaturated fatty acid (PUFA) composition in serum phospholipids. Significant annual rhythms were detected in the long-chain PUFAs arachidonic acid (C20:4*n*–6), eicosapentaenoic acid (C20:5*n*–3), and docosahexaenoic acid (C22:6*n*–3) [8]. Comparing these data with their previous seasonal findings, the group found a significant correlation between, on the one hand, the changes over the previous 2 weeks in arachidonic acid and eicosapentaenoic acid, and, on the other hand, the mean weekly number of violent, but not non-violent, suicide deaths in Belgium. (There was also a significant correlation between the PUFAs arachidonic acid and docosahexaenoic acid, and the B_{max} [^{3}H]-paroxetine binding to platelets.)

This Belgian group also studied serum phospholipids in post-fasting samples in 36 patients with DSM-III-R major depression, 14 patients with DSM-III-R adjustment disorder with depressed mood and dysthymia, and 24 normal controls [9]. Compared with the normal controls, the major depression group showed a higher ratio of arachidonic acid to eicosapentaenoic acid, and lower eicosapentaenoic acid levels in the serum cholesteryl fraction and in phospholipids.

Adams et al. [10] from Australia published an important study of 20 moderately to severely depressed patients, diagnosed using research diagnostic criteria and excluding known bipolar mood disorder and reactive depression, in which they investigated relationships between severity of depression and levels and ratios of *n–3* and *n–6* PUFA in plasma and erythrocyte phospholipids. Severity of depression was measured using the 21-item Hamilton Depression Rating Scale and a second linear rating scale of severity of depressive symptoms that omitted anxiety symptoms. They reported a significant correlation between the ratio of erythrocyte phospholipid arachidonic acid to eicosapentaenoic acid and severity of depression as rated by both scales. A significant negative correlation was also found between erythrocyte eicosapentaenoic acid and the linear rating scale of severity of depressive symptoms. The arachidonic acid to eicosapentaenoic acid ratio in plasma phospholipids and the ratio of erythrocyte long-chain (C20 and C22 carbon) *n–6* to long-chain *n–3* PUFAs were also significantly correlated with this linear rating scale. The authors commented that their findings did not appear to be simply explained by differences in dietary intake of eicosapentaenoic acid. They further suggested that their findings provided a basis for studying the effect in depressed patients of nutritional supplementation aimed at reducing the ratio of arachidonic acid to eicosapentaenoic acid in tissues on the severity of depression. (Clearly this could be achieved by increasing the intake of eicosapentaenoic acid.)

Similar findings were reported in the British study by Edwards et al. [11] in which erythrocyte membrane fatty acid levels, dietary PUFA intake, and the level of depressive symptomatology (assessed using Beck Depression Inventory) were measured in 10 depressed patients and 14 matched healthy control subjects. They reported a significant depletion of erythrocyte membrane *n–3* PUFAs in the depressed patients which was not the result of reduced omega–3 fatty acid intake. Furthermore, the

severity of depression was found to correlate negatively with erythrocyte membrane levels and with dietary intake of *n–3* PUFAs. In line with the paper by Adams et al., in this paper the authors suggested that their findings raised the possibility that depressive symptoms may be alleviated by *n–3* PUFA supplementation.

Based on the above evidence, our group became the first actively to treat major depression with eicosapentaenoic acid. The first published case of a patient with depression to be treated with eicosapentaenoic acid underwent cerebral 31-phosphorus magnetic resonance spectroscopy at baseline and at 79-month follow-up [12]. The patient was a 21-year-old male student with a 7-year history of unremitting depressive symptoms. In this severe case of treatment-resistant depression, no conventional antidepressant treatment or combination of conventional treatment (for example, a selective serotonin re-uptake inhibitor with a lithium salt) has proved effective. However, the addition of ultra-pure eicosapentaenoic acid, in the form of its ethyl ester, was followed by clinical improvement. This included the cessation within 1 month of previously unremitting severe suicidal ideation, and also a marked improvement in previously troubling social phobia symptomatology. The improvement continued and by 9 months the patient's depressive symptoms had disappeared altogether, without any apparent adverse side effects from taking the nutritional supplementation of pure eicosapentaenoic acid. During this 9-month period, the relative phosphomonoester peak value, expressed as a ratio to the total 31-phosphorus signal, increased markedly from 9.23 to 14.11%, representing a 52.9% increase. This 31-phosphorus spectroscopy peak contains contributions from freely mobile phosphomonoesters, including phosphocholine and phosphoethanolamine, and small contributions from inositol phosphate, glycerophosphate, phosphothreonine, and L-phosphoserine. It also contains contributions from phosphomonoester-containing molecules which are less mobile, including some phosphorylated proteins, and from neuronal cytoskeletal protein components. Therefore the area under this peak indexes the level of membrane phospholipid anabolism. In contrast, over the same 9-month period the relative phosphodiester peak value, again expressed as a ratio to the total 31-phosphorus signal, decreased from 46.60 to 41.99%, representing a 9.8% increase in value. This 31-phosphorus phosphodiester spectroscopy peak contains contributions from freely mobile phosphodiesters, including glycerophosphocholine and glycerophosphoethanolamine, as well as contributions from less mobile phosphodiester-containing molecules including some involved in cell membrane structure, the latter including not just the outer cell membrane but also intracellular organelle membranes. Therefore, the area under this phosphodiester peak indexes the level of membrane phospholipid catabolism. Taken together, these neurospectroscopy results were consistent with the hypothesis that nutritional supplementation with pure eicosapentaenoic acid was associated with a reduction in neuronal phospholipid turnover, with increased cerebral membrane phospholipid biosynthesis and decreased cerebral membrane phospholipid breakdown. They were also in line with the finding that the volumetric niacin response, which indexes arachidonic

acid-related signal transduction [13], showed an increase of 30% over the same 9-month period.

High-resolution structural MRI scanning was also carried out at the same time as the 31-phosphorus neurospectroscopy. The structural data obtained underwent subvoxel registration using a sinc interpolation function. The sinc function is defined as:

$$\text{sinc } z = (\sin z)/z, \text{ where } z \in \mathbb{C}$$

This is used as it is the appropriate in-plane point spread function for magnetic resonance image data acquired in the Fourier domain over a bounded region of *k*-space, with band-limited frequency content [14]. The right-hand side of the above equation may worry some readers as, clearly, z may need to take the value $0 \in \mathbb{C}$, and this may lead to the concern that a singularity is being produced through the division of sin 0 (which equals zero) by zero. However, since the numerator may be expressed as a Taylor series, as $z - z^3/3! + \ldots$, it follows that sinc z is given by:

$$\text{sinc } z = 1 - z^2/3! + \ldots \; (z \in \mathbb{C})$$

and hence sinc 0 is unity and so the domain of the function is the whole of $\mathbb{C}$, including zero. Following subtraction of the subvoxel-registered images (follow-up minus baseline), detailed analysis revealed definite structural changes in the brain. In particular, during the 9-month initial period of nutritional supplementation with the ultra-pure eicosapentaenoic acid, not only had the patient's previous treatment-resistant depressive symptomatology markedly improved, but his lateral ventricles had actually shrunk in size [12, 15], again consistent with the 31-phosphorus neurospectroscopy changes described above.

Subsequent randomized, double-blind, placebo-controlled supplementation trials in depression with either ultra-pure eicosapentaenoic acid or a mixture of eicosapentaenoic acid and docosahexaenoic acid have generally been associated with clinically positive results [16].

Huntington's Disease

Huntington's disease, also known as Huntington's chorea, is a progressive inherited neurodegenerative disease which is associated with abnormal involuntary movements, psychiatric disturbance, and cognitive deterioration with progression to dementia and death over 10–20 years. The movement disorder typically includes choreiform movements in head, face, and arms, and a wide-spaced gait with sudden lurching. The psychiatric disturbance is variable but common. Initial insight may lead to depression, while prodromal personality changes, antisocial behavior with substance misuse, affective or schizophreniform disorders may occur. The initial

insight may give way to mild euphoria, explosive outbursts, irritability, and rage. Unfortunately there is no effective treatment for Huntington's disease, although tetrabenazine, a benzoquinolizine derivative initially developed in the 1950s as an antipsychotic medication, may help with the movement disorder [17]. Huntington's disease is inherited as an autosomal dominant disorder, with complete penetrance, caused by an expansion in the number of CAG repeats in the gene for huntingtin on chromosome 4p16.3, coding for polyglutamine [18]. Huntingtin interacts with many other proteins, including calmodulin (a Ca^{2+}-binding regulatory protein), CREB-binding protein (a transcriptional coactivator), mSin3a (a transcriptional co-repressor), cystathionine B-synthase (a metabolic enzyme), GAPDH (a glycolytic enzyme and translational regulator), HAP-1 (involved with membrane trafficking), HAP-40 (unknown function), HIP-1 (a pro-apoptotic protein involved in actin organization and endocytosis), HIP-2 (ubiquitin-conjugating enzyme), HYP-A (a WW-domain protein involved in mRNA splicing), HYP-B (a WW-domain protein which acts as a transcription factor), HYP-C (a WW-domain protein involved in mRNA splicing), MLK2 (JNK activator), N-CoR (nuclear receptor co-repressor), p53 (a transcription factor), SH3GL3 (involved with clathrin-mediated endocytosis and the recycling of synaptic vesicles), Shc (a signaling protein), and EGF receptor (a signaling protein).

Several converging lines of evidence lent support to the suggestion by the first author of this article that ultra-pure eicosapentaenoic acid might be therapeutic in Huntington's disease. These included two single-case studies, findings relating to membrane phospholipid metabolism from a post-mortem study, impaired phospholipid-related signal transduction, a study of the effects of fatty acids on a transgenic mouse model of the disease, and the cerebral effects of eicosapentaenoic acid in other neuropsychiatric disorders. We shall now outline each of these in turn.

The two single-case studies in Huntington's disease were published by Vaddadi [19]. These 2 Australian patients showed improvement in some of their symptomatology following supplementation with fatty acids, mainly from evening primrose oil, and therefore mainly containing linoleic acid and γ-linolenic acid.

Ellison et al. [20] carried out measurements of both phosphoethanolamine and ethanolamine in post-mortem brain samples from patients with Huntington's disease using high-performance liquid chromatography with electrochemical detection. The concentrations of phosphoethanolamine were significantly reduced by 76% in the caudate, 53% in the putamen and 48% in the nucleus accumbens, while ethanolamine concentrations showed similar but smaller reductions. Since both phosphoethanolamine and ethanolamine are involved in phospholipid metabolism, these findings suggested that phospholipid metabolism might be impaired in Huntington's disease.

The first author of this paper carried out a study of the volumetric niacin response, which indexes phospholipid-related signal transduction [13], in patients with advanced (stage III) Huntington's disease. Compared with age- and sex-matched control subjects, who had a mean volumetric niacin response of 28.3 mol s l^{-1}, the

Huntington's disease patients were found to have a reduced mean volumetric niacin response of 16.3 mol s l^{-1} [21].

Clifford et al. [22] described the systematic effects of treatment with fatty acids or placebo, given throughout life, on the emergence and progression of phenotype in the R6/1 transgenic mouse model of Huntington's disease using assessment techniques which included a novel, ethologically based, approach to dissect neurological impairment into topographical domains of function at a naturalistic level. Transgenic R6/1 mice incorporate a human genomic fragment containing promoter elements exon 1 and a portion of intron 2 of the huntingtin gene and they develop late-onset neurological deficits, as occurs with the motor abnormalities of Huntington's disease. In this study, R6/1 and normal mice were randomized to receive a mixture of fatty acids, containing linoleic acid, γ-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid, or placebo on alternate days throughout life. Over mid-adulthood, topographical assessment of behavior revealed the R6/1 transgenic mice to evidence progressive shortening of stride length, with progressive reductions in locomotion, elements of rearing, sniffing, sifting and chewing, and an increase in grooming, deficits which were either not evident or markedly diminished in the R6/1 transgenic mice receiving the fatty acids. The latter also showed reductions in body mass and in brain dopamine D_1-like and D_2-like quantitative receptor autoradiography which were unaltered by the fatty acid supplementation. The authors concluded that these findings indicated that early and sustained treatment with fatty acids were able to protect against motor deficits in R6/1 transgenic mice expressing exon 1 and a portion of intron 2 of the huntingtin gene, and they suggested that fatty acids may have therapeutic potential in (human) Huntington's disease.

The final strand of evidence related to the cerebral effects of eicosapentaenoic acid in neuropsychiatric disorders other than Huntington's disease. These effects in relation to depression have been described above. Similar benefits were also found in schizophrenia, with clear evidence of a reversal of the previously increasing ventricle-to-brain ratio accompanying clinical benefits in the first patient with schizophrenia to be treated solely with ultra-pure eicosapentaenoic acid (in the absence of any other medication, including antipsychotics) [23, 24].

Based on the above evidence, we carried out the first pilot study of ultra-pure eicosapentaenoic acid in Huntington's disease. This consisted of a 6-month randomized, placebo-controlled study of the ethyl ester of eicosapentaenoic acid in 7 inpatients with advanced (stage III) Huntington's disease (3 on eicosapentaenoic acid, 4 on placebo; no significant difference in age or sex between the groups) [25]. At 6-month follow-up all the patients treated with eicosapentaenoic acid were found to have improved on the orofacial component of the Unified Huntington's Disease Rating Scale while all the patients on placebo deteriorated on this scale ($p < 0.03$). Following subvoxel registration of follow-up 3D MRI brain scans with baseline scans, subtraction images showed that while the placebo was associated with progressive cerebral atrophy, the eicosapentaenoic acid supplementation was associated with a reverse process. From

this pilot study we concluded that treatment with eicosapentaenoic acid appeared to be associated with beneficial motor and MRI changes.

As a result of this pilot study, a larger, phase III, multicenter, double-blind, placebo-controlled trial was carried out by us [26]. A total of 135 patients with either stage I (a decline in functional capacity to work with impaired motor function and emotional control, including irritability and depression, but fully able to maintain his or her own activities of daily living) or stage II (unable to work or drive a car, reliant on family or other carers for activities of daily living but with assistance can remain at home, increasing dysarthria, dysphasia, loss of motor control and cognitive function) Huntington's disease entered into the study to receive either ultra-pure eicosapentaenoic acid (as the ethyl ester) or placebo daily. All the patients had symptomatic, genetically confirmed Huntington's disease or had a family history of Huntington's disease. The inclusion criteria also included having a score from 50 (24-hour supervision appropriate: assistance required for bathing, eating, toileting) to 90 (no physical care needed if difficult tasks are avoided) on the Independence Scale component of the Unified Huntington's Disease Rating Scale. Pharmacotherapy with depot antipsychotics was not allowed. The primary efficacy variable was the Total Motor Score 4 subscale of the Unified Huntington's Disease Rating Scale. The items of this motor subscale are: maximal dystonia (five locations); maximal chorea (seven locations); ocular pursuit (two tests); tongue protrusion; retropulsion pull test; finger tapping (right and left); pronation and supination of the hands (right and left); rigidity (right and left), and tandem walking. A total of 121 patients completed 12 months, and 83 did so without protocol violations (the per protocol cohort). Intent-to-treat analysis revealed no significant difference between eicosapentaenoic acid and placebo on the Total Motor Score 4 subscale. In the per protocol cohort, however, eicosapentaenoic acid proved significantly better than placebo on this Total Motor Score 4 subscale. Exploration of moderators of the efficacy of eicosapentaenoic acid on the motor signs of the Total Motor Score 4 subscale showed a significant interaction between treatment and a factor defining patients with high versus low CAG repeat number at 4p16.3. Reported adverse events were distributed equally between treatment arms.

Two potential mechanisms of action of eicosapentaenoic acid in Huntington's disease have recently been proposed [27, 28]. They are based on two pathways which might be of pathophysiologic significance in this disease. In the first pathway, interleukin-1β (IL-1β), lipopolysaccharide, and glutamate may induce nuclear factor-κB (NF-κB), a transcription factor which is a critical regulator of neuronal survival, via an IKK complex, p65, IκBα, and p50, while in the second pathway huntingtin, lipopolysaccharide, and radiation may activate c-jun N-terminal kinases (JNK), which may mediate neuronal degeneration via JNK-AP-1. Eicosapentaenoic acid appears to inhibit both NF-κB and JNK-AP-1, and this may therefore help explain its efficacy in Huntington's disease. Our finding that the clinical efficacy of eicosapentaenoic acid in Huntington's disease varied according to the CAG repeat number is more difficult to explain at present, but a partial explanation may lie in the fact that

the CAG repeat length determines the extent of mitochondrial sensitivity towards (metabolic) challenges; further research is clearly indicated.

Myalgic Encephalomyelitis

Myalgic encephalomyelitis, also known as chronic fatigue syndrome, is a devastating disease which, according to the Revised CDC (Centers for Disease Control and Prevention) Criteria, include the following symptoms and signs in addition to chronic fatigue: impaired memory or concentration; sore throat; tender cervical or axillary lymph nodes; myalgia; multi-joint pains; new headaches; unrefreshing sleep, and post-exertion malaise [29]. The etiology of myalgic encephalomyelitis is currently unknown. However, there is evidence that LC-PUFAs may have an important role to play [30]. A key part of the evidence for this came from proton neurospectroscopy studies.

The first such systematic proton neurospectroscopy study was carried out by our group, in which 8 patients with myalgic encephalomyelitis (chronic fatigue syndrome), diagnosed according to the Revised CDC Criteria, were compared with 8 matched control subjects [31]. The key finding was an increased level of cerebral choline (the area of the peak associated with free choline-containing compounds) in the myalgic encephalomyelitis patients. The second systematic proton neurospectroscopy study was carried out by Chaudhuri et al. [32] in Scotland, using the same number of patients and controls as we had in our study. Again, this group reported a significant increase in the area of the peak associated with free choline-containing compounds in the myalgic encephalomyelitis patients compared with the matched control subjects. A series of 3 cases of juvenile myalgic encephalomyelitis was reported by Tomoda et al. [33] from Japan, and again included the finding of a raised level of the choline peak on proton neurospectroscopy.

We have hypothesized that the raised level of choline-containing compounds in the brain in myalgic encephalomyelitis may be the result of reduced incorporation of the choline polar head group in phospholipid molecules (at the Sn3 position) in both outer cell membranes and intracellular organelle membranes in neurons and glial cells [30]. In turn, this might be the result of impaired biosynthesis of membrane phospholipid molecules in the brain, caused by reduced biosynthesis of long-chain PUFAs (at the Sn2 position of phospholipids) by putative viral infectious inhibition of the first LC-PUFA biosynthetic step catalyzed by Δ6-desaturase.

Intriguingly, there exist other lines of evidence pointing to a possible viral etiology for myalgic encephalomyelitis [30]. One is that many clinical features of epidemics of myalgic encephalomyelitis-like illnesses, such as the Los Angeles County Hospital epidemic of 1934 and the Royal Free Hospital (in London, UK) epidemic of 1955, are consistent with viral infections. Another is that the general pattern of immune system changes in myalgic encephalomyelitis is consistent with a pre-existing long-term viral infection. Again, direct analysis of blood fatty acids has shown decreased levels

of certain long-chain PUFAs in myalgic encephalomyelitis patients compared with matched control subjects.

The recent report by Kerr's group [34] of up-regulation of the mitochondrial translation initiation factor EIF4G1 transcript variant 5 in myalgic encephalomyelitis is consistent with a persistent virus infection. Virus infections, through their inhibition of Δ6-desaturase, might then cause a deficiency of long-chain PUFAs.

Two placebo-controlled, double-blind trials of the use of fatty acids in chronic fatigue patients have been published. The first, by Behan et al. [35], showed a significant benefit, while the second, by Peet's group [36], proved negative. In the first neuroimaging study of fatty acid supplementation in a patient with myalgic encephalomyelitis, our group carried out high-resolution MRI brain scanning at baseline and after just 16 weeks in a female patient in her mid-20s who had a 6-year history of unremitting symptoms of this illness [37]. The fatty acid supplementation was associated with both clinical improvement and, upon subvoxel registration of the structural MRI scans, a reduction in the ventricle-to-brain ratio, with the baseline lateral ventricular volume of 28,940 mm^3 decreasing to just 23,660 mm^3 at 16-week follow-up. This is clearly an area that requires further research.

Forensic Patients with Schizophrenia

We have already mentioned the first case report of the successful use of ultra-pure eicosapentaenoic acid (in the absence of any other form of pharmacotherapy) in the treatment of a patient with a long history of positive and negative symptoms of schizophrenia; the rapid clinical improvement was accompanied by improvements in indices of cerebral structure, including a reduction in the lateral ventricular volume and in the associated ventricle-to-brain ratio [23, 24]. These results were consistent with the membrane hypothesis of schizophrenia, formulated largely by the late David Horrobin.

However, schizophrenia is a heterogeneous disorder. While the majority of structural brain-scanning studies of schizophrenia have indicated that the disorder is associated with ventricular enlargement, a study of first-episode schizophrenia, carried out by our group, that utilized the monomodal rigid-body subvoxel registration technique outlined earlier, showed that lateral ventricular volumes can alter in either direction early on in the course of the illness [38]. Moreover, the direction of change may relate to schizophrenia syndromes. Compared with the control subjects, the schizophrenia patients categorized as suffering from the withdrawn syndrome showed progressive ventricular enlargement, with an increase in ventricle-to-brain volume ratio, whereas the schizophrenia patients suffering from the active syndrome showed a reduction in ventricle-to-brain volume ratio, with a change opposite in sign and smaller in magnitude [39]. The active syndrome consists of raised activity levels, accelerated cognition, positive thought disorder, positive labile affect, and affective

delusions, and has been said to be associated with a greater left than right functional activation, while the opposite pattern of functional hemispheric imbalance has been said to be associated with the withdrawn syndrome, which comprises the essential negative features of schizophrenia [40]. In studying schizophrenia, therefore, it is helpful to select a group which is relatively well circumscribed clinically. We have chosen to do so by studying those patients with schizophrenia who have seriously and violently offended while psychotic; we were interested to ascertain whether the subgroup of patients with schizophrenia who are violent offenders may suffer from abnormal membrane phospholipid metabolism.

Accordingly, we carried out 31-phosporus neurospectroscopy on 15 male patients with DSM-IV schizophrenia who were all inpatients in the largest European Medium Secure Unit and on a comparison group of 13 healthy normal age-matched male controls with no known medical or psychiatric disorder [41]. Expert psychiatric opinion, accepted in court, was that all the patients had violently offended directly as a result of schizophrenia prior to admission. These offences consisted of homicide, attempted murder, or wounding with intent to cause grievous bodily harm. Interestingly, we found no direct neurospectroscopic evidence of a membrane phospholipid abnormality in the brain in this cohort of patients, which underlines the heterogeneous nature of the illness. However, the level of β-nucleotide triphosphate (β-NTP) was found to be lower, and the level of γ-nucleotide triphosphate (γ-NTP) higher, in the forensic schizophrenia patient group compared with the control group. While β-NTP indexes ATP (adenosine triphosphate), the majority of ADP (adenosine diphosphate) is nuclear magnetic resonance-invisible, although the γ-NTP signal overlaps with signals from β-ADP, although the signal of γ-NTP is much stronger. Therefore, our results were consistent with increased cerebral energy metabolism taking place in this particular group of schizophrenia patients.

We then decided to examine the association of arachidonic acid-related signal transduction, as quantified using the volumetric niacin response [13], with cerebral metabolism, measured using 31-phosphorus neurospectroscopy, in this group of schizophrenia patients [42]. There was a strong, and negative, correlation between the volumetric niacin response and the cerebral metabolite concentration of inorganic phosphate expressed as a ratio of the total 31-phosphorus signal. There was also a trend towards a negative correlation between the volumetric niacin response and the metabolite concentration of α-NTP expressed as a ratio of the total 31-phosphorus signal. This suggests that reduced phospholipid signal transduction may be related to higher cerebral energy metabolism in this group of schizophrenia patients. However, since the volumetric niacin response is reduced in patients with schizophrenia compared with normal controls, this suggests that a lower response is likely to be associated with increased severity of illness. Hence, these results lead to the possibility that patients with schizophrenia who have violently offended and have poor phospholipid-related signal transduction may have higher levels of cerebral energy metabolism.

Discussion and Conclusions

In this paper we have demonstrated the utility of applying serial structural MRI analysis and proton and 31-phosphorus magnetic resonance spectroscopy to the investigation of cerebral fatty acids in neurologic and psychiatric disorders. While most of these applications have thus far been mainly related to advancing our understanding of the basic scientific foundations of the relationship of lipids to the pathophysiology of the disorders described, it seems probable that they will increasingly contribute to the study of the effects of dietary supplementation in these disorders.

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Omega–3 Fatty Acid Docosahexaenoic Acid Is the Precursor of Neuroprotectin D1 in the Nervous System

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Omega–3 fatty acids cannot be made de novo in the human body. They are derived from linolenic acid (18:3,n–3) and are an essential part of the human diet. A substantial body of research exists indicating the beneficial effects of increased dietary intake of essential omega–3 fatty acids on cardiovascular and brain health. They are required to maintain membrane integrity and necessary for optimal cellular function [1].

Docosahexaenoic acid (DHA, 22:6,n–3) is an essential omega–3 fatty acid obtained from the diet mostly in the form of fish oil and marine algae. It is highly concentrated in membrane phospholipids of the retina and brain [2]. Lipids comprise 22% of the cerebral cortex and DHA is the most abundant omega–3 fatty acid in the phospholipid bilayer of neurons. DHA has been shown to be essential for proper maturation and maintenance of the visual cortex and retina [3]. Free DHA has been shown to limit oxidative-stress induced apoptosis via the production of neuroprotectin D1 (NPD1), a transient, yet powerful, signaling molecule.

As the name suggests, NPD1 is a potent anti-inflammatory, anti-apoptotic agent with great neuroprotective utility in multiple disease processes. D1 indicates that NPD1 is the first of presumably several neuroprotective signaling molecules derived from DHA in circumstances of oxidative stress and cellular damage (fig. 1). Enhancing our understanding of and influence on DHA's endogenous anti-inflammatory signaling potential, including the synthesis of NPD1, will provide a means for developing therapeutic strategies aimed at decreasing the morbidity and mortality of many devastating diseases, including retinal degenerative diseases, stroke, Alzheimer's disease (AD), and epilepsy.

T.D.N. and D.T.S. contributed equally to this work.

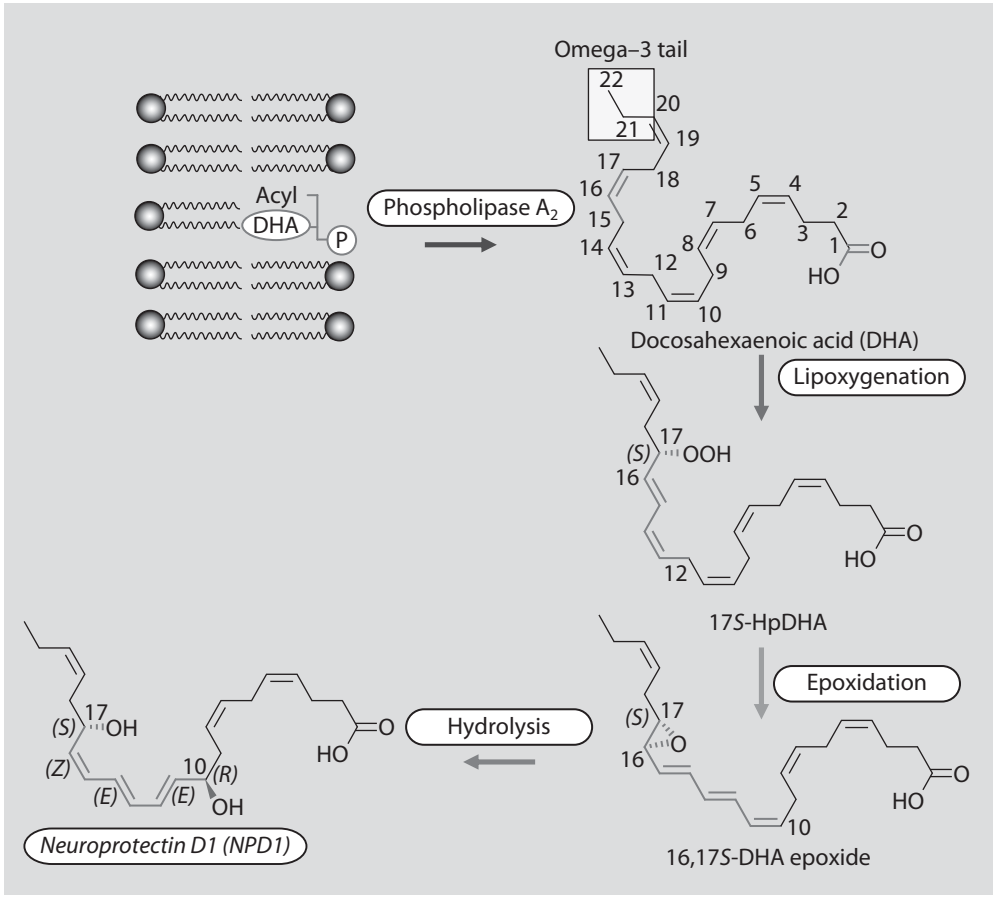

Fig. 1. Biosynthesis of NPD1. Phospholipase A_2 hydrolyzes membrane phospholipids releasing free DHA. Lipoxygenation is then followed by epoxidation and hydrolysis to generate NPD1 (reprinted from Bazan [46] with permission of The Association for Research in Vision and Ophthalmology).

Adequate Stores of DHA Are Required for Proper CNS Function

The consumption of large amounts of DHA has been associated with multiple health benefits. DHA has been shown to be involved with brain and retinal development, aging, memory formation, synaptic membrane function, photoreceptor biogenesis and function, and neuroprotection [2, 4]. DHA-supplemented infant formula enhances maturation of retinal function, visual acuity, and mental performance in preterm and term infants [2]. In age-related macular degeneration, there is an inverse relationship between diets high in DHA and risk for the disease [5]. Finally, epidemiologic studies indicate that diets enriched with DHA are associated with reduced risk of cognitive impairment and slow the progression of dementia and AD [6].

Certain individuals with low dietary intake of DHA, such as vegans, vegetarians, and the elderly, have been shown to have less brain DHA [7]. The fatty acid

composition of brain lipids can be modified by diet. While lack of DHA in the diet will eventually result in loss of DHA content from brain and retina, both the brain and retina actively conserve DHA pools displaying a striking ability to retain DHA even after prolonged dietary deficiencies [8]. In cases of prolonged essential omega–3 fatty acid deficiency, decreased amounts of DHA in neuronal membranes alter membrane fluidity and signaling properties [6].

Depleted stores of DHA are associated with several health risks. It has been suggested that a lack of specific dietary nutrients, such as essential omega–3 fatty acids, may significantly contribute to cognitive decline and increased risk and severity of brain injury. When rats are fed low-DHA diets for one or more generations, clear deficits in cognition are observed [6]. Blood DHA levels are decreased in various forms of retinitis pigmentosa, in Usher's syndrome, and in animal models of inherited retinal degeneration [2].

Aging is associated with decreased levels of DHA in both rat and human brains, especially the frontal cortex [6]. In addition to decreased dietary intake and reduced liver fatty acid desaturase capacity, age-related defects in antioxidant systems result in an increase in lipid peroxidation that further reduces DHA levels [6]. Therefore, the elderly population is specifically at risk for the cognitive and cellular impairments associated with depleted DHA levels.

DHA Is Delivered to the CNS via the Bloodstream

DHA is concentrated in the brain and retina. DHA and its lipid precursor, linolenic acid (18:3,n–3), are provided by the diet. Linolenic acid is elongated and desaturated in liver hepatocytes to form DHA. Hepatic stores of DHA are then activated (22:6-CoA) and acylated into phospholipids and released as lipoproteins from the liver into the bloodstream for distribution to the central nervous system (CNS) [9].

Once processed in the liver and released into the blood, retinal pigment epithelial (RPE) cells take up newly elongated DHA via the choriocapillaris [2]. Intraperitoneal injection of [^{3}H]DHA demonstrates that DHA first accumulates in RPE cells prior to being incorporated into the inner segments of photoreceptors [10, 11]. Under in vitro conditions, the retina has been shown to incorporate approximately 60–90% of physiologically meaningful administrations of [^{3}H]DHA (i.e., nanomolar range) into the retina in the form of esterified phospholipids within 4 h [12].

Phospholipids Containing DHA Are Incorporated into Photoreceptors

RPE cells are derived from the neuroectoderm and are closely associated with photoreceptors. In humans, approximately 23 photoreceptors interact with each RPE cell. They are specialized to maintain optimal photoreceptor health by providing nutrition,

protection, synthesis/secretion of neurotrophic growth factors, and phagocytosing/processing of aged photoreceptor outer segment tips [13]. After RPE cells absorb DHA from the choriocapillaries, they release DHA into the interphotoreceptor matrix, where it is preferentially taken up by the myoid region of the photoreceptor inner segment and activated by coenzyme A. DHA is then esterified into both the SN-1 and SN-2 position of phosphatidic acid and triacylglycerol, ultimately producing multiple DHA-containing membrane phospholipids including phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine [14–17].

Previous research has indicated that photoreceptors are rich in a supraenoic form of the phospholipid phosphatidylcholine. This phospholipid incorporates a DHA in position SN-2 and a 24-6-36:6 elongation product of DHA in position SN-1 that tightly bind rhodopsin [18]. The SN-1 tail functions to 'curl' and restrict rhodopsin motion [19]. This interaction between DHA and rhodopsin is required for photoreceptor function and vision. In healthy individuals, DHA must be provided in adequate quantities for successful photoreceptor formation during prenatal and perinatal development; DHA must be provided over the lifetime of the individual in order to carry out photoreceptor repair and membrane turnover.

Photoreceptors have outer segments composed of stacks of photosensitive disks that respond to light exposure. These photosensitive membrane disks are continuously formed in the basal portion of the photoreceptor outer segment with a proportional amount shed from the outer segment tips. As more and more membrane disks are assembled and accumulate in the basal region, older disks are advanced toward the photoreceptor tip where they are ultimately shed and phagocytosed by closely associated RPE cells. The rate of membrane shedding and the rate of membrane biogenesis is identical, resulting in successful outer segment renewal/turnover with no modification in outer segment length [10].

Electron microscopic autoradiography, using labeled [^{3}H]DHA, tracks disk labeling and progression. [^{3}H]DHA is not observed at the tips of the photoreceptors prior to labeled disk migration into the tip region. In addition, the density of [^{3}H]DHA incorporated into each disk membrane in the basal region of the photoreceptor outer segment remains stable throughout the life of the disk membrane [10]. Thus, there is no further redistribution of DHA once it is loaded onto a disk membrane at the basal region of the outer segment [10]. Rather, photoreceptors incorporate DHA into disks at the base of the outer segment where DHA molecules will remain until eventual outer segment shedding and phagocytosis by the RPE cells [10].

The body actively conserves DHA stores in order to prevent DHA depletion. This is especially true in the retina. After RPE cells phagocytize shed photoreceptor outer segment tips during routine photoreceptor maintenance, DHA, like vitamin A, is recycled back to the inner segments of the photoreceptor via the interphotoreceptor matrix. In one experiment, frogs were injected with [^{3}H]DHA until the entire photoreceptor outer segment was heavily labeled (~30 days after injection). During subsequent shedding of photoreceptor tips and the uptake of those phagosomes into the RPE cells, a pulse of labeled

[^{3}H]DHA was observed entering the RPE cell. Once inside the RPE cell, the transient oil droplets containing [^{3}H]DHA-labeled phospholipids immediately underwent degradation and dissipated. It was observed that during the experiment, 12% of the labeled [^{3}H]DHA was phagocytized into the RPE cell, while total retinal [^{3}H]DHA remained unchanged [10]. The stable [^{3}H] signal in photoreceptors indicates that [^{3}H]DHA is not lost during outer segment shedding. Rather, RPE cells must successfully recycle [^{3}H]DHA back to the photoreceptor inner segment for subsequent use in new membrane disks [10]. Although this efficient recycling of DHA back and forth from the RPE cells to the photoreceptors via the interphotoreceptor matrix (IPM) has been observed, it is still poorly characterized regarding the mechanisms of DHA uptake into the photoreceptor inner segment as well as a detailed description of the specific form of DHA transported across the IPM.

DHA in Neurodegenerative Disease

DHA has been implicated as a risk modifier for several CNS pathologies. Some prospective and case-control epidemiological studies have suggested that increased dietary intake of DHA lowers the long-term risk for developing AD and that a low concentration of DHA in the blood is an AD risk factor [20]. Post-mortem studies have shown that DHA is reduced in the hippocampi of AD patients [21]. Additionally, animal models of AD are more vulnerable to DHA depletion than controls, and DHA reduces several pathological hallmarks of AD (e.g., synaptic dropout, τ protein hyperphosphorylation, and Aβ peptide aggregation) [20].

In AD, abnormal accumulation of the Aβ peptides results in cell death associated with Ca^+ excitotoxicity and a general oxidative stress response. We have recently shown that DHA downregulates Aβ peptide secretion from aging human neural (HN) cell cultures [21]. This response is associated with a concomitant increase in NPD1 synthesis. The soluble amyloid precursor protein-α (sAPPα) is a product of β-amyloid precursor protein (β-APP) cleavage by the α-secretase pathway and is known to reduce Aβ peptide secretion. Application of sAPPα to HN cells also significantly increases NPD1 synthesis, and application of a small concentration (50 nM) of exogenous NPD1 protects HN cells from Aβ42 peptide-induced apoptosis. Exogenous NPD1 exerts this effect by activation of a neuroprotective program that counteracts oxidative stress by upregulation of anti-apoptotic Bcl-2 family proteins (Bcl-2; Bcl-xl; Bfl-1/A1), downregulation of pro-apoptotic Bcl-2 family proteins (Bad; Bax; Bid; Bik), and inhibition of pro-inflammatory gene expression (COX-2 [prostaglandin synthase-2, cyclooxygenase-2]; CEX-1 [chemokine exodus protein-1]; B-94 [TNF-α-inducible pro-inflammatory element]). Moreover, in the same study, both unesterified DHA and NPD1 were found to be significantly reduced in the hippocampi from AD patients as compared to age-matched controls. These data suggest that DHA acts to induce an anti-apoptotic, neuroprotective gene-expression program that counteracts Aβ42-induced inflammation by serving as a precursor to the potently bioactive oxygenation product NPD1.

DHA in Epilepsy, Epileptogenesis, and Synaptic Plasticity

Alterations in fatty acid metabolism have been proposed to contribute to the efficacy of the ketogenic diet used to treat refractory epilepsy in children [22–24]. Ketogenic diets have been shown to increase plasma DHA in rats and humans [25, 26], and rats consuming a ketogenic diet showed a 15% increase in brain DHA as compared to controls [22]. Multiple mechanisms have been proposed for DHA's role in modulation of seizure activity and pathological sequelae, including attenuation of neuronal membrane excitability via direct interaction with ion channels, regulation of expression and activity of mitochondrial uncoupling proteins, and regulation of genes linked to energy metabolism [24]. However, strong evidence for reduced seizure activity in patients fed DHA-enriched diets has remained elusive [27, 28].

DHA added to hippocampal slices may elicit anti-epileptogenic activity, modulate ion channels and neurotransmitter receptors, and in turn regulate synaptic plasticity. There are conflicting observations on the effects of DHA using extracellular and intracellular recordings of hippocampal slices. DHA facilitates *N*-methyl-D-aspartate (NMDA) responses [29], and it blocks delayed-rectifier K^+ channels [30, 31]. Thus a consequence of these DHA actions would be enhancement of neuronal excitability and worsening of seizure activity. In contrast, DHA added to hippocampal slices significantly reduced the frequency of evoked action potentials in CA1 neurons, hyperpolarized the resting membrane potential, and raised the stimulatory threshold for action potential generation [32]. At lower stimulation frequencies than in the studies described in previous reports [30, 31], it was found that DHA does not exert actions on multiple spikes induced by bicuculline or in Mg^{2+}-free medium, indicating that DHA does not directly interact with NMDA or non-NMDA receptors [33]. However, DHA may attenuate synaptic transmission and epileptiform activity in rat hippocampis by frequency-dependent Na^+ channel blockade [33]. Most of these electrophysiologic studies have used added DHA at relatively high concentrations (e.g., 50 μM). In contrast, we reported that 50 nM of added NPD1 exerts potent bioactivity [21, 34–36]. We now propose a new approach to define the significance of DHA in epilepsy: that is the finding that very small amounts of systemically infused DHA elicit profound down-regulation of kindling-induced epileptogenesis [unpubl. observations] due to the fact that DHA is used as a precursor for NPD1. Brain DHA is the substrate for the synthesis of NPD1 in the hippocampus during kindling, and this offers a different explanation for the involvement of DHA in epileptogenesis.

COX-2 regulates neuronal excitability and activity-dependent induction of long-term potentiation in the hippocampus via synthesis of prostaglandin E_2 (PGE_2), which is derived from arachidonic acid (AA) (20:4,n–6), the major omega–6 fatty acid in CNS cellular membranes. PGE_2 increases neuronal excitability by modulating K^+ channels, hyperpolarization-activated cation channels, and tetrodotoxin-resistant Na^+ channels [37–39], and it facilitates back-propagation of axonal action potentials required for long-term potentiation [40]. COX-2 and phospholipase A_2, which cleaves

AA (and DHA) from membrane phospholipids, become activated during kindling epileptogenesis, and the resulting prostaglandin synthesis is believed to mediate the development of aberrant neuronal connectivity [41].

Interestingly, in hippocampal synaptosomal fractions, bicuculline-induced status epilepticus promotes the accumulation of free (unesterified) DHA, which is also cleaved from membrane phospholipids by PLA_2 [40]. In turn, seizures elicit the synthesis of NPD1, indicating activation of an auto-neuroprotective signaling after seizures that counteracts seizure-induced increases in COX-2 expression [unpubl. observations].

DHA in Ischemia-Reperfusion Injury

In ischemia-reperfusion injury, pathological events stemming from energy depletion lead to non-specific glutamate release and glutamate reuptake inhibition, intracellular Ca^{2+} overload, mitochondrial dysfunction, and generation of reactive oxygen species. Increases in unesterified DHA are triggered by ischemia-reperfusion injury [41–43]. Under these circumstances, DHA can contribute to cellular injury through its non-enzymatic conversion to lipid peroxides. However, we showed, using a mouse model of ischemia-reperfusion injury, that DHA is also converted to NPD1 upon its release from membrane phospholipids [44]. In the presence of aspirin, DHA was converted into a series of 17R-hydroxy-containing DHA oxygenation products (D series resolvins), which possess pro-resolution bioactivity in inflammatory processes [45]. After infusing exogenous NPD1 into the third ventricle of mice undergoing ischemia-reperfusion injury, we found a dramatic decrease in polymorphonuclear leukocyte infiltration into infarcted areas, and the volume of infarction was greatly reduced as compared to vehicle-treated controls. These changes were associated with NPD1 attenuation of ischemia-induced up-regulation of COX-2 expression. In the same study [44], NPD1 was shown to inhibit IL-1β-mediated COX-2 expression. These findings have important implications for potential new experimental therapeutics for stroke, as well as other neurologic diseases sharing cytokine-mediated inflammatory processes.

Future Directions

In spite of all that has been discovered, many questions remain concerning DHA and its product NPD1. First, during situations of cellular stress, how do the retina and brain signal for the release of lipoproteins containing DHA from the liver? It is known that the liver is capable of releasing DHA when the CNS is threatened, but how does the CNS communicate that message to the liver (perhaps a plasma messenger)? Second, it is known that certain neurotrophins induce the synthesis of NPD1 (such as PEDF), but which receptor do they bind and how does that receptor signal for the release of free DHA and synthesis of NPD1? Finally, once synthesized, how

does NPD1 traverse the nuclear membrane in order to modulate gene expression? Also, what is the nature of the signal that 'turns off' this gene expression? In time, we hope to discover the answers to these and many more questions in order to harness NPD1's extraordinary neuroprotective potential and find beneficial therapies in the treatment of retinal disorders and neurologic diseases.

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Docosahexaenoic acid and Amyloid-β Peptide Signaling in Alzheimer's Disease

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Docosahexaenoic acid (22:6,n–3, DHA), an essential marine-derived omega–3 polyunsaturated fatty acid, is selectively concentrated in neuronal, synaptic and retinal membranes. 60% of fatty acids esterified in neocortical neuronal cell membrane phospholipid stores consist of DHA, so brain cells have a convenient and readily accessible reserve of DHA, that through phospholipase activities, liberate membrane-bound DHA into unesterified (free) DHA that serves in neural signaling, survival, and cell fate pathways.

Stereospecific oxygenated derivatives of DHA created through lipoxygenase action on free DHA further generate neuroprotectin D1 (NPD1) that elicits potent cyto- and neuroprotective effects. The neurophysiological actions of esterified DHA occur in part through the maintenance of neuronal plasma membrane integrity and lipid bilayer biophysics. The beneficial actions of free DHA and NPD1 occur (a) through the repression of the induction of inflammatory signaling mediators such as the inducible cyclooxygenase-2 (COX-2) enzyme, (b) through the recruitment of anti-apoptotic members of the Bcl-2 gene family, and (c) through the repression of pro-inflammatory and pro-apoptotic signaling genes and their translation products. DHA is essential for prenatal brain development and normal, homeostatic brain function. Dietary deficiencies in DHA are associated with retinal and neurological dysfunction and visual and cognitive decline. Deficits in DHA and NPD1 abundance are associated with the neurodegenerative mechanisms that characterize Alzheimer's disease (AD), the leading cause of neurodegeneration and cognitive impairment in our society. AD exhibits a progressive deposition of ragged amyloid-β (Aβ) peptides derived from the β-γ secretase pathway that processes β-amyloid precursor protein (βAPP) into the more toxic forms of βAPP-derived fragments. Aβ peptides themselves, and downstream consequences of Aβ peptide signaling, are pro-oxidative, neurotoxic, pro-inflammatory and pro-apoptotic. The enzymatic generation, speciation and trafficking of βAPP and Aβ peptides in AD

and in experimental AD models are impacted by DHA abundance, the bioavailability of unesterified (free) DHA, and by derivatives of DHA such as NPD1.

This paper will review the interplay of DHA, NPD1, βAPP and Aβ peptide-related oxidative mechanisms in homeostatic brain function and in the development and progression of AD, currently the most prevalent neurological dysfunction in aging Western and Asian populations.

β-Amyloid Precursor Protein, Amyloid-β Peptides, Oxidative Stress and Apoptosis in Alzheimer's Disease

Significant molecular, genetic and epidemiological data support the idea that βAPP-derived peptide- and cytokine-induced oxidative stress, and the generation of reactive oxygen species (ROS), play important roles in aging and in the development and progression of neurodegenerative disease [1–20]. These pathogenic processes exhibit two key features: (a) they act in an accumulative fashion over the lifespan of the organism and (b) once begun, their deleterious effects on brain cell structure and function exhibit positive feedback, often perpetuating until the substrate is consumed or until the oxidative reactions are quenched or terminated. Mitochondrial dysfunction and focused oxidative damage, including primary peroxidation of cell components by ROS, appears to be among the earliest events in pathological aging and in the onset, development and progression of AD [14–22]. Free radical oxidative damage to brain plasma membrane lipids, which contain a high proportion of DHA, is thought to be one of the early critical and determining events involved in initiating brain cell membrane instability, dysfunction and degeneration. A related hallmark event in AD is the evolution from βAPP of soluble Aβ peptides into dense, fibrous insoluble deposits of extracellular congophilic, agyrophilic, neuropathological lesions called neuritic plaques. Neuritic plaques accumulate in the extracellular space and around the endothelial cell walls of cerebral blood vessels and especially in brain microvasculature [5, 6, 9, 21, 23–27]. Importantly, the generation of Aβ peptide oligomers, thought to be a 'priming' event in the pathogenesis of AD, appears to precede the formation of τ-positive paired helical filaments in neurofibrillary tangles [23, 24, 28, 29]. The βAPP holoprotein, comprising the substrate of the 'γ-secretase complex' that consists of presenilin 1 and/or 2 (PS1/PS2; essential components of γ-secretase) and nicastrin, gives rise to neurotoxic Aβ peptides 37–43 amino acids in length (Aβ37–Aβ43). The 'γ-secretase complex' thereby contains both peripheral and trans-membrane domains intimately associated with the lipid bilayers of neuronal lysosomal, Golgi, endoplasmic reticular and plasma membranes. The interaction of DHA with specific components of the 'γ-secretase complex' is not well understood, however, DHA-induced alterations in synaptic plasma membrane fluidity may contribute to learning-related memory retention in Aβ-peptide infused rats (see below) [23, 24, 30]. Both Aβ peptides and mature neuritic plaques support oxidative stress, partly through an incompletely understood direct action

through metal-ion induced Fenton chemistries, and indirectly through a brain-specific glial-mediated inflammatory response [4–17]. Aβ peptide accumulation in the cerebral vasculature disturbs homeostatic functions of the blood-brain barrier, alters blood vessel neurophysiology, obstructs regional cerebral blood flow, and generates directly or further facilitates the production of ROS and oxidative stresses that progressively contribute to neuronal dysfunction and cognitive decline [5, 8–10, 31, 32]. Peripheral sources of Aβ peptides and inflammatory lipids, either by transversing a damaged or leaky blood-brain barrier, may further contribute to Aβ peptide deposition in the AD brain [4, 19, 25, 26]. The presence of neuritic plaques triggers an immune attack by brain microglia, resulting in a robust release of microglia-derived cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and excessive local production of ROS, actions which may in part be catalyzed by trace amounts of neurotoxic metal ions [1, 4, 6, 15, 17, 33, 34]. The state of aggregation and compactness of Aβ peptides and maturation stage of neuritic plaques are associated with their potential for ROS generation and their ability to induce oxidative stress and to promote apoptotic signaling [5, 17, 20, 35, 36]. Apoptosis in turn is in part regulated by pro-apoptotic or anti-apoptotic proteins of a growing 20+ member Bcl-2 gene family [37–40]. Pro-apoptotic members such as Bad, Bax and Bik trigger apoptosis via their translocation into the mitochondrial membrane while promoting loss of calcium homeostasis and cytochrome c release. In response to apoptotic stimuli, mitochondria can also release caspase-independent cell death effectors such as apoptosis inducing factor (AIF), a flavoprotein induced by noxious stimuli, neurotoxic metals and by the redox state of the cell [20, 41, 42]. In contrast, anti-apoptotic members of the Bcl-2 gene family such as Bcl-2, Bcl-xl and Bfl-1(A1) form heterodimeric complexes with pro-apoptotic family members and thereby neutralize their activity [42–44]. Imbalances between the rates of synthesis and clearance of Aβ peptides and their pro-inflammatory and pro-oxidative effects, and also between pro-apoptotic and anti-apoptotic factions of the Bcl-2 gene family, are therefore important contributors to the onset, development and progression of degenerative mechanisms in neurological disease. Interestingly, in both in vitro and in vivo studies, antioxidants have beneficial effects in reducing markers for brain oxidation and apoptosis, suggesting that exogenously applied bioavailable factors can effectively moderate neural cell destruction and be part of a useful armamentarium in pharmacological strategy to treat neural degeneration [20, 45–50].

Alzheimer's Disease as an Inflammatory Brain Disease – DHA and Oxidative Stress

While the degree to which brain inflammation plays an initiator role in AD is controversial, there is abundant evidence that pathogenic inflammatory signaling contributes significantly to the maintenance and progression of the AD process. The enhancement in AD brain of ROS, activated microglia and astrocytes, complement proteins, pro-inflammatory cytokines such as IL-1β and TNF-α and their association

with aggregated Aβ peptides and neuritic plaques suggests that early-to-late stage AD represents a progressive state of neural inflammation [19, 33, 34, 51–57]. A key genetic element that regulates the inducible generation of pro-inflammatory mediators, including prostaglandins, is COX-2, an oxido-reductase up-regulated in AD brain and a useful neocortical marker for the stage or degree of AD pathophysiological change [4, 19, 53, 58–61]. Free radicals and major pro-inflammatory cytokines in the brain such as IL-1β and TNF-α strongly activate both COX-2 and βAPP gene transcription in cultured brain cells and in models of brain injury that involve ischemia-reperfusion [54–62]. Recent findings that IL-1β can substitute for key aspects of hypoxia-mediated oxidative stress signaling further support a strong neurochemical connection between up-regulation of pro-inflammatory cytokine signaling, oxidative stress, apoptosis and neural cell death [5–8, 63–65]. AD brain can therefore be characterized biochemically by increases in the products and consequences of oxidative and inflammatory stress – accumulation of neurotoxic Aβ peptides, lipofuscin pigments, F4 neuroprostane (from non-enzymically oxidized DHA), acute phase reactants and other components of the complement cascade, increases in hydroxynonenol (HNE), decreases in membrane fluidity, and depletion of DHA content [47, 58, 66].

DHA exerts anti-oxidative, anti-inflammatory and anti-apoptotic actions by reducing the cellular levels of ROS, in part by suppressing pro-inflammatory mediators and inducible COX-2 expression, and by maintaining higher levels of anti-oxidative enzyme activities such as those for glutathione [67–69]. Specific increases in HNE, a neurotoxic free radical aldehyde of fatty acid oxidation, may be one of the earliest biochemical markers for the development of AD [4–6, 65]. DHA treatment of cell cultures reduces HNE-induced oxidative stress and apoptosis [5, 6, 14, 65, 70]. Interestingly, COX-2 up-regulation in AD brain may have a direct bearing on γ-secretase activity and increased generation of amyloidogenic Aβ peptides, strengthening the pathological connection between brain inflammation and oxidative burden [19, 49, 50, 71]. Combined with extensive epidemiologic studies suggesting that non-steroidal anti-inflammatory drugs (NSAIDs), as inhibitors of inducible COX-2 activity, retard AD onset or severity, these data cumulatively suggest that inflammation-mediated oxidative stress and/or inflammatory cascades fuelled by oxidative stress are important factors that contribute to the progressive brain cell dysfunction and degeneration characteristic of AD brain [26, 33, 34, 72].

Therapeutic strategies aimed at effectively retarding Aβ peptide-, cytokine- and oxidation-induced brain cell stress are urgently needed to treat the insidious onset and progression of neurodegenerative disorders such as AD that have both oxidative stress and inflammatory components [4, 14, 17, 18, 49, 50]. The up-regulation of intrinsic, anti-apoptotic neurotrophic signals represents one such strategy. As further described below, DHA and NPD1 biosynthesis are key elements of a potent endogenous signaling system that effectively counteracts Aβ peptide and IL-1β-mediated oxidative stress, promoting neuroprotective signaling that supports brain and retinal cell function and survival [19, 38, 59–61, 73–75].

DHA in CNS Injury Oxidation and Oxygenation Pathways

As previously mentioned, DHA, the most abundant polyunsaturated fatty acid in the CNS, and obtained through the diet primarily from marine sources such as fish oil and algae, preferentially accumulates in the phospholipids of brain synaptic membranes and retinal photoreceptors. In rodent and non-human primate studies dietary deprivation of omega–3 fatty acids must be prolonged over one generation to reduce DHA content in the CNS. While this remarkably tenacious retention ability suggests that DHA is critical for neural and visual function, specific molecular mechanisms through which DHA elicits these positive, supportive bioactivities are just beginning to become understood. DHA incorporated into nuclear membranes and DHA exogenously applied to human neural cells in primary culture represses the expression of genes related to inflammation [4, 14, 38, 59–61, 76, 77]. Alterations in membrane architecture, function and fluidity coupled with specific repression of pro-inflammatory gene expression may contribute to the beneficial action of n–3 polyunsaturated fatty acids on cognition [57, 68, 69, 78]. The anti-inflammatory gene expression effects of DHA may arise in part through the inhibition of NF-κB-DNA binding response elements of the inducible nitric oxide synthase, COX-2 and other pro-inflammatory and/or pro-oxidative gene promoters [79–84].

Gene transcripts displaying changes in abundance in human fetal retinal explants supplemented with DHA encode for proteins involved in neurogenesis, neurotransmission, and the consolidation and refinement of synaptic connectivity [38, 85]. Because of the high concentration of DHA in the brain relative to other organs, DHA peroxidation is a major outcome of free radical-mediated oxidative brain injury induced by either acute trauma or more chronically through age-related neurodegenerative mechanisms. ROS directly attack membrane-esterified DHA in situ, generating an array of damaged pathogenic lipids and oxidized DHA intermediates [5, 6, 54, 65, 86, 87]. Lipid peroxidation is a self-propagating and self-sustaining neurodestructive process capable of extensive, progressive and prolonged neural tissue damage [5, 6, 16]. DHA esterified into phospholipids at the carbon two position of the glycerol backbone is liberated by phospholipase (PLA_2) to yield free DHA (fig. 1). The bioavailability of free unesterified DHA is a tightly regulated event and free DHA pools, normally at very low levels under basal conditions, become significantly increased during cerebral ischemia due to up-regulation of PLA_2 activity [54, 60, 61, 64]. Up-regulation of PLA_2 activity is also observed in Aβ peptide- and IL-1β-stressed human neural cells in primary culture and in AD neocortex and hippocampus [15, 18, 64, 88].

During oxidative stress, DHA may be oxidized non-enzymatically into F4-, D4-, E4-, A4- and J4-neuroprostanes, prostaglandin-like compounds formed independently of cyclooxygenase that further support oxidative stress [4, 14–16, 86, 87]. Synthesis of F4-neuroprostane-containing aminophospholipids may adversely affect neuronal function as a result of alterations they induce in the biophysical properties of neuronal plasma membranes [86, 87]. The abundance and speciation of F4

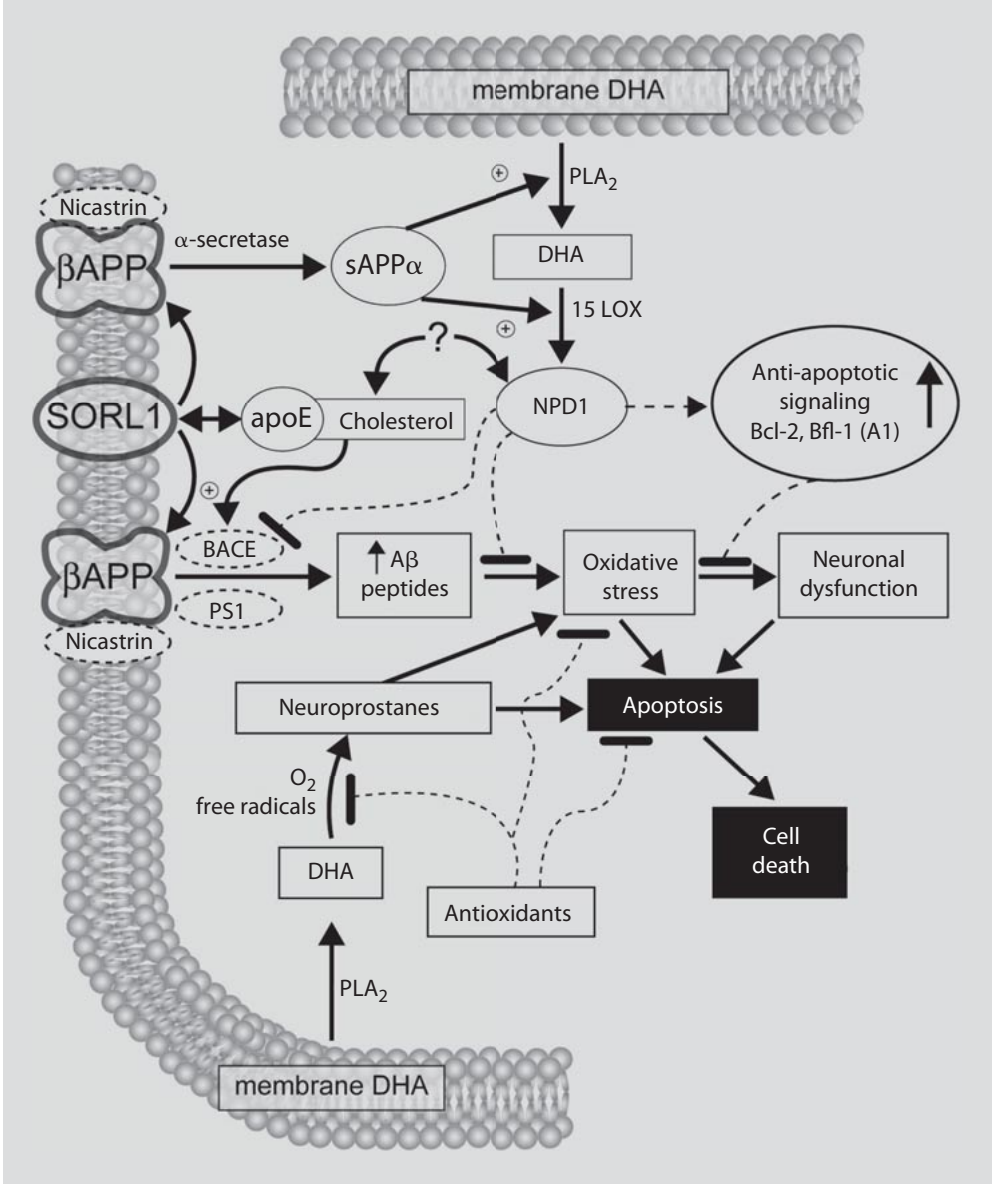

Fig. 1. Essentials of βAPP-Aβ peptide-DHA-NPD1 signaling, including enzymatic and non-enymatic processing pathways for DHA. DHA from the omega–3 essential fatty acid family preferentially accumulates within neuronal and retinal phospholipids of central nervous system membranes, concentrating specifically within neuronal synapses and retinal photoreceptors. Free DHA derived from membrane DHA stores (upper) is liberated via a stringently regulated phospholipase A_2 (PLA_2), and may subsequently be converted into the 10,17S-docosatriene neuroprotectin D1 (NPD1) through an enzyme-mediated lipoxygenation via a 15-lipoxygenase (15-LOX) or 15-LOX-like enzymes. The neurobiological activity of DHA-derived NPD1 in cultured human retinal pigment epithelial and human neural cells has been characterized as a potent cyto- and neuroprotective oxygenated lipid mediator [38, 59–61, 89, 73–75, 134, 135]. Membrane DHA (lower) is also rapidly oxidized non-enzymatically by molecular oxygen (O_2) and free radicals to form F4 neuroprostanes, a class of peroxidized lipids that further support oxidative stress and brain cell apoptosis leading to cellular dysfunction and cell death.

neuroprostanes and HNE, which reflect the general state of lipid peroxidation and oxidative stress, and may be useful biomarkers for the therapeutic efficacy of anti-oxidation drugs [5, 6, 11, 14, 16, 65, 86, 87].

Alternatively, membrane-esterified DHA can be liberated and oxygenated via enzymatic pathways, such as by tandem PLA_2-15-LOX action on free DHA, to generate the 10,17S-docosatriene or NPD1 (fig. 1). NPD1 elicits highly potent anti-oxidative and neuroprotective functions in brain and retinal cells [59–61, 73–75, 89]. The nature of the switch from membrane disruptive to neuroprotective roles for DHA, such as the generation of F4 neuroprostanes versus NPD1 and other DHA-derived neuroprotectins, is under intense research study. Changes in the redox balance of brain cells, modulated in part by bioavailable antioxidants and/or neurotoxic metal chelators, may affect the kinetics of these DHA-processing systems [17, 20].

β-Amyloid Precursor Protein Processing, DHA and Cholesterol

βAPP holoprotein, an ~110 kDa integral type-1 transmembrane glycoprotein and key player in AD neuropathology, is imbedded within lysosomal, Golgi, endoplasmic reticular, retinal and neuronal plasma membrane lipid bilayers, normally functioning in transport, intracellular contact and nuclear signaling [90–94]. βAPP can be sequentially processed via the β-amyloid cleavage enzyme (BACE; β-secretase), and subsequently by PS1/2 (γ-secretase), into ragged amyloidogenic Aβ peptides 37–43 amino acids in length, the most neurotoxic of which appear to be Aβ40 and Aβ42 peptides [23, 24, 95, 96]. Interestingly, the cleavage domains of βAPP targeted by β- and γ-secretase are membrane proximal, suggesting that βAPP-processing enzymes

Fig. 1 Continued These non-enzymatic reactions may be quenched by specific antioxidants and free radical scavengers [49, 50, 86, 87, 137]. The integral membrane protein β-amyloid precursor protein (βAPP) gives rise to sAPPα via a neurotrophic, non-amyloidogenic, α-secretase-mediated pathway that stimulates both PLA_2 and 15-LOX enzymes. Alternately, βAPP also generates neurotoxic Aβ peptides via a β-amyloid cleavage enzyme (BACE)-presenilin 1 (PS1) amyloidogenic pathway (β-γ secretase pathway) [23, 24, 103, 138]. The transmembrane protein nicastrin and the sorting receptor sortilin-1 (SORL1) direct trafficking of βAPP, and down-regulation of SORL1 may lead to activation of the amyloidogenic pathway and increased generation of Aβ peptides as is observed in AD brain [88]. SORL-1 interacts with the type E apolipoprotein carrier (apoE) which functions in part as the major cholesterol transporter in the brain [38, 97, 102]. Cholesterol also increases Aβ peptide production via stimulation of BACE and the β-γ secretase pathway [97, 103, 139–141]. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) both lower cholesterol and reduce intracellular and extracellular abundance of Aβ peptides in primary neuronal and neuronal-glial co-cultures and in clinical trials [109, 142–146]. Interactions between cholesterol, statins, DHA and NPD1 are not well understood although DHA supplementation in combination with statin therapy has shown significant health benefits in patients with hyperlipidemia, and demonstrate a further reduction of serum cholesterol than by statin treatment alone [147, 148].

operate directly at the membrane interface or within the membrane itself. The fatty acid composition of neuronal membranes, including DHA and cholesterol content, and cholesterol transporters, have direct bearing on whether βAPP is processed into neurotrophic or the more neurotoxic species (fig. 1) [97–102]. βAPP processing and Aβ peptides derived from βAPP in turn appear to be regulators of both lipid homeostasis and cholesterol biosynthesis [103–106]. DHA has been reported to suppress the age-related Aβ peptide shedding from human neural cells in primary culture [38], to repress Aβ peptide-related pathology in a Tg2576 transgenic cell model of AD [68], and to stimulate non-amyloidogenic βAPP processing and reduce both intracellular and extracellular levels of Aβ peptide in SH-SY5Y cells [107].

The effects of DHA and cholesterol on the activity of β-γ secretase enzymes are not well understood but are a tremendously active area of contemporary medical research. Initial interest in the cholesterol-βAPP-Aβ peptide connection came from the observation that cholesterol levels were found to positively correlate with Aβ peptide load in the brains of AD patients [57, 101, 108]. A class of inhibitors of the rate-limiting enzyme in cholesterol biosynthesis 3-hydroxy-3-methylglutaryl-coenzyme A reductase, collectively known as statins, have been repeatedly shown to lower serum cholesterol while reducing Aβ peptide abundance, both in vitro and in vivo [97, 103–109]. Cholesterol-rich regions of neuronal membranes known as lipid rafts appear to alter the distribution of βAPP-cleaving secretases within the membrane, resulting in production of the more amyloidogenic species of Aβ peptides and a decreased generation of the more neurotrophic forms of βAPP, such as the soluble sAPPα (see below, fig. 1) [38, 101, 108, 110, 111]. An unusual γ-secretase cleavage site within the hydrophobic trans-membrane domain of βAPP suggests that pathological events which alter or disorganize lipid bilayer structure or fluidity contribute to Aβ40 and Aβ42 peptide generation [98, 99, 111]. Cholesterol has significant biophysical effects on membrane lipid fluidity, curvature and the translocation, orientation or positioning of the βAPP holoprotein within lipid raft domains [101, 106, 112]. The neuronal membrane-enriched ATP-binding cassette transporters ABCA1 and ABCG1 play a significant role in the regulation of neuronal cholesterol trafficking and efflux, and in suppression of βAPP processing to generate Aβ peptides, hence their role in promoting cholesterol mobility remains an attractive Aβ peptide-reducing strategy [100, 113, 114]. Gross disorganization of the lipid bilayer and aberrant processing of βAPP may be further orchestrated by peroxidation of DHA, cholesterol, or by neurotoxic metal-ion catalyzed free radical damage to membrane lipid constituents [5, 6, 17, 20, 65]. While the interaction of cholesterol and statins with DHA are just beginning to become understood, DHA supplementation in combination with statin therapy demonstrates a significant enhancement in the reduction of serum cholesterol than is observed with statin treatment alone [cf. 147, 148].

Gene mutations in βAPP, BACE, PS1 or PS2 each drive the production of the more neurotoxic species of Aβ peptides but their individual contributions to neuronal plasma membrane dynamics and membrane biophysics remain elusive [29, 115–117]. The particularly neurotoxic peptide Aβ42, a 'sticky' 42-amino-acid self-aggregating peptide

not only supports oxidative stress by contributing directly to neuronal dysfunction, apoptosis and eventual cell death, but are also 'secreted' into the extracellular space via the β-γ secretase pathway to trigger extraneuronal effects that support the extracellular generation of ROS [23, 24, 88, 97, 103, 118, 119]. While βAPP can generate toxic Aβ peptides, this integral membrane glycoprotein and can be alternatively processed via a membrane-bound α-secretase into a soluble form of APP (sAPPα), which is neuritogenic and neurotrophic. In fact, sAPPα supports normal neuronal function and also protects neurons from the toxicity of Aβ peptides [23, 24, 95, 96, 120]. A less abundant related protein, sAPPβ, cleaved by the proteolysis of βAPP by β-secretase, serves non-amyloidogenic functions [38, 51, 52]. A significant portion of the neuroprotective activity of DHA may be derived not only through its support of neural and retinal plasma membrane integrity and function, but also through its role as substrate for the generation of other oxygenated neuroprotective molecules. The roles of DHA and NPD1 in the membrane-bound enzymes involved in βAPP-processing pathways are just beginning to become understood. In APP/PS1 doubly transgenic mice, diets enriched in DHA were found to lower hippocampal Aβ peptide levels, and dietary supplementation with DHA in a triply transgenic AD mouse model reduced intraneuronal accumulation of Aβ peptide levels via a decrease in the steady-state levels of PS1 [98, 99, and unpubl. observations]. DHA has been found to attenuate both Aβ40 and Aβ42 peptide secretion in primary human neural cells, an effect accompanied by the formation of NPD1 [38, 59–61]. Neurotrophins such as pigment epithelium-derived factor (PEDF) induce concentration-dependent DHA-mediated NPD1 synthesis in retinal pigment endothelial cells. DHA and PEDF appear to synergistically modify the expression of Bcl-2 family members by activating anti-apoptotic proteins, by decreasing pro-apoptotic proteins, and by attenuating caspase-3 activation during oxidative stress [73–75, 121, 122]. DHA and NPD1 also induce a gene-expression program in human primary neural cells that up-regulates the production of anti-apoptotic Bcl-2 family members such as Bcl-2 and Bfl-1 (A1) that also promote neuronal cell survival. DHA and DHA-derived neuroprotectins are stimulated by sAPPα through up-regulation of PLA_2 and 15-LOX or 15-LOX-like activities. Specific direct actions of DHA or NPD1 on BACE (β-secretase) and/or PS1/PS2 (γ-secretase) activities, thereby down-regulating neurotoxic Aβ peptide production and subsequent ROS generation are currently not known.

Conclusion

The primary pathogenic events that initiate neurodegenerative disorders such as AD are multifaceted and multifactorial. Current clinical, epidemiological, molecular-genetic and neuropathological evidence suggests that this prototypic human neurodegenerative disease evolves from a complex interplay of genetic and environmental factors against a background of normal brain aging. Recent biochemical and epidemiological evidence suggests that dietary lipids such as cholesterol and DHA are causally

involved in the aberrant neurochemical and physiological processes that initiate the neurodegenerative process [4, 10, 18, 19, 27, 39, 97, 123–127]. The hallmark pathological process in AD remains the progressive condensation and aggregation of fibrillar Aβ peptides into neuritic plaques that support oxidative stress, pro-inflammatory and pro-apoptotic signaling, resulting in neuronal dysfunction and irreversible loss of brain cell homeostasis. Mechanisms responsible for generating Aβ peptides, and their neurotoxic consequences such as driving brain cell oxidation, increase with age, and may potentially predispose aging humans to neurological dysfunction [23, 128–130]. The chronic nature of AD suggests that neuroprotective and survival factors are progressively lost, switching from an up-regulation in the expression of anti-apoptotic factors to increases in the expression of pro-apoptotic members of the Bcl-2 gene family [43, 44, 131, 132]. Unlike the actions of excessive cholesterol, the neuroprotective lipid DHA, and DHA-derived NPD1, decrease the rate of AB peptide generation, aggregation, and its consequences. DHA and NPD1 may also influence apoptosis-induced brain cell damage in part by shifting the balance from the expression of pro-apoptotic factors toward the expression of anti-apoptotic, survival-promoting members of the Bcl-2 gene family [38, 134–136].

Several important unanswered questions remain. The impact of DHA and NPD1 on the secretase-mediated cleavage mechanism of βAPP is still not well characterized. DHA and NPD1 as potential modulators of cholesterol biosynthesis, trafficking and apolipoprotein-mediated transport, βAPP processing, Aβ peptide speciation, generation and secretion during aging and in cytokine- and oxidation-stressed human brain cell models of AD are also not well understood [38, 98]. sAPPα, a modulator of DHA-derived NPD1 biosynthesis in cultured human neural cells and its bioactivity in young, adult and aging human brain, and in areas of the brain, such as the limbic system and association neocortex, targeted by AD neuropathology as compared with the relatively spared occipital cortex, is probably an important factor in modulating both DHA and NPD1 signaling. Further mechanistic studies on how DHA and NPD1 promotes neuroprotection through the up-regulation of anti-apoptotic Bcl-2 family proteins should further unravel how endogenously-derived lipid mediators promote brain cell survival that rally host defenses against oxidative stress and inflammation-triggered neuronal decline.

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Administration of Docosahexaenoic Acid, Uridine and Choline Increases Levels of Synaptic Membranes and Dendritic Spines in Rodent Brain

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That docosahexaenoic acid (DHA), an omega–3 polyunsaturated fatty acid (PUFA), can affect brain function and behavior no longer is controversial: deficiencies in this compound are associated with impairments in cognitive development, correctable by its repletion [1] and the consumption of DHA or fish oil by humans reportedly slows cognitive decline in the aged [2] and in subjects with early Alzheimer's disease [3] and promotes mental development in infants [4]. Some of DHA's effects on brain have been shown to occur with 'physiologic' doses which raise its plasma concentrations significantly but keep them within their normal range [5]; others probably are pharmacologic. Some are shared with eicosapentaenoic acid (EPA), another omega–3 PUFA, or with the omega–6 fatty acid arachidonic acid (AA), and others with both or neither of these compounds.

In general, nutrients and drugs that modify brain function or behavior tend to do so by affecting synaptic transmission [6]: they modulate the quantities of particular neurotransmitter molecules within synaptic clefts, or act directly on the transmitter's receptors or on downstream signal-transduction molecules. Is this also the case for DHA? Hypotheses attempting to explain how DHA affects brain function have, in general, been based on its incorporation into membrane phospholipids and consequent effects on membrane fluidity [7]; on proteins affecting transcription (RXR [8]) or neurite outgrowth (syntaxin-3 [9]); on increasing phosphatidylserine (PS) production [10]; on suppression of neuronal apoptosis [11]; or on the neuroprotective actions of its product 10,17S-docosatriene [12]. Little has been known concerning possible changes DHA might produce in synaptic transmission.

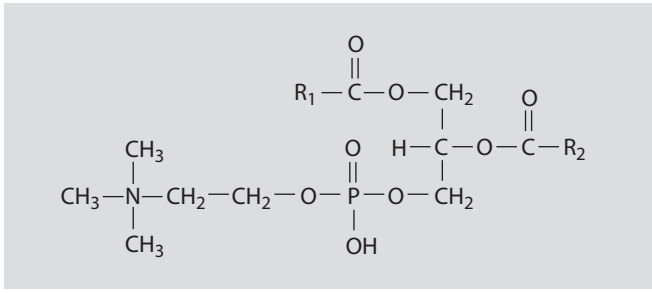

Fig. 1. Structure of phosphatidylcholine.

Now it can be stated that DHA does indeed affect important components of brain neurotransmission: it increases the synthesis and levels of phosphatides, the main constituents of synaptic membranes, and of specific pre- and postsynaptic proteins [13, 14], and it promotes the formation of dendritic spines [15], where most excitatory synaptic transmission takes place. In doing these things, DHA acts in concert with two other circulating compounds, uridine and choline [13]. Like DHA, these compounds also cross the blood-brain barrier (BBB) via specific transport mechanisms and also affect the substrate saturation of the enzymes that utilize them for synthesizing phosphatides. The effects of administering all three phosphatide precursors together tend to be greater than the summed effects of giving each alone. Both uridine (via its product uridine-5′-triphosphate [UTP]) and DHA may also promote membrane phosphatide synthesis by interacting with specific neuronal proteins, such as the P2Y receptor for UTP [16] and syntaxin-3 for DHA [9].

This article summarizes available information concerning mechanisms by which DHA affects synaptic membrane levels, synapse formation, and brain neurotransmission. Because DHA's effects on synaptic membrane depend to such a great extent on its interactions with brain uridine and choline, the article also describes the metabolism of these compounds in some detail.

Biosynthesis of Membrane Phosphatides

All cells utilize DHA and other fatty acids, uridine, and choline to form the phosphatide subunits (e.g. phosphatidylcholine [PC]) (fig. 1) which, when aggregated, constitute the major components of their membranes. PC, the principal subunit in brain, is synthesized from these precursors by the cytidinediphosphocholine (CDP-choline) cycle or 'Kennedy cycle' [17] (fig. 2). PC, in turn, provides the phosphocholine moiety for synthesizing sphingomyelin (SM), another major choline-containing brain phospholipid. The phosphatide phosphatidylethanolamine (PE) also is synthesized via the Kennedy cycle, utilizing ethanolamine instead of choline, while the third major structural phosphatide, PS, is produced by exchanging a serine molecule for the choline in PC or the ethanolamine in PE [18].

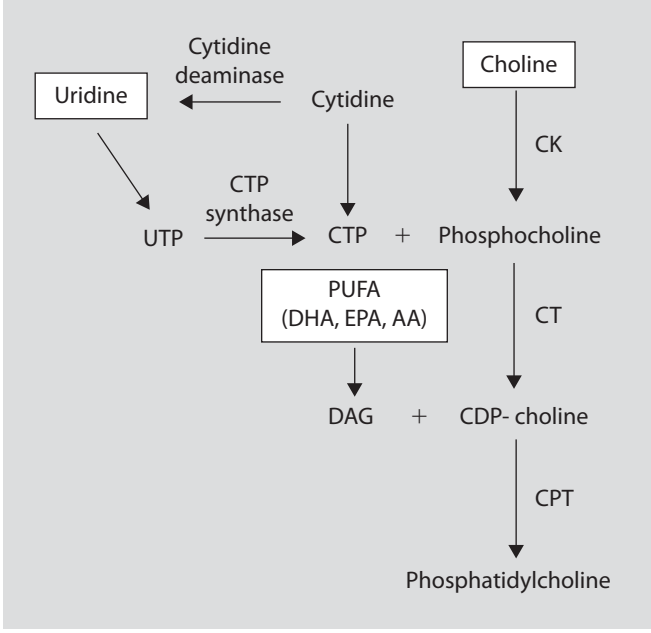

Fig. 2. Phosphatidylcholine (PC) biosynthesis via the Kennedy cycle [17]. In rats, cytidine is the major circulating pyrimidine [95]; in humans [19] and gerbils [20] the primary circulating pyrimidine is uridine. Only small amounts of circulating cytidine are converted to brain CTP, since the blood-brain barrier (BBB) high-affinity transporter for pyrimidines (CNT2) has a very low affinity for cytidine [96–98]; uridine, in contrast, readily enters the brain via CNT2, yielding UTP which can be converted to CTP by CTP synthase [89]. CTP then reacts with phosphocholine to form CDP-choline, which combines with diacylglycerol (DAG), preferentially species containing PUFAs like DHA, EPA or AA to form PC. Boxes indicate the compounds that are obtained from the circulation. Synthesis of PE via the Kennedy cycle utilizes ethanolamine instead of choline [data from 14].

The CDP-choline cycle involves three sequential enzymatic reactions (fig. 2). In the first, catalyzed by choline kinase (CK), a monophosphate is transferred from ATP to the hydroxyl oxygen of the choline, yielding phosphocholine. The second, catalyzed by CTP:phosphocholine cytidylyltransferase (CT), transfers cytidylylmonophosphate (CMP) from cytidine-5′-triphosphate (CTP) to the phosphorus of phosphocholine, yielding cytidylyldiphosphocholine (also known as CDP-choline or as citicoline). As discussed below, much of the CTP that the human brain uses for this reaction derives from circulating uridine [19]. The third and last reaction, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), bonds the phosphocholine of CDP-choline to the hydroxyl group on the 3-carbon of diacylglycerol (DAG), yielding the PC. All three precursors of PC must be obtained entirely or in large part from the circulation, and because the PC-synthesizing enzymes that act on all three have low affinities for them, blood levels of all three can affect the overall rate of PC synthesis [13, 20].

Thus, choline administration increases brain phosphocholine levels in rats [21] and humans [22], because CK's K_m for choline (2.6 mM [23]) is much higher than usual

Table 1. Effects of UMP-containing diet and/or DHA on brain phospholipid levels [data from 13]

Treatments	Total PL	PC	PE	SM	PS	PI
Control diet + Vehicle	351	152	65	45	33	21
UMP diet + Vehicle	367	171*	84*	52	35	31**
Control diet + DHA	392	185*	78*	56*	39	32**
UMP diet + DHA	442***	220***	113***	73***	46***	36***

Groups of 8 gerbils were given either a control or a UMP-containing (0.5%) diet, and received orally (by gavage) DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for phospholipids. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared with values from Control diet + Vehicle group. Data are presented as nmol/mg protein.

brain choline levels (30–60 μM) [24–26]. Most commonly the second, CT-catalyzed reaction is most rate-limiting in PC synthesis, either because not all of the CT enzyme is fully activated by being attached to a cellular membrane [27] or because local CTP concentrations are insufficient to saturate the CT [26]. Thus, when brain CTP levels are increased by giving animals uridine [20], CTP's circulating precursor in human blood [19], PC synthesis is accelerated [20]. The activity of CPT and the extent to which this enzyme is saturated with DAG can also control the overall rate of PC synthesis, as has been shown, for example, in permeabilized HeLa cells exposed to glycerol-3-phosphate and acyl-CoA [28], or in PC12 cells extending neurites after exposure to the nerve growth factor (NGF) [29]: in PC-12 cells, NGF increased DAG levels 5-fold, CPT activity by 70%, and the incorporation of choline into PC by 2-fold. As discussed below, DAG species containing DHA or other PUFAs on the middle carbon apparently are preferentially utilized for phosphatide synthesis compared with the amounts utilized for producing triglycerides [30]. (This does not explain, of course, why giving DHA and presumably increasing levels of DHA-containing DAG would also increase the levels of PC in a cell, e.g. in table 1).

If rodents are given a standard diet that also contains both choline and uridine (as its monophosphate, UMP) and, by gavage, DHA, brain PC synthesis rapidly increases [13, 20], and absolute levels of PC per cell (DNA) or per mg protein increase substantially (e.g., by 40–50% after several weeks of daily treatment [13] (table 1). This treatment also increases the levels of each of the other principal membrane phosphatides (table 1), as well as those of particular proteins known to be localized within synaptic membranes (for example, synapsin-1 [31], PSD-95 [32] and syntaxin-3 [9]), but not the ubiquitously-distributed brain protein β-tubulin (fig. 3) [13, 14]. As discussed below, treatment with DHA, UMP and choline also promotes the formation of dendritic spines in adult gerbil hippocampus [15] (see section 6), and improves hippocampus-dependent cognitive behaviors in rats reared in a socially-deprived

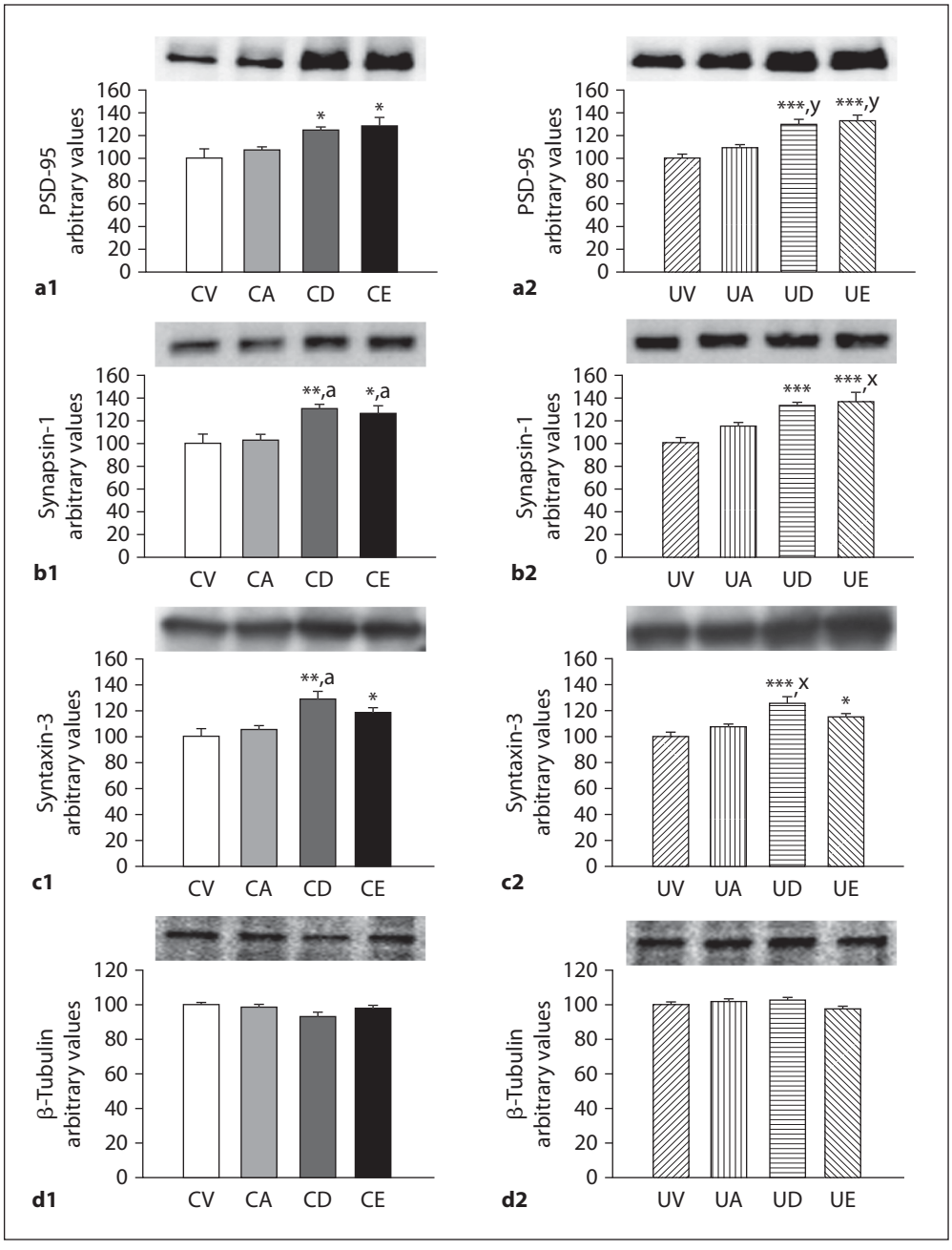

Fig. 3. Effects of AA, DHA or EPA, alone or in combination with a UMP-supplemented diet, on levels of the pre- or postsynaptic proteins PSD-95 (**a1**, **a2**); synapsin-1 (**b1**, **b2**) and syntaxin-3 (**c1**, **c2**). CV = Control diet + vehicle; CA = control diet + AA; CD = control diet + DHA; CE = control diet + EPA; UV = UMP-supplemented diet + vehicle; UA = UMP-supplemented diet + AA; UD = UMP-supplemented diet + DHA; UE = UMP-supplemented diet + EPA. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared with CV, and $^{a}p < 0.05$ compared with CA on the left-sided columns (**a1**, **b1**, **c1**) using one-way ANOVA. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared with UV, and $^{x}p < 0.05$ and $^{y}p < 0.01$ compared with UA on the right-sided columns (**a2**, **b2**, **c2**) using one-way ANOVA [data from 14].

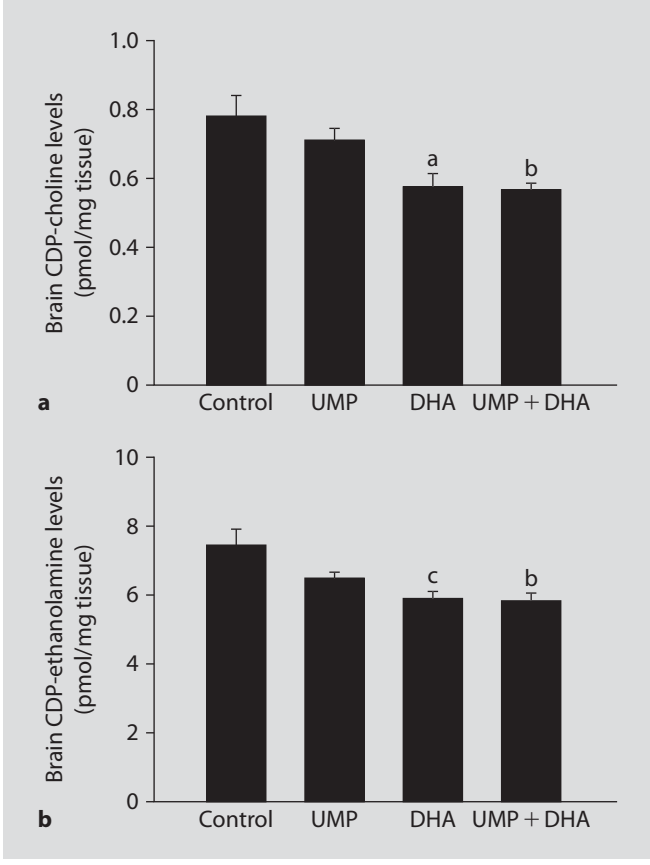

Fig. 4. Effects of DHA on brain CDP-choline or CDP-ethanolamine levels. Groups of 8 gerbils received either a control or a UMP-containing (0.5%) diet and, by gavage, DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle, for 28 days. On the 29th day their brains were harvested and assayed for (**a**) CDP-choline or (**b**) CDP-ethanolamine. $^{a}p < 0.05$ and $^{c}p < 0.01$ when compared with the values for control diet + vehicle group; $^{b}p < 0.05$ when compared with values for UMP diet + vehicle group [data from 13].

environment [33] (see section 7). Thus, the production, levels, and functional properties of PC, other phosphatides, and proteins in brain membranes, are found to depend to a surprising extent on blood levels of PC's three circulating precursors. Providing supplemental UMP or DHA without the other also increases brain phosphatide levels, but by less than when all three precursors are presented. (Choline is included in all of the diets.)

In studies designed to affirm that the increases in brain phosphatide levels caused by giving DHA, with or without UMP, reflect actual increases in phosphatide synthesis (and not, for example, inhibition of phosphatide degradation), brain levels of CDP-choline and CDP-ethanolamine, the immediate precursors of PC and PE, have also been measured. It was postulated that if DHA acted by generating more DHA-containing DAG, and if this compound then combined with endogenous CDP-choline or CDP-ethanolamine to form additional PC or PE, then DHA administration might concurrently reduce brain CDP-choline and CDP-ethanolamine levels while increasing those of PC and PE. This expectation was confirmed [13] among animals receiving DHA, or DHA + UMP; CDP-choline and CDP-ethanolamine levels fell significantly while those of PC and PE rose (fig. 4).

Properties of the Enzymes That Mediate Brain Phosphatide Synthesis

As discussed above, the ability of each of the circulating phosphatide precursors to affect the overall rate of phosphatide synthesis results from the relatively low affinities of these enzymes for their substrates. This unusual property is discussed below.

Choline Kinase

The synthesis of PC (fig. 2) is initiated by the phosphorylation of choline, in which, as described above, CK (EC 2.7.1.32) catalyzes the transfer of a monophosphate group from ATP to the hydroxyl oxygen of the choline. In some neurons choline is also used for synthesizing the neurotransmitter acetylcholine (ACh), a process catalyzed by choline acetyltransferase (ChAT), which mediates the transfer of an acetyl group from acetyl-CoA to the hydroxyl oxygen of the choline. The ACh is then stored, largely within synaptic vesicles, for future release. Like CK, ChAT has a very low affinity for its choline substrate [34, 35]. The K_m's of these enzymes in brain (which describe the choline concentrations at which the enzymes operate at only half-maximal velocity) are reportedly 2.6 mM [23] and 540 μM [36], respectively, whereas brain choline levels are only about 30–60 μM [24–26]. Hence, the rates of both phosphocholine and ACh synthesis are highly responsive to treatments which raise or lower brain choline levels.

The ability of choline administration to increase the synthesis and brain levels of phosphocholine was first noted in 1982 [21] and its similar effect on ACh in 1975 [37, 38]. It had previously been shown that the production of another brain neurotransmitter, serotonin, was increased among animals receiving physiologic doses of its circulating precursor, tryptophan [39, 40]. This was shown to be because tryptophan hydroxylase, the enzyme that determines the overall rate at which tryptophan is converted to serotonin, has a very low affinity for this substrate. Inasmuch as the affinities for choline of CK and ChAT had also been found, in in vitro studies, to be low, it seemed reasonable to enquire as to whether choline availability could also control the syntheses of phosphocholine or ACh.

Even though brain choline concentrations shared with those of tryptophan the ability to control the rates at which the precursor is used for neurotransmitter synthesis, choline and tryptophan differed in an important respect: although both are used by certain neurons for two purposes – tryptophan for conversion to serotonin and incorporation into proteins, and choline for conversion to ACh and incorporation into phospholipids – in the case of tryptophan these two processes are segregated into different parts of the neuron, the nerve terminal and perikaryon, whereas for choline both can take place within the nerve terminal inasmuch as that structure contains both ChAT and CK. Hence, the acetylation and phosphorylation of choline sometimes compete for available substrate [41, 42]: When cholinergic neurons are forced to fire frequently and maintain the rapid release of ACh, choline's incorporation into PC decreases [41] and the breakdown of membrane PC increases ('autocannibalism'), liberating additional choline for ACh synthesis [43–45]. However, when

the utilization of choline to form PC is increased (by providing supplemental uridine and an omega–3 fatty acid, as described above), ACh synthesis is not diminished, probably because so little choline is used in cholinergic neurons for phosphatide formation, relative to the amount needed for ACh synthesis [42].

CTP: Phosphocholine Cytidylyltransferase

CTP: phosphocholine cytidylyltransferase (CT; EC 2.7.7.15) catalyzes the condensation of CTP and phosphocholine to form CDP-choline (fig. 2). CT is present in both the soluble and particulate fractions of the cell [46]; the cytosolic form is reportedly inactive and the membrane-bound form active [27, 47]. Increases in the association of CT with membranes reportedly correlate with increases in CT activity and in the net synthesis of PC in vitro [48–50]. Some other lipids (e.g. PS) [51] and DAG [48, 52] also stimulate the translocation of CT from the cytosol to membranes in vitro, thereby activating the enzyme. However, translocation is clearly not the sole mechanism for CT activation, inasmuch as increases in the activity of membrane-bound CT often do not correlate with decreases in that of the cytosolic enzyme [53] (as would be expected if translocation were the only means whereby CT become activated). The phosphorylation state of CT may also be important [54] as well as the enzyme's substrate saturation with CTP and perhaps with phosphocholine, as described below.

CT has been purified to homogeneity [55], and has been cloned from rat liver [56] and from a human erythroleukemic cell line [57]. The purified form exists as an elongated dimer [58]. Mammalian CT proteins contain four functional domains: an N-terminal nuclear targeting sequence, a catalytic domain, a membrane-lipid binding domain, and a C-terminal phosphorylation domain. The K_m's of CT for CTP and phosphocholine in brains of laboratory rodents and humans are reportedly 1–1.3 mM and 0.30–0.31 mM [26, 59], respectively, while brain levels of these compounds are only 70–110 μM [20, 60, 61] and 0.32–0.69 mM [21, 25, 62] respectively. Hence, brain CT normally is highly unsaturated with CTP, and only about half-saturated with phosphocholine in vivo, suggesting that its degrees of substrate saturation, particularly with CTP, exert important limiting roles in PC synthesis. In fact, treatments that increase cellular CTP (e.g. administration of a uridine or cytidine source) have been shown to enhance CDP-choline and PC synthesis in poliovirus-infected HeLa cells [63]; undifferentiated PC12 cells [64, 65]; slices of rat corpus striatum [66], and gerbil brain in vivo [20].

CDP-Choline: 1,2-Diacylglycerol Cholinephosphotransferase

CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT; EC 2.7.8.2) catalyzes the final reaction in the Kennedy cycle; it transfers the phosphocholine moiety from CDP-choline to DAG, thus yielding PC (fig. 2). CPT, an integral membrane protein, is present primarily in the endoplasmic reticulum [67]. The enzyme protein has been solubilized and partially purified from microsomes of rat liver [68, 69], rat

brain [70] and hamster liver [71]. A human cDNA has been isolated which codes for an enzyme with both cholinephosphotransferase and ethanolamine-phosphotransferase (EPT) activities (hCEPT1 [72]), and a different human cDNA has also been isolated, the product of which exhibits only cholinephosphotransferase-specific activity (hCPT1 [73]). CPT may be a reversible enzyme, synthesizing CDP-choline from PC and CMP in microsomal preparations from liver [74, 75] or brain [76–78].

The choline phosphotransferase reaction also is unsaturated with the enzyme's substrates: its K_m values for CDP-choline and DAG in rat liver are 200 and 150 μM [79] respectively, while the concentrations of these compounds in liver are approximately 40 μM [80] and 300 μM [81]. (A DAG concentration of at least 1,000 μM thus would probably be needed to saturate the enzyme.) Brain CDP-choline and DAG levels are even lower, i.e., about 10–30 μM [20, 82] and 75 μM [61], respectively. Levels of cellular DAG have been shown to limit PC synthesis in permeabilized HeLa cells [28], cultured rat hepatocytes [83], and PC12 cells (described above) [29]. None of these studies distinguished between the enzyme which acts on both choline and ethanolamine (PECT1) and the enzyme that acts only on choline (PCT1). A more recent report, using cloning and expression methods, described the K_m of human PECT1 for CDP-choline, as being 36 μM [84], which would probably still be too high for the enzyme to be saturated with this substrate in brain. The K_m of PCT for its substrates might also be affected by the fatty acid composition of the DAG molecule; for example, incubating mouse liver microsomes with DAG molecules that contained two oleic acids (1,2-dioleoyl-sn-glycerol [Di-C18:1 (cis-9)]) rather than two palmitic acids (1,2-dipalmitoyl-sn-glycerol [Di-C16:0]), increased its K_m's for DAG from 86 ± 6 to 1,860 ± 39 μM and its K_m for CDP-choline from 41 ± 2 to 1,000 ± 141 μM [85]. Hence the enzyme's affinity for its substrates declined by 20- to 25-fold. Apparently no data are available demonstrating that fatty acids (e.g., DHA) most able to promote PC synthesis [86] do so because they enhance cellular levels of DAG species which CPT binds most effectively or on which it has greatest catalytic activity.

Uptake of Uridine into Brain and Its Conversion to UTP and CTP

Since brain uridine can, by elevating CTP levels, modulate the effect of DHA on synaptic membrane formation, the enzymes and uptake proteins that mediate blood uridine's effect on brain CTP are discussed here.

Uridine and cytidine are transported across cell membranes, including the BBB, via two families of transport proteins, i.e. the Na^+-independent, low-affinity, equilibrative transporters (ENT1 and ENT2) [87] and the Na^+-dependent, high-affinity, concentrative (CNT1, CNT2, and CNT3) [88] nucleoside transporters [89]. The two ENT proteins, which transport uridine and cytidine with similar affinities, have been cloned from rat [90] and mouse [91]. Inasmuch as their K_m values for the pyrimidines are in the high micromolar range (100–800 μM [92]) they probably mediate BBB pyrimidine uptake only when plasma levels of uridine and cytidine have been elevated experimentally. In contrast, CNT2, which transports both the pyrimidine

uridine and such purines as adenosine, probably do mediate uridine transport across the BBB under physiologic conditions. K_m values for the binding of uridine and adenosine to this protein (which has been cloned from rat BBB [93]) are in the low micromolar range (9–40 μM in kidney, intestine, spleen, liver, macrophage and monocytes [94]), while plasma uridine levels are subsaturating, i.e. 0.9–3.9 μM in rats [95], 3.1–4.9 μM in humans [95], and around 6.5 μM in gerbils [20]. Cytidine has not been thought to be a substrate for CNT2 [88], however recent studies suggest that CNT2 can also transport this compound, but with a much lower affinity than that for uridine [96–98].

It should be noted that, while both of the pyrimidines, uridine and cytidine, are present in the blood of laboratory rats, human blood contains unmeasurably low quantities of cytidine [95] even among individuals consuming a cytidine source like oral CDP-choline [19]; the cytidine is quantitatively deaminated to uridine in the human liver. Hence circulating uridine, and not cytidine, is the precursor of the brain CTP utilized for phosphatide synthesis. Gerbil blood contains both pyrimidines, but proportionately less cytidine than blood of rats; hence, gerbils are often used as a model for studying the effects of exogenous uridine sources on the human brain [99].

Like other circulating compounds, pyrimidines may also be taken up into brain via the epithelium of the choroid plexus (CP) and the ENT1, ENT2 and CNT3 transporters [87, 88]; all of these proteins have been found in CP epithelial cells of rats [90, 100, 101] and rabbits [102, 103]. However, the surface area of BBB is probably 1,000 times that of the CP epithelium (i.e., 21.6 vs. 0.021 m^2 in humans [104]), hence the BBB is the major locus at which circulating uridine enters the brain.

Uridine and cytidine are converted to their respective nucleotides by successive phosphorylations catalyzed by various kinases. Uridine-cytidine kinase (UCK) (ATP:uridine 5′-phosphotransferase, EC 2.7.1.48) phosphorylates uridine and cytidine to form UMP and CMP, respectively [105–107]. Several different forms of UCK exist, possibly as isoenzymes [108, 109]. Humans have two such isoenzymes, UCK1 and UCK2, both of which have been cloned [110, 111]. UMP-CMP kinase (UMP-CMPK) (ATP:CMP phosphotransferase, EC 2.7.4.14) [112–114] then converts UMP or CMP to UDP or CDP. These nucleotides in turn are further phosphorylated to UTP and CTP, by nucleoside diphosphate kinases (NDPK) (nucleoside triphosphate:nucleoside diphosphate phosphotransferase, EC 2.7.4.6) [115, 116]. mRNAs for UCK1 [111] and UMP-CMPK [117] have been described in brain [118, 119], as has NDPK activity.

Various interconversions between uridine and cytidine, and between their respective nucleotides, are known to occur in mammalian cells. Cytidine and CMP can be deaminated to uridine and UMP [120], while UTP is aminated to CTP by CTP synthase (UTP:ammonia ligase [ADP-forming], EC 6.3.4.2) [121, 122]. This enzyme acts by transferring an amide nitrogen from glutamine to the C-4 position of UTP, thus forming CTP [123]. CTP synthase activity has been demonstrated in rat brain [124].

All of the enzymes described above apparently are unsaturated with their respective nucleosides or nucleotides in brain and other tissues. For example, the K_m's for uridine and cytidine of UCK prepared from various tissues varied between 33 and 270 μM [106, 107, 125, 126], and the K_m for uridine of recombinant enzyme cloned from mouse brain was 40 μM [127, 128]. Brain uridine and cytidine levels are about 22–46 pmol/mg wet weight [20, 129] and 6–43 pmol/mg wet weight [20, 130], respectively. Hence, the syntheses of UTP and CTP, and the subsequent syntheses of brain PC and PE via the Kennedy pathway, depend on available levels of their pyrimidine substrates. Indeed, increasing the supply of uridine or cytidine to neuronal cells, in vitro [16, 65, 66] or in vivo [20, 99], enhanced the phosphorylation of uridine and cytidine, and elevated levels of UTP, CTP, and CDP-choline.

Availability of DHA and Other PUFAs to Brain Cells

As discussed extensively elsewhere in this volume, the omega–3 PUFAs DHA and EPA, and the omega–6 fatty acid AA are essential for humans and other animals, and thus must be obtained from the diet either as such or as their also-essential precursors, α-linolenic acid (ALA) and linoleic acid (LA).

Although the processes by which circulating PUFAs are taken up into the brain and, subsequently, into brain cells await full characterization, they are thought to include both simple diffusion (also termed 'flip-flop' [131]) and protein-mediated transport [132]. One such transport protein (B-FATP) [133] has been cloned [134]. DHA, EPA and AA are then transported from the brain's ECF into cells, and can be activated to their corresponding CoA species (e.g., docosahexaenoyl-CoA; eicosapentaenoyl-CoA; arachidonoyl-CoA) and acylated to the sn-2 position of DAG [135] to form PUFA-rich DAG species [136, 137]. DHA is acylated by a specific acyl-CoA synthetase, Acsl6 [138] which exhibits a low affinity for this substrate (K_m = 26 μM [139] relative to usual brain DHA levels (1.3–1.5 μM) [140]). Hence, treatments that raise blood DHA levels rapidly increase its uptake into and retention by brain cells.

EPA can be acylated to DAG by the Acyl-CoA synthetase [141] or it can be converted to DHA by brain astrocytes [142], allowing its effects on brain phosphatides and synaptic proteins, described below, to be mediated by DHA itself. Exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides [143, 144], as well as into other lipids, e.g. the plasmalogens [145, 146]. AA shares with DHA the ability to activate syntaxin-3 [9], however, as described below, its oral administration to laboratory rodents apparently does not promote synaptic membrane synthesis nor dendritic spine formation.

DHA and AA are major components of brain membrane phospholipids [147]. While AA is widespread through the brain and is abundant in phosphatidylinositol (PI) and PC, DHA is concentrated in synaptic regions of gray matter [148] and is especially abundant in PE and PS [149]. In contrast, EPA is found only in trace

amounts in brain phosphatides, mostly in PI [150]. No significant differences have been described between the relative proportions of ingested omega–3 and omega–6 PUFAs that actually enter the systemic circulation [151, 152]. Moreover, the rates at which radioactively-labeled DHA and AA are taken up into brain and incorporated into phospholipids following systemic injections also are similar [143, 153]. (To our knowledge, no study has compared the brain uptake of EPA with that of another PUFA in rodents or humans, however exogenously administered EPA does increase brain EPA levels in vivo [154].) On the other hand, the half-lives of the omega–3 PUFAs in the blood (20 ± 5.2 h for DHA and 67 ± 14 h for EPA [155]) are substantially higher than that for AA (3.8 s [156]). Similarly, the half-life of DHA in brain PC (22.4 ± 2.9 h), but not in PI or PE, is much longer than that of AA (3.79 ± 0.12 h) [157]. Thus, a considerable proportion of AA may be cleared from plasma or oxidized before it is utilized for PC synthesis, or, once incorporated into phosphatides, may be liberated by hydrolysis (mediated by phospholipase A_2 [158]), and then oxidized.

It should be noted that the ability of orally-administrated DAG, given daily for several weeks, to increase brain phosphatide levels does not necessarily imply that, concurrent with such increases, the quantities of DHA in the phosphatides, relative to those of other fatty acids, also are increased. Indeed this has not been demonstrated.

Effects of DHA and Other PUFAs on Synaptic Protein and Phosphatide Levels in Gerbils

In experiments designed to compare the effects of administering each of the three PUFAs, DHA, EPA, or AA, on brain phosphatide levels, animals received 300 mg/kg daily by gavage of one of the fatty acids for 4 weeks, with or without dietary UMP and with choline as noted previously. Giving DHA without uridine increased PC, PI, PE and PS levels significantly, by 18, 20, 22, and 28% respectively (table 2), throughout the brain (e.g. in cortex, striatum, hippocampus, brain stem and cerebellum) (table 3). Giving EPA also increased brain PE, PS, and PI levels significantly, by 21, 24 and 27%, respectively (table 2). In contrast, AA administration failed to affect brain levels of any of the phosphatides (table 2) [14].

Consuming the UMP-supplemented diet alone increased brain PS and PC levels significantly (by 15 and 16%, respectively) (table 2) compared with those in control gerbils. Among gerbils receiving both UMP and DHA, brain PC, PE, PS, and PI levels rose significantly by 12, 26, 34, and 38%, respectively (table 2). Similarly, among gerbils receiving both UMP and EPA, brain PC, PE, PS, and PI levels rose significantly by 13, 30, 41 and 56%, respectively (table 2). In contrast, giving UMP with AA failed to increase levels of any brain phosphatide above those found in gerbils receiving UMP alone (table 2). Total brain phospholipid levels were also elevated significantly, by 16 and 23% following treatment with UMP + DHA, or with UMP + EPA, respectively (table 2), but not by treatment with UMP + AA [14]. Essentially similar results

Table 2. Effects of various PUFAs, given with a control diet (**a**) or a UMP-supplemented diet (**b**), on gerbil brain phosphatide levels [data from 14]

a

	Total PL	PC	PE	PS	PI
Control diet + Vehicle	322	113	63	251	15
Control diet + AA	326	114	65	281	16
Control diet + DHA	344	133*	77*	32***	18*
Control diet + EPA	347	125	76*	31**	19**, a
UMP diet[1] + Vehicle	332	131*	701	29*	16

Groups of gerbils were given a control diet, and received by gavage AA, DHA, or EPA (each 300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for phosphatides as described in the text. Data are given as means ± SEM. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared to Control diet + Vehicle group, and $^{a}p < 0.05$ compared to Control diet + AA group by one-way ANOVA.

[1]Data from gerbils receiving the UMP diet but no PUFAs are included in table 2a to illustrate that uridine alone also affects phosphatide levels. Data are presented as nmol/mg protein.

b

	Total PL	PC	PE	PS	PI
UMP diet + Vehicle	332	131	70	29	16
UMP diet + AA	379	132	81	31	20
UMP diet + DHA	384*	147**, y	88**	39**	22**
UMP diet + EPA	407***	148**, y	91***	41**, x	25***

Groups of gerbils were given a UMP-containing (0.5%) diet, and received by gavage AA, DHA, or EPA (each 300 mg/kg; in a vehicle of 5% gum Arabic solution) or just its vehicle for 28 days. On the 29th day their brains were harvested and assayed for phosphatides as described in the text. Data are given as means ± SEM. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared to Control diet + Vehicle group, and $^{x}p < 0.05$ and $^{y}p < 0.01$ compared to UMP diet + AA group by one-way ANOVA.

were obtained whether data were expressed per μg DNA or per mg protein (data not shown).

Giving gerbils, as above, DHA or EPA alone significantly increased brain levels of the postsynaptic density protein PSD-95, by 24 or 28% (fig. 3a1). When this treatment was combined with dietary UMP the observed increases in PSD-95 were 29 or 33% greater than those found after UMP supplementation alone (fig. 3a2). AA failed to affect brain PSD-95 levels either when given alone or in combination with the UMP-supplemented diet (fig. 3a). Similar to PSD-95, levels of synapsin-1, a presynaptic vesicular protein, were also significantly increased, by 31 or 27% respectively, by DHA or EPA treatment alone (fig. 3b1) or by 33 or 36% when the PUFA was combined with

Table 3. Effects of giving UMP-supplemented diet (0.5%) and DHA (300 mg/kg) on phosphatide levels in different gerbil brain regions [data from 14]

	Cortex	Striatum	Hippocampus	Brain Stem	Cerebellum
Total PL					
Control diet + Vehicle	267	265	264	450	270
UMP diet + DHA	316**	339***	314**	521**	317**
PC					
Control diet + Vehicle	94	100	102	114	98
UMP diet + DHA	122***	126*	117***	139***	111***
PE					
Control diet + Vehicle	58	60	61	117	64
UMP diet + DHA	80**	85***	81***	156***	85***
PS					
Control diet + Vehicle	24	24	24	30	24
UMP diet + DHA	30***	29*	28***	35***	29**
PI					
Control diet + Vehicle	10.6	7.6	8.8	9.3	10.4
UMP diet + DHA	13.2**	11.9***	11***	11.8*	11.5*

Groups of gerbils were given a UMP-containing (0.5%) diet and, received by gavage, DHA (300 mg/kg; in a vehicle of 5% gum Arabic solution) or just the vehicle, for 28 days. On the 29th day various brain regions were harvested and assayed for phosphatides as described in the text. Data are presented as nmol/mg protein. *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$ compared to Control diet + Vehicle group using Student's t test.

UMP (fig. 3b2). Again, AA failed to affect brain synapsin-1 levels when given alone or in combination or concurrently with a UMP-supplemented diet (fig. 3b).

Also similarly to PSD-95 and synapsin-1, brain levels of syntaxin-3, a plasma membrane SNARE (soluble *N*-thylmaleimidesensitive-factor attachment protein receptor) protein which reportedly mediates the stimulation by PUFAs of neurite outgrowth [9], and exocytosis [159], in cultured cells, were significantly increased in animals receiving DHA or EPA by 29 or 19%, respectively (fig. 3c1); whether or not they also received UMP, but AA was without effect alone or in combination with UMP (fig. 3c).

None of the PUFA, given alone or with UMP, changed brain levels of the structural protein β-tubulin, perhaps reflecting its ubiquity in brain; hence, β-tubulin was used as the loading control for Western blot assays of synaptic proteins (fig. 3d).

The mechanism that allows the omega–3 fatty acids DHA and EPA, but not the omega–6 fatty acid AA to increase synaptic membrane is unclear. As discussed above, exogenously administered AA, like DHA, is preferentially incorporated into brain phosphatides [143, 144], as well as into other brain lipids (e.g. the plasmalogens [145, 146]), and AA shares with DHA the ability to activate syntaxin-3 in vitro [9].

Mechanisms that could underlie the differential effects of omega–3 and omega–6 PUFAs on membrane synthesis might include, among others, different efficacies for their uptakes into brain or their acylation; different half-lives in the circulation; different affinities for enzymes that control their incorporation into DAG and phosphatides (apparently not the case [14]; differences in the rates at which the PUFAs are removed from phosphatides by deacylation; the differential activation of genes encoding proteins needed for membrane synthesis [5], or the tendency of AA to be incorporated into phospholipids by the acylation of 1-acyl-2-lyso-snglycerophospholipids, not via the Kennedy cycle [160].

Effects of DHA and Other PUFAs on Dendritic Spine Formation and Synaptogenesis

Dendritic spines are small membranous protrusions extending from postsynaptic dendrites in neurons most of which eventually form synapses with presynaptic axon terminals. The dendritic spines compartmentalize postsynaptic responses, and their numbers are thought to reflect the density of excitatory synapses within regions of the central nervous system [161–163]. Oral supplementation with DHA to adult gerbils increases the number of dendritic spines in the hippocampus, particularly if the animals are also supplemented with UMP (fig. 5). This effect is associated with parallel increases in levels of membrane phosphatides and of various pre- and postsynaptic hippocampus proteins, as described above. Oral DHA may thus increase the number of brain synapses, particularly when co-administered with UMP [15].

Gerbils that received daily doses of DHA for 4 weeks (100 or 300 mg/kg, by gavage) exhibited increased dendritic spine density (i.e. the number of spines per length of dendrite) in CA1 pyramidal neurons (fig. 6); the increases were 12% ($p = 0.04$) with the 100 mg/kg/day dose, and 18% ($p < 0.01$) with the 300 mg/kg/day dose. These effects were amplified when gerbils received both DHA (300 mg/kg/day, by gavage, as above) and UMP (0.5%, via the standard choline-containing diet) for 4 weeks, DHA supplementation alone increasing spine density by 19% ($p < 0.04$; fig. 5) and administration of both precursors did so by 36%, or approximately double the increase produced by DHA alone ($p = 0.008$) (fig. 5). (Giving UMP alone did not affect dendritic spine density significantly (fig. 5), however it did increase spine density when all dendritic protrusions were included for statistical analysis, including the filopodia, which are precursor forms of dendritic spines.) The effect on dendritic spine density of giving both DHA and UMP was already apparent after 1 week of treatment ($p = 0.02$), and continued for as long as animals were treated (4 weeks) (fig. 5). DHA + UMP did not affect the length nor width of individual dendritic spines, only their number.

In the above experiments the increases in hippocampal phospholipids after DHA alone were: PC 8%, PE 26%, PS 75%, and PI 29% (all $p < 0.5$ except for PC), and after

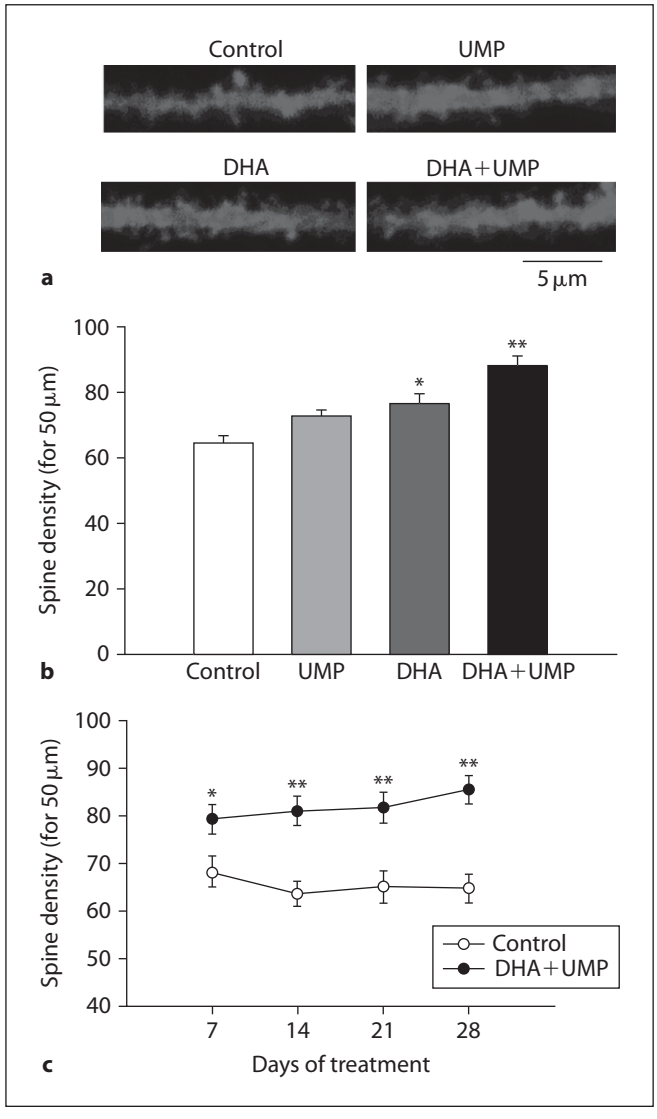

Fig. 5. Effects of DHA, alone or in combination with a UMP-supplemented diet, on dendritic spine formation in adult gerbil hippocampus. Animals received UMP (0.5%), DHA (300 mg/kg) or both daily for 4 weeks; control gerbils received neither. **a** Apical dendrites of CA1 pyramidal neurons. **b** Animals supplemented with DHA exhibited a significant increase in spine density (by 19%, *p = 0.004 vs. Control); those receiving both DHA and UMP exhibited a greater increase (by 36%, **p < 0.01 vs. Control or by 17%, p = 0.008 vs. DHA). n = 20–25 neurons from 4 animals per group. One-way ANOVA followed by Tukey's test. **c** The effect of DHA + UMP on spine density was apparent by 1 week after the start of the treatment. The treated groups received both UMP (0.5%) and DHA (300 mg/kg) daily for 1, 2, 3 or 4 weeks; the control groups were given only a regular diet. n = 12–20 neurons from 2 animals per group. Two-way ANOVA followed by Tukey's test. *p = 0.02; **p < 0.01 [data from 15].

DHA + UMP were: PC 28%, PE 59%, PS 160%, and PI 100% (all $p < 0.01$ vs. their controls). Comparable increases were noted in the pre- and postsynaptic proteins examined in the contralateral hippocampus of the same animals. Expression levels of PSD-95 [164] and GluR-1 [165, 166] are known to be highly associated with the growth of dendritic spines, and also with the intensity of the physiological responses of the postsynaptic neurons. Synapsin-1, on the other hand, is expressed in presynaptic terminals, and apparently anchors synaptic vesicles to the actin cytoskeleton for exocytosis or synaptogenesis [167, 168]. The increases in PSD-95, synapsin-1, and GluR-1 (the metabotropic glutamate receptor subunit) after treatment with DHA alone were 42, 37, and 29% (all $p \leq 0.05$), while the increases after treatment with

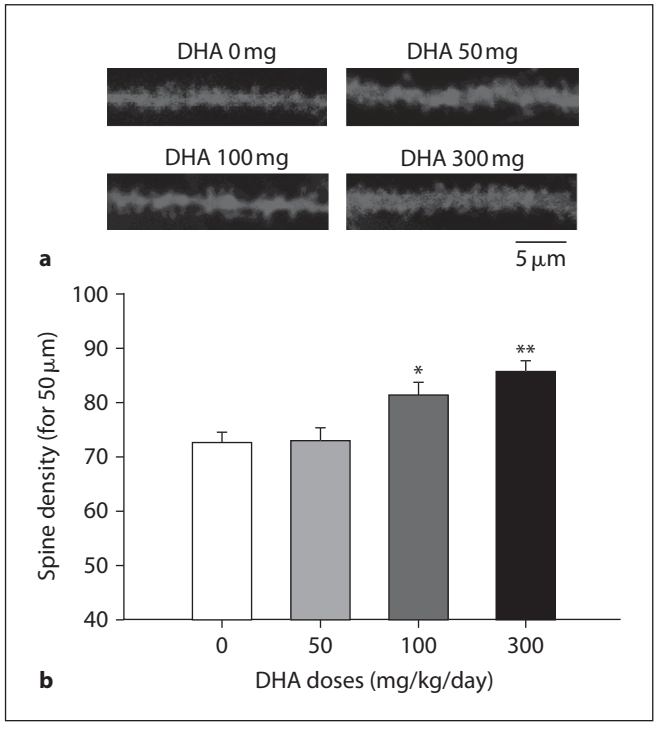

Fig. 6. Effects of oral supplementation with various doses of DHA on dendritic spine density in adult gerbil hippocampus. Animals were supplemented with 0, 50, 100 or 300 mg/kg of DHA daily for 4 weeks. **a** Apical dendrites of CA1 pyramidal neurons. **b** Animals supplemented with 100 or 300 mg/kg/day showed increased spine density: a 12% increase after the 100 mg/kg/day dose (*p = 0.04) and an 18% increase after the 300 mg/kg/day dose (**p < 0.01 vs. 0 mg/kg/day). n = 16–20 neurons from 2 animals per group [data from 15].

DHA + UMP were by 44, 57, and 37%, respectively (all $p < 0.01$). Treatment with DHA or with DHA + UMP also elevated brain levels of actin, a cytoskeletal protein which can directly regulate the morphology of dendritic spines and which is implicated in such manifestations of synaptic plasticity as long-term potentiation (LTP) and depression (LTD) [161–163, 166, 169]. Actin levels rose by 60% after DHA, and by 88% in animals receiving DHA + UMP.

In contrast, levels of β-tubulin, a cytoskeletal protein that is not specifically localized within synaptic structures, were unaffected by the treatments [15].

Oral supplementation with AA failed to affect dendritic spine density in the CA1 region of the adult gerbil hippocampus even though, like DHA, AA does affect synaptic plasticity in cultured neurons [170–172]. As described above, AA also failed to affect hippocampal levels of phosphatides or of synaptic proteins [15].

The mechanisms through which DHA, with or without uridine, increases dendritic spine formation may also involve presynaptic processes. Results from various model systems indicate that both DHA [9, 173, 174] and uridine [16, 175, 176] can promote axonal growth and exocytosis in cultured cells. As mentioned previously, DHA can activate the SNARE protein syntaxin-3 [9] while uridine, through UTP, can activate P2Y receptors [16], which are expressed in hippocampal neurons [177] and are implicated in presynaptic induction of LTP [178]. Formation of dendritic spines and synaptogenesis in mammalian brains can be induced or initiated by presynaptic

neurons, and this process may involve calcium [161–163, 179]. The increases in spine density with DHA and UMP treatment (fig. 5) may thus result from potentiation of pre- or postsynaptic mechanisms.

Effects of Uridine on Neurotransmitter Release

Consumption by rats of a diet containing uridine (as UMP) and choline can increase dopamine (DA) and ACh levels in, and – as assessed using in vivo microdialysis – their release from, corpus striatum neurons. Apparently no data are available on the effects on neurotransmitter production or release of giving DHA alone or with the other two phosphatide precursors. Dietary supplementation of aged male Fischer 344 rats with 2.5% UMP for 6 weeks, ad libitum, increased the release of striatal DA that was evoked by potassium-induced depolarization from 283 ± 9% in control rats to 341 ± 21% in those receiving the UMP ($p < 0.05$) [175]. In general, each animal's DA release correlated with its striatal DA content, measured postmortem. The levels of neurofilament-70 and neurofilament-M proteins, two markers of neurite outgrowth, were also increased after UMP treatment, to 182 ± 25% of control levels for the neurofilament-70 ($p < 0.05$) and to 221 ± 34% ($p < 0.01$) for the neurofilament-M [175].

In a similar microdialysis study, ACh release, basally as well as after administration of atropine (a muscarinic antagonist which blocks inhibitory presynaptic cholinergic receptors), was found to be enhanced following UMP consumption. Among aged animals consuming a UMP-containing diet (2.5%, w/w) for 1 or 6 weeks, baseline ACh levels in striatal microdialysates rose from 73 to 148 fmol/min after 1 week of treatment ($p < 0.05$), and to 197 fmol/min after 6 weeks ($p < 0.05$) [176]. Dietary UMP (0.5%, 1 week) also amplified the increase in ACh release caused by giving atropine (10 μM, via the artificial CSF); atropine alone increased ACh concentrations from 81 to 386 fmol/min in control rats and from 127 to 680 fmol/min in those consuming UMP ($p < 0.05$). Young rats eating the UMP-containing diet exhibited similar responses. These data suggest that giving a uridine source may enhance some cholinergic functions, perhaps by increasing synaptic membrane or the ACh stored in synaptic vesicles.

Additional evidence that treatment with UMP alone or with UMP + DHA can affect brain neurotransmission comes from a few behavioral studies [33, 180]. Among socially-impoverished rats DHA (300 mg/kg by gavage) or DHA + dietary UMP (0.5%) treatment for 4 weeks reversed the deficits in hippocampal-dependent learning and memory performance [33] (fig. 7). Similarly, chronic dietary administration of UMP (0.1%) alone for 3 months also ameliorated this impairment among the impoverished rats [180]. Since in these small studies both UMP alone and UMP + DHA completely restored learning and memory in the socially-impoverished animals, these data do not allow comparisons to be made on the relative efficacies of the two treatments.

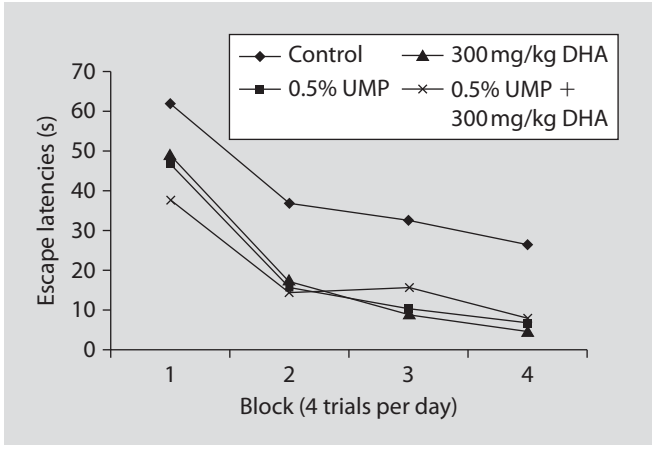

Fig. 7. Rats were allowed to eat 16% protein chow, or the same diet supplemented with 0.5% UMP and/or by gavage 300 mg/kg DHA. Rats began to eat the UMP-supplemented chow, and to receive the daily gavage of DHA, at approximately 4 weeks of age, 4 weeks prior to testing in a water maze, which continued throughout testing. Also beginning at 4 weeks of age and continuing for a duration of 4 weeks, rats were placed in impoverished conditions (IC). Impoverished conditions required that rats be housed individually without toys, and exercised 3 times a week for 15 min in an empty room to avoid weight gain. Following exposure to 4 weeks of impoverished environmental conditions, rats' learning and memory was tested using the hidden version of the Morris water maze (8 animals in each group) [data from 33].

Conclusions

Brain phosphatide synthesis requires three circulating compounds: DHA, uridine, and choline. Administration of these compounds orally increases the levels of phosphatides in brain cells, and also those of synaptic proteins and the numbers of dendritic spines; EPA but not AA reproduces these effects of DHA. This treatment may thus enhance neurotransmission.

Administering these phosphatide precursors may be useful in clinical situations in which the number of particular brain synapses and/or neurons is inadequate because of a disease process (e.g. neurodegenerative disorders like Alzheimer's or Parkinson's diseases) or a developmental disturbance (e.g. prematurity). In some of these situations patients already may be treated with supplemental DHA [3, 181]. Available data suggest that the beneficial effects of giving this PUFA will be enhanced if patients also receive adequate amounts of uridine. (They may also benefit from supplemental choline, although at least in infants, plasma choline levels are already manifold higher than they are in adults [182, 183], and breast milk is particularly rich in choline sources [183].) Clinical testing will be needed to determine whether this approach is effective.

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An Assessment of Dietary Docosahexaenoic Acid Requirements for Brain Accretion and Turnover during Early Childhood

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Brain development begins shortly after conception. In humans, the brain approaches adult mass within the first 2 years of life. Within this time, the foundation for the neural circuitry of the brain is established. As the body's central command center, the brain is responsible for orchestrating physiological actions in response to environmental stimuli. Normal brain operation is therefore critical for optimizing survivability in the animal kingdom and socioeconomic potential for humans. Early access to an adequate supply of substrates for neural development is a key requirement for preventing irreversible cognitive deficits [1–3].

The omega–3 and omega–6 long-chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA, 22:6n–3) and arachidonic acid (AA, 20:4n–6) are the major n–3 and n–6 PUFAs in the membrane structural lipids of the white and gray matter of the brain, and retina [4, 5]. In humans, AA is present throughout the gray and white matter of the brain at the sn-2 position of phosphatidylinositol and phosphatidylethanolamine [6]. DHA is highly enriched in the synaptic regions of gray matter [7] at the sn-2 position of phosphatidylethanolamine and phosphatidylserine [6]. In nervous tissue, both DHA and AA serve as precursors of signal mediators [8, 9]. They are also needed for vesicle fusion [10] and synaptic neurotransmission [11]. In addition, DHA affects several aspects of brain development and function. In particular, neuron proliferation [12], gene expression [13], size [14, 15], connectivity [16, 17], synapse turnover [18], neurotransmitter levels [19, 20], and survivability [21]. The effects of DHA on neural activities ultimately influence cognition [22] and behavior [23, 24].

LC-PUFAs are synthesized from the respective essential fatty acid precursors, α-linolenic (ALA) and linoleic acid. In humans, ALA to DHA conversion efficiency is estimated to be less than 1% [25]. Therefore, in periods of rapid brain growth [26],

Table 1. Brain weight and content of DHA by age [data from 26]

Age years	Days post-conception	Brain weight g	Brain concentration of DHA, nmol/g	Total brain DHA (calculated, mg)
–0.028	Beginning of 3rd trimester	99	2,826	92
0 (birth)	104	413	5,292	718
0.44	160.60	756	7,255	1,802
1.15	420	1,100	8,714	3,149
2.28	832	1,180	9,669	3,748

adequate dietary LC-PUFA supplementation is needed in order to obtain the minimal levels required to support optimal neurological development [27, 28]. For this reason numerous investigators have sought to determine the dietary requirement for DHA during infancy and throughout life [27, 29–31]. A number of studies have considered dietary DHA requirements based upon brain accretion rates of DHA [32], tissue concentration [33], and turnover or metabolism of DHA in the brain [31]. Initial estimates by Cunnane et al. [27] indicated that during the first 6 months of life, DHA accumulates in the brain at a rate of about 10 mg/day in breast-fed infants. They proposed that in order to support this rate of accretion or accumulation, a minimum dietary intake of 20 mg/day of DHA is needed. The accretion rate of dietary DHA in the brains of infants was therefore estimated to be about 50% of intake [27]. Based on isotope tracer information from neonatal primates, Su et al. [29, 30] reported a much lower estimate for the rate of DHA accretion. According to their estimate, only 1.7% of dietary DHA is accumulated in the primate brain, whereas only 0.23% of brain DHA accretion was attributable to conversion of its precursor, ALA. In another study, Rapoport et al. [31, 34] described aspects of human brain metabolism for both DHA and AA (20:4n–6). These authors considered rates and mechanisms of incorporation of these LC-PUFAs from plasma to replace the amounts lost by turnover. However, dietary requirements and accretion rates for specific age groups were not reported.

In the present assessment, we consider the data for DHA accretion, concentration, and turnover from previously published studies and propose a general model for human brain DHA homeostasis. An estimate for the dietary requirement of DHA during early childhood (1–2 years of age) is provided.

Methods

DHA Content in the Brain

Based on autopsy information, Martinez [26] reported the amount of DHA (nmol/g) present in the brain at different ages (e.g., third trimester to 2.28 years of age, table 1). From the third trimester of life to 2.3 years of age there is about a 12-fold increase in brain size [35], and the concentration of

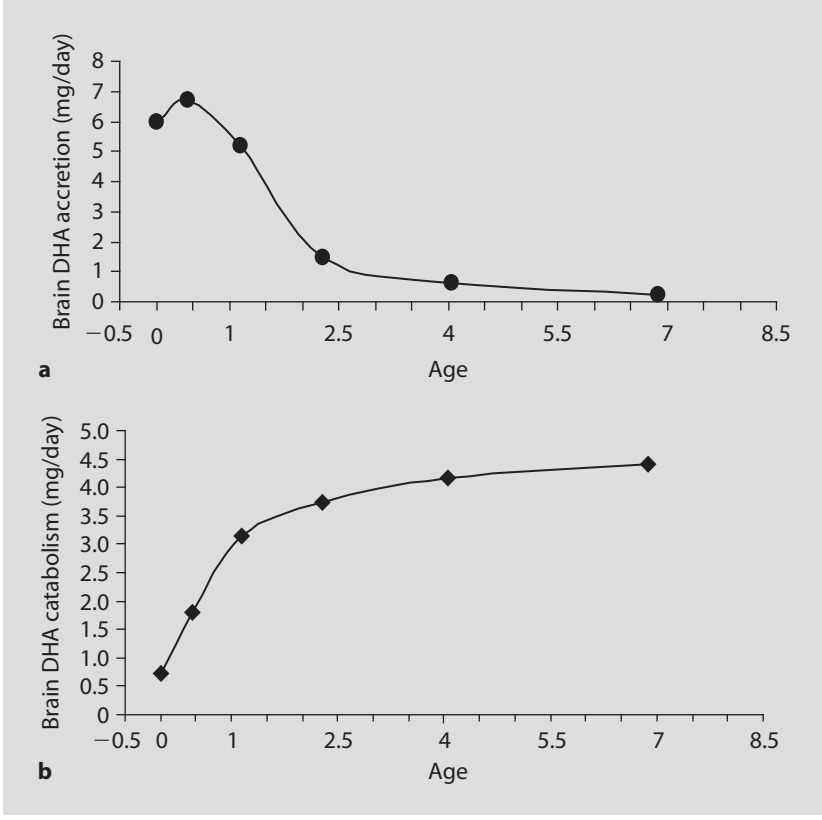

Fig. 1. DHA accretion (**a**) and estimated turnover (**b**) rate by the brain during early development.

DHA increases from 2,826 to 9,669 nmol/g [26]. To provide an estimate of the daily accretion of DHA between 1 and 2 years of age as described below, we used the information for children 1.15 and 2.28 years of age as provided by Martinez [26] (fig. 1a).

Total brain DHA for a typical 1-year-old was determined to be 3.15 g by using the molecular weight of 329 g/mol for DHA, and a brain weight of 1,100 g (table 1) (8,714 nmol × 329 ng/nmol × 10^{-6} = 2.86 mg DHA/g brain tissue; 2.86 mg DHA × 1,100 g brain = 3.2 g DHA). Total brain DHA for a 2-year-old was estimated similarly by using a brain weight of 1,180 g to calculate 3.75 g at 2.28 years of age. The amount of DHA accumulated daily in the brain between 1.15 and 2.28 years is 1.46 mg/day (3.75–3.15 g = 0.6 g or 600 mg; 832–420 days = 412 days; 600 mg/412 day = 1.46 mg DHA/day).

DHA Turnover in the Brain

In addition to the content of DHA in the brain, information about daily turnover is needed to assess DHA uptake by the brain. Turnover is defined as the quantity of unesterified DHA lost to efflux or catabolism by the brain, and is equivalent to the amount incorporated from circulation for adults [31]. Incorporation data from recently reported radioisotope-labeling studies [36] and the total content of brain DHA calculated above were used. To measure incorporation, an adult human male was administered radiolabeled DHA [36]. An accretion rate of 4.6 mg/day (14 μmol/day) was reported [36]. Incorporation at this rate for a 1,500 g brain containing 4,773 mg (9.7 μmol/g) of

DHA, according to Rapoport's described method [31] was then used. From this, an estimated turnover of 0.1%/day was derived [(14 μmol/1,500 g/day)/9.7 μmol/g] ×100).

Assuming a constant turnover throughout this time period, the estimated rate of DHA turnover for a 1- to 2-year-old child with a total amount of 3.75 g of DHA in the brain is 3.75 mg/day (0.001/day × 3.75 g) (fig. 1b).

Daily Dietary Requirement of DHA during Early Childhood

The dietary requirement of DHA is the sum of two components: daily accretion (1.45 mg/day) and turnover, i.e. catabolism (3.75 mg/day). Thus, during the period of about 1–2 years of age, 5.2 mg/day of DHA (1.45 mg + 3.75 mg/day) is needed by the brain.

Dietary DHA is available to the brain from either circulating preformed DHA or from the conversion of ALA to DHA. Following absorption, hepatic metabolism and redistribution determine LC-PUFA availability for other tissues via the circulation [37]. In the liver, 50–80% of the ALA from the diet undergoes β-oxidation [38]. In contrast, preformed dietary DHA bypasses the β-oxidation pathway and is much more bioavailable for accretion by various tissues relative to ALA derived DHA [39].

In the USA, the average intake of ALA is ~700 mg/day between 1 and 3 years of age [40]. Assuming that only 0.23% [29, 41] (0.0023 × 700 mg/day = 1.6 mg/day) of this amount is available for use by the brain, the remaining 3.6 mg/day (5.2–1.6 mg/day) must be provided by preformed dietary DHA. Because only 1.7% of dietary preformed DHA reaches the brain [29, 41], 212 mg/day (3.6 mg/day/0.017) is necessary to maintain DHA homeostasis in the brain between 1 and 2 years of age.

Discussion and Conclusions

Our calculations show that 212 mg/day of DHA is required to support the net requirement composed of brain accretion and catabolism components of 5.2 mg/day of DHA between 1 and 2 years of age. Notably, the value for brain DHA accretion and turnover that this estimate is based on is nearly identical to the estimate of about 5 mg/day previously reported by Cunnane et al. [27]. During perinatal development, brain growth is rapid and DHA plays an important role in cognitive function and visual acuity [42]. Additional amounts of DHA may be needed to prevent potential losses due to disease, infection, surgery, undernutrition or other conditions that adversely affect metabolism [27, 34].

A recommended dietary intake has not been established for DHA. Instead, recommendations at present have been based on adequate intake values established for ALA, the precursor of DHA. The Institute of Medicine (IOM) recommends that dietary intake of the omega–3 LC-PUFAs DHA and/or eicosapentaenoic acid make up 10% of the adequate intake of ALA [43]. Accordingly, the current IOM recommendation for DHA for the 1- to 3-year-old child is only 70 mg/day. Notably, the fact that eicosapentaenoic acid does not accumulate in the brain of humans [26] and primates [30, 44] is not mentioned in the IOM report [43]. Recommendations by an expert panel indicated that an intake level of 0.35% DHA, or about 80–200 mg/day, was adequate between birth and 6 months of age [45]. The World Health Organization

recommends 20 mg/kg/day of DHA for term infants based on neurological benefits [46]. In terms of dietary intake, this amounts to 172–248 mg/day for infants between the 5th and 95th percentile of body weight at 1 year of age, a level that is in agreement with the daily recommendation provided here.

Presently, the amounts of DHA added to commercially available infant formulas are based upon median LC-PUFA concentrations in Western human milk [47]. As a result, dietary concentrations of DHA in infant formulas range from 0.15 to 0.32%, or 8.1–17 mg DHA/100 kcal. Throughout the world, DHA intake by nursing infants varies widely because of differences in maternal dietary habits and access to DHA-enriched foods [47]. The mean DHA content of human milk in Japan is close to 1% compared to only 0.06% availability for nursing infants in Pakistan [48]. From this it is clear that nutrient availability in breast milk depends largely upon environmental resources. Based upon the rate of DHA accretion by the brain during the early phases of development [26] a dietary DHA requirement near 1% has been estimated by this assessment.

Other sources of LC-PUFAs for infant brain development include tissue stores and synthesis. Careful estimates indicate that in term infants peripheral body compartments are used as reserves to support brain substrate requirements when the availability of preformed DHA is inadequate [27]. Indeed, a recent study of deuterated EFA metabolism showed evidence of DHA efflux from certain tissue reserves and concomitant maintenance by the brain, retina, and other high priority systems [49]. Human infants are capable of synthesizing DHA from ALA [50]. Recent studies indicate that neonatal DHA synthesis levels of ~12 mg/kg/day are reduced to about 2 mg/kg/day as toddler age nears [51]. However, the preponderance of evidence supports a wide gap between DHA accrual by the brain due primarily to endogenous synthesis, versus preformed dietary sources [52–55]. Ultimately, the combination of limited synthesis and storage capacities by peripheral tissues necessitates preformed DHA in the diet in order to maintain circulating levels [28, 56]. In the USA, the transition from breast milk to complementary foods typically occurs between 4 and 6 months of age. Infants are typically weaned long before the brain growth spurt has been completed. DHA intake by children between 7 months and 3 years old is about 30 mg/day [57]. This amount is less than one-third of the dietary intake level from human milk, at 6 months of age.

In order to provide adequate DHA for the growing infant brain, requirements based upon brain metabolism and function are needed. Evidence of this has recently been demonstrated in non-human primates by a nearly 3-fold higher dietary DHA requirement to saturate DHA levels in the cerebral cortex, relative to mother-reared controls (63 vs. 21.3 mg DHA/100 kcal) [24]. However, the potential health benefits gained from nutritional DHA seems to persist well beyond early infancy [25–29]. In fact, a recently reported clinical study, preterm infants supplemented with 1% DHA showed improved neurodevelopment after 18 months relative to those supplied with 0.2–0.3% [58].

To better define dietary DHA requirements for optimal brain and neurophysiological health, more information about DHA concentration in the brain and overall metabolism during growth and development is necessary. Additional autopsy analyses of tissue DHA, and brain DHA uptake and turnover studies using radiotracers and other techniques are needed for accurately estimating dietary requirements for humans.

In this assessment, data for brain metabolism of DHA was derived from human and animal models. This was obviously due to technical limitations related to characterizing LC-PUFA metabolism and distribution in different tissues in humans. Other considerations include the use of data from adults for estimating DHA turnover. Reports of the DHA content in the adult brain indicate values of 4.6–5.3 g [31, 34, 59]. There are many possible reasons to explain this variation of DHA content. Since ideal DHA accumulation levels in the adult human brain have not been identified, the significance of these amounts is unknown. Moreover, studies of DHA metabolism by the human brain should be accompanied by information about biomarkers of DHA status. Such information is indeed relevant due to potential influences on the rate of DHA, and possibly total 22-carbon LC-PUFA, turnover [60]. A clearer understanding of each of these factors is important to facilitate determination of metabolic requirements for DHA by humans.

The model presented here for the dietary requirement of DHA during 1–2 years of age is based on the physiological demands of the brain rather than on an estimate derived from the caloric contribution of ALA or LC-PUFA status. The limited conversion of ALA to DHA necessitates the use of pre-formed dietary DHA. A recommendation for DHA intended for normal brain development should reflect physiological requirements throughout life. According to our estimates, 212 mg/day of dietary preformed DHA are required to support DHA accretion and turnover in the brain during (between 1 and 2 years of age) early childhood. More work is needed in this area, in order to better understand the nutritional LC-PUFA needs of the growing brain.

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Variation in Lipid-Associated Genes as They Relate to Risk of Advanced Age-Related Macular Degeneration

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Conceptual Basis of Inquiry: Points at Which Gene Variants May Impact Lipid Age-Related Macular Degeneration Relationships

Age-related macular degeneration (AMD) is a major cause of visual impairment and blindness in people of Western European ancestry [1]. Approximately 3.4 million, 1.8 million, and 130 thousand people are now living with sight-threatening AMD in Europe, the USA, and Australia, respectively; this number is expected to increase by 50% by 2020 if preventive interventions are not developed [2]. AMD manifests tissue and cellular dysfunction in the forms of abnormal angiogenesis and proliferative neovascularization [3], excessive vascular permeability [4], immunoregulatory dysfunction [5], alterations in physiologic reduction-oxidation balance [6], and neuronal/retinal pigment epithelium (RPE) cell degeneration [4]. Lipid-based compounds, their precursors, cleavage and biosynthetic enzymes, and metabolites have the capacity to modulate processes and systems implicated in AMD pathogenesis [7–14]. A number of bioinformatic and analytic methods exist to guide investigations on joint actions of genes encoding elements of biologic pathways. These have been adapted to analysis of data from genome-wide association studies [15].

An emerging evidence base indicates a lower likelihood of advanced AMD among people reporting the highest levels of eicosapentaenoic acid (EPA, 20:5ω–3,), and docosahexaenoic acid (DHA, 22:6ω–3), EPA + DHA, or fish intake [12, 16–26]. DHA, a major dietary omega–3 (ω–3) long-chain polyunsaturated fatty acid (LCPUFA), is also a major structural lipid in sensory [27] and vascular [28, 29] retina. EPA is a major dietary ω–3 LCPUFA, the precursor to both DHA and a family of potent

paracrine and autocine effectors with vaso- and immunoregulatory actions [30, 31]. While EPA is not detected in appreciable quantities within retinal phospholipids, it circulates in vascular tissue and is rapidly used for eicosanoid biosynthesis in various cellular and tissue compartments. The most efficient way to alter retinal DHA concentrations is through consumption of dietary DHA. Fish are the main dietary source of EPA and DHA [32–34]. Pioneering mechanistic studies informing inference on ω–3 LCPUFA-AMD relationships have been conducted over the past decade [7, 8, 10, 11, 14, 35–44], and breadth of findings from applied clinical research designs is now expanding.

Our central premise that *dietary and retinal fatty acids and their metabolites affect and are affected by metabolic and environmental factors and processes implicated in pathogenesis of neural and vascular retina* was developed from a number of extant reports and texts [13, 27, 45]. Elements of our conceptual framework exist in table 1. ω–3 LCPUFAs and their metabolites exhibit cytoprotective and reparative actions contributing to a number of anti-angiogenic [11] and neuroprotective [9, 46] mechanisms within the retina. AMD pathogenesis is associated with ischemia, chronic light exposure, oxidative stress, inflammation, cellular signaling mechanisms, and aging [3, 6]. ω–3 LCPUFAs operate within complex systems to impact production and activity of vaso- and immonoregulatory compounds classified as eicosanoids, angiogenic factors, matrix metalloproteinases, reactive oxygen species (ROS), cyclic nucleotides, neurotransmitters and neuromodulators, pro-inflammatory and immunoregulatory cytokines, and inflammatory phospholipids involved in these processes [reviewed in 13, 47]. Effects and actions of metabolic and environmental bioactivators and bioactive molecules include, but are not limited to, activation of phospholipase A_2 (PLA_2), cyclooxygenase (COX), and lipoxygenase (LOX), and fatty acid anhydrase. Balance and concentration of dietary ω–3 and ω–6 LCPUFA intake and tissue status impacts this enzyme system [34] to yield a pool of LCPUFAs and potent autocoids [8].

The sections that follow contain information on observed and putative relationships of ω–3 LCPUFAs with metabolic- and environment-based activators and bioactive compounds in the context of AMD pathogenesis models. We first present the body of evidence implicating LCPUFAs as key modulators of processes influencing AMD pathogenesis. We then apply empirical and inference-based methods for examining the relationship of genetic variation in LCPUFA -associated molecules with sight-threatening AMD. Our general conclusions are that: (1) there is consistent evidence to suggest that ω–3 LCPUFAs may act in protective roles for AMD; (2) whole-genome scanning technology may be applied to efficiently elucidate novel pathway and gene set-based associations with complex diseases like AMD, and (3) a knowledge-based approach to exposure ascertainment (in this case, identification and annotation of lipid-associated genes) is valuable in planning, implementation, analytic inference efforts to elucidate novel associations in complex systems.

Table 1. Central elements of our conceptual framework

Description and physiologic significance of LCPUFAs [reviewed in 45, 48–50]

- EPA has 20 carbons and 5 methylene-interrupted double bonds
- DHA has 22 carbons and 6 methylene-interrupted double bonds
- DHA, EPA are ω–3 LCPUFAs of physiologic significance; they act as constituents of lipid-protein complexes, substrates for bioactive autocoids and natural ligands to nuclear transcription factors

LCPUFA metabolism, intake, transport, and accretion [reviewed in 27, 45, 51]

- LCPUFAs may be of dietary or cellular origin
- Humans may not have capacity to meet retinal tissue needs for LCPUFA through biosynthesis
- Retinal LCPUFA tissue status is modifiable by and dependent on dietary intake
- The hepatocyte is the major site of LCPUFA biosynthesis
- LCPUFAs are esterified as components of triglycerides and phospholipids, integrated with chylomicrons or very low density lipoproteins before transport to the choriocapillaris
- LCPUFA-rich phospholipids are hydrolyzed and bind to a high affinity, receptor at the choroid-RPE interface. They are transported through the interphotoreceptor matrix to the photoreceptor inner segment. Esterified DHA-phospholipid compounds are then hydrolyzed, actively transferred to the inner segment cytosol and re-esterified into phospholipids, incorporated into photoreceptor disc membranes and transported to the outer segment. Discs migrate to the apical tip of the photoreceptor and are then shed and phagocytized by RPE cells. DHA is then stored within oil droplets in the RPE. DHA is efficiently recycled to the inner segment via a receptor-mediated process
- LCPUFAs may be biosynthesized on neural and vascular retinal endoplasmic reticulum and peroxisomes
- ω–3 LCPUFA-rich foods are based mainly in marine and lacrustine products [32, 33]
- DHA is accreted selectively and retained efficiently in photoreceptors [52]

LCPUFAs in retinal structure and function

- DHA is a major structural component of retinal membranes [27]
- DHA tissue status insufficiency is associated with altered visual processing capacity [53]
- DHA affects retinal cell signaling mechanisms in phototransduction [54–56]
- LCPUFAs influence gene expression [50, 57–59] and retinal cell differentiation, and survival [37–44, 60]

Impact of metabolic and environmental factors on LCPUFAs and implications for AMD pathogenesis [reviewed in 12, 13]

- PLA_2 cleaves LCPUFAs from their esterified form within membranes and lipoproteins to a free form capable of acting as a substrate for synthesis of potent autocrine and paracrine lipid mediators [7, 9–11, 14, 31, 46, 61]. PLA_2 is activated by light exposure, ischemia, oxidative stress, apoptosis, inflammation, cell-signaling molecules, and aging. Retinal diseases are associated with PLA_2 activity and with these metabolic and environmental factors
- COX and LOX catalyze conversion of LCPUFAs to eicosanoids. COX and LOX are activated by ischemia, oxidative stress, light exposure, apoptosis, inflammation, cell-signaling molecules, and aging. Retinal diseases of public health significance are associated both with COX/LOX activity and with these metabolic and environmental factors
- LCPUFAs demonstrate anti-angiogenic, anti-vasoproliferative, and neuroprotective actions on factors and processes implicated in the pathogenesis of vasoproliferative and neurodegenerative retinal diseases
- These actions affect eicosanoids, angiogenic factors, reactive oxygen species, matrix metalloproteinases, cyclic nucleotides, neurotransmitters and neuromodulators, pro-inflammatory and immunoregulatory cytokines, and inflammatory phospholipids

LCPUFAs and retinal diseases of public health significance [reviewed in 12, 13]

- LCPUFAs have the capacity to affect pathogenic processes implicated in retinal neovascularization
- LCPUFAs have the capacity to affect pathogenic processes implicated in retinal neural degeneration

The Nature of Essential Fatty Acids, LCPUFAs, and Their Distribution in Retina

Fatty acids are synthesized via condensation of malonyl coenzyme A (CoA) units by a fatty acid synthase complex. Two families of essential fatty acids (EFAs) exist, i.e. ω–3 and ω–6. Humans do not have capacity for de novo biosynthesis of EFAs (α-linolenic acid (ALA, 18:3ω–3) and linoleic acid (LA, 18:2ω–6), because of a natural absence of Δ15- and Δ12-desaturase enzymes. LCPUFAs may be obtained directly through the diet or formed from ALA (ω–3 LCPUFAs) or LA (ω–6 LCPUFAs).

ω–3 and ω–6 LCPUFAs contain a carboxyl head group and a carbon chain (≥18 carbons) with two or more methylene-interrupted double (unsaturated) bonds. EFAs and LCPUFAs are structurally classified by the number of carbons, double bonds, and proximity of the first double bond to the methyl (ω) terminal of the fatty acid acyl chain. Fatty acids of the ω–3 family contain a double bond at the third carbon and those of the ω–6 family contain a double bond at the sixth carbon. EPA contains 20 carbons, 5 double bonds, and has a molecular weight of 302.451. DHA has a carbon chain length of 22, 6 double bonds, and a molecular weight of 328.488. Arachidonic acid (AA, 20:4-ω6) is a major dietary ω–6 LCPUFA with 20 carbons, 4 double bonds, and a molecular weight of 304.467.

In humans, LCPUFA stores exist mainly as esterified complexes in the *sn-2* position of glycerophosphates (also known as glycerophospholipids or phospholipids) or trihydric glycerols (also known as triacylglycerols or triglycerides). Within the neural retina, phospholipids are the predominant LCPUFA-rich lipid class; these compounds act mainly as structural elements of membranes. Phosphatidylcholine (PC) represents 40–50% of retinal phospholipids and is localized mainly to the outer membrane leaflet. Phosphatidylethanolamine (PEA) and phosphatidylserine (PS) represent 30–35 and 5–10% of retinal phospholipids, respectively; both species tend to orient within the cytoplasmic leaflet. PEA and PS respectively represent 30–35 and 5–10% of retinal phospholipids; both tend to orient within the cytoplasmic leaflet. Phosphatidylinositol (PI) represents 3–6% of retinal phospholipid membrane domains [47].

Highest body concentrations of DHA per unit weight exist in phospholipids of retinal photoreceptor outer segments and the overall percent of DHA (30% of all retinal fatty acids) is 50 mol% greater than in the next most concentrated tissue [52]; DHA is also found in substantial amounts within retinal vascular tissue and glia. PEA and PS are the dominant retinal DHA-containing phospholipid species. AA is a major fatty acid of vascular and neural retinal tissue. The highest concentrations of AA are found in PC and PEA. EPA exists in vascular tissue and blood components, but does not accrue appreciable quantities to retinal tissue – it is quickly used in DHA or eicosanoid biosynthesis (reviewed in Nelson [62]).

The lipid composition of retinal photoreceptor outer segments is unique: 80–90% of structural lipids are phospholipids and 8–10% are neutral lipids [27, 63]. Neutral lipid species are mainly cholesterol, although free fatty acids can be detected as well. Retinal phospholipids are also unique because of their polyenoic nature. Polyenoic phospholipids contain PUFAs in the *sn*-1 or *sn*-2 positions of the molecule's glycerol

backbone. Phospholipids in the outer segments are predominantly dipolyenoic [64–66]. Dipolyenoic species are known to increase rate of rhodopsin activation (metarhodopsin II formation) in model membrane systems [67]; this process is essential in phototransduction.

Fliesler and Anderson [27] provide a detailed review on chemistry and metabolism of lipids in the vertebrate retina. Vertebrate retinal phospholipid species include: (1) PEA as ~40% of outer segment lipids; (2) PS as ~12%, and (3) PC as ~10% of total outer segment lipids. PC, PE, PS, and PI respectively represent ~48, 32, 9, and 4% of retinal phospholipids. DHA exists as approximately 20% of the fatty acids for outer segment PC, and ~30% for each of PE and PS [27, 68]. Half of all PC fatty acids are saturated (~30% palmitic acid and ~20% stearic acid); in PE these values are ~10 and 36%, respectively. 30% of PS fatty acids are saturated, with the greatest proportion being stearic acid (~28%).

EPA, DHA, and AA are fatty acids of physiologic significance; they act as:

- *Key structural constituents of phospholipid membranes.* DHA and AA are major fatty acids of neural and vascular retinal tissue.
- *Ligands to transcription factors* for genes influencing: (a) cellular differentiation and growth; (b) lipid, protein, and carbohydrate metabolism. DHA and EPA affect gene expression through regulation of transcription factor activity and concentration within the nucleus. Transcription factors containing a LCPUFA-binding domain include peroxisome proliferator-activated receptors (PPARs), the retinoid X receptor (RXR), nuclear factor-κB (NF-κB), and sterol regulatory element-binding proteins (SREBPs). In some cases, metabolites of the EPA and DHA also act directly as ligands.
- *Effectors of signal transduction pathways regulating gene transcription*. These pathways include enzyme-based LOX, COX, protein kinase C (PKC), and sphingomyelinase. LCPUFAs may also regulate pathways affecting serine-threonine and tyrosine kinase-linked- and G-protein receptors.
- *Substrates for eicosanoid or endocannabinoids* of in inter- and intracellular signaling cascades influencing vascular, neural, and immune function.

EFA and LCPUFA Metabolism, Transport, and Accretion to the Retina

A detailed discussion of LCPUFA metabolism, transport, and accretion appears in Chow [45]. LCPUFAs are obtained through diet or derived from EFAs. EFAs may be desaturated (by insertion of double bonds) and elongated (by addition of 2-carbon units) to LCPUFAs on the hepatic or retinal endoplasmic reticulum (ER). ALA is the dietary precursor to EPA and DHA. LA is the dietary precursor to AA. Conversion from 24- to 22-carbon LCPUFAs requires β-oxidation in the peroxisome. Because ω–3 and ω–6 EFA families compete for the same desaturases and elongases, dietary lipid balance and composition will affect production and tissue accretion of these nutrients [33, 69, 70]. Although biosynthesis of LCPUFAs from EFAs is possible, the

efficiency of tissue accretion is highest when they are ingested in the preformed state [71]. Photoreceptors are constrained in their capacity to synthesize DHA [72, 73]. RPE [73], retinal endothelium [74], and brain astrocytes [75] are able to synthesize DHA.

Gordon and Bazan [47] and Rodriguez deTurco et al. [76] present pathways through which LCPUFAs enter the retina. Within foods, EFAs and LCPUFAs exist mainly in esterified forms as triacylglycerols (TGs). During early phases of absorption, free fatty acids are hydrolyzed within the intestine from the *sn-1* and *sn-3* positions of TGs by pancreatic lipase. DHA is most likely to occupy the *sn-2* position of the resulting 2-monoglyceride. EPA exists mainly at the *sn-3*, and to a lesser extent, the *sn-1* position [32]. Free LCPUFAs and LCPUFA-monoacylglycerol complexes are subsequently re-esterified to phospholipids and TGs within enterocytes of the intestinal epithelium. TGs and phospholipids are next integrated to chylomicrons and very low density lipoproteins (VLDL), secreted into the lymphatic system, and circulated to the liver from the thoracic duct via blood. The bulk of DHA biosynthesis from ALA occurs in liver [77]. ALA enters the hepatocyte through a receptor-mediated process and is activated by CoA. The fatty acid complex subsequently enters the smooth ER where it is elongated and desaturated to DHA-CoA. DHA-CoA enters the rough ER and is esterified to phospholipids and then complexes with apoproteins. The complex arrives to the Golgi bodies in vesicular form where they are assembled into lipoproteins and secreted [78]. DHA of cellular and dietary origin is transported via VLDL lipoproteins to the choriocapillaris. Lipoprotein lipase hydrolyzes chylomicrons remnants and VLDL within the choriocapillaris. The great majority of lymph-borne esterified LCPUFAs are transported in TGs of the chylomicron and VLDL fractions; they exist to a lesser extent as free fatty acids and within PC, cholesterol ester, monoglyceride, and diglyceride pools. DHA is accreted mainly to phospholipids species composing membranes (PEA, PC, PS) in the retina. Within the circulation, LCPUFAs on chylomicron-bound TGs are hydrolyzed to their free forms by capillary-endothelial-cell-derived lipoprotein lipase. These free fatty acids may subsequently form non-covalent bonds with albumin in blood plasma for delivery to tissues.

Retinal biosynthesis of DHA is slow and may be insufficient to sustain the needs of photoreceptors [72, 73]. In addition to photoreceptors, cellular sources of DHA exist in RPE [73], retinal endothelium [74], and brain astrocytes [75]. Scott and Bazan [77] and Li et al. [79] present evidence that the liver is a key site for LCPUFA biosynthesis. Hepatic biosynthesis determines availability and distribution of LCPUFAs in plasma lipids and lipoproteins, lipoprotein assembly, and tissue uptake [47].

DHA transport via the choriocapillaris to the RPE and inner segments is likely to be mediated by a high affinity receptor-mediated uptake. Hepatectomized rats exhibit rapid accretion of LCPUFAs in neural tissue [80], suggesting that transport systems and specific binding proteins for these compounds operate effectively within the mammalian nervous system. DHA travels from the choriocapillaris via the RPE cells

and interphotoreceptor matrix (an extracellular region between the RPE and outer limiting membrane). The hydrophobic nature of fatty acids requires specific cytoplasmic transport mechanisms, binding proteins, and receptors to transport LCPUFAs to the photoreceptors. As there is no direct contact between choroidal circulation and photoreceptors, adjacent cell types including RPE cells, astrocytes, and Müller cells must aid in the process.

LCPUFA Delivery to Subcellular Membrane Systems in the Photoreceptor. LCPUFA-containing phospholipids enter the RPE and photoreceptor inner segment via a receptor-mediated transport process involving a high affinity fatty acid-binding protein with a lipoprotein lipase [47]. LCPUFAs enter the photoreceptor inner segment in a smooth ER-dense area adjacent to the base of the outer segment. After enzymatic degradation of the DHA-containing triglyceride in the inner segment, activation of fatty acid co-enzyme A initiates esterification of DHA to phosphatidic acid. De novo phospholipid, di- and triglyceride biosynthesis occurs following this process. DHA-containing phospholipids are subsequently integrated as structural constituents of photoreceptor disc membranes and are retained in proximity to rhodopsin molecules across the lifespan of the photoreceptor disc. Efficient mechanisms of repair for oxidized DHA exist and this condition allows the molecule to remain intact within photoreceptor discs. Properties of DHA retention and repair are unique among photoreceptor lipids. As discs migrate to the outer segment-RPE interface, are shed, and phagocytized the photoreceptor, the DHA content is not altered appreciably. Phagosomes are degraded in the RPE to form oil droplets containing DHA-rich triglycerides. These triglycerides are then transported back to the myoid of the inner segment for re-uptake. RPE cytosol remains virtually free of DHA-containing lipid and lipoprotein species and this condition may have important consequences for AMD prevention.

LCPUFAs in the Vascular Retina. Lecompte et al. [29] reported on fatty acid composition of isolated bovine retinal microvessels. DHA and AA each represent approximately 10% of total fatty acids in purified intact vessels. In primary cultures (confluent endothelial cell/pericyte monolayers) the value for DHA was reduced to approximately 2%; the value for AA did not change. DHA levels were restored in the cell culture with 10 μM supplementation of unesterified DHA. In endothelial cells, AA concentration was unchanged; in the case of pericytes, AA concentration was reduced. Levels of EPA in both systems were more than 10 times less than those of DHA and AA. While substantial variation existed across model systems, the mol% of EPA in retinal microvessels was 5-fold higher than that in non-vascular retina (0.5 vs. 0.1%). This is an important issue, as retroconversion of DHA to EPA is estimated at 9–11% [81, 82] in human serum [81, 82]. Also, endothelial cultures from bovine macrovascular networks (aorta) exhibit considerable retroconversion. In the Lecompte et al. [29] report, retroconversion was negligible, demonstrating specificity of fatty acid metabolism that is based on the origin of vascular tissue.

Delton-Vandenbroucke et al. [74] reported that cultured bovine retinal endothelial cells are capable of biosythesizing DHA via desaturation of docosapentaenoic

acid (DPA, 22:5) of the ω–3 family. Although EPA was the major metabolite of DPA ω–3 desaturation, DPA has been shown to represent 2 mol% of isolated and purified bovine microvessels [29].

EFA and LCPUFA Intake and Supplementation

Hibbeln et al. [33] provide estimates of ω–3 and ω–6 fatty acid intake across more than 30 countries. US residents typically consume approximately 1.6 g/day (approximately 0.7% of total energy intake) of ω–3 fatty acids [83]. The bulk of this is ALA. EPA and DHA usually represent 6–12% of total ω–3 intake (0.1–0.2 g/day). The main sources of ALA are vegetable oils. Linseed, canola, and soybean oils contain the highest levels. EPA and DHA are concentrated in fatty fish and this is the main source in the Western diet. Roughly 10% of DHA is typically derived from eggs. Products fortified with DHA and EPA are now entering the US consumer market. These products usually contain <75 mg/serving. ω–3 LCPUFAs are available as dietary supplements. Capsules typically contain 120 mg DHA and 180 mg EPA [84]. In these products EPA is usually provided from fish oil. DHA may be derived from single-celled organisms or fish oil. A list of commercially available supplements containing DHA and/or EPA, the nutrient composition of these supplements, and the supplement manufacturers exists at The Natural Medicines Comprehensive Database (http://www.naturaldatabase.com). Data from the 2003–2004 National Health and Nutrition Survey indicate that approximately 1 in 20 US residents aged ≥20 years is now taking a supplement containing ω–3 LCPUFAs.

The American Heart Association statement on ω–3 fatty acids and cardiovascular disease reviews safety of ω–3 fatty acids and fish [84] and cites dietary intake recommendations of 0.3–0.5 g/day of EPA + DHA from the World Health Organization, North Atlantic Treaty Organization, and National Health Ministries of Australia, Canada, Japan, Sweden, and the United Kingdom. Gebauer et al. [85] provide a history of recommendations. The US Food and Drug Administration (FDA) [86] stated that consumption of up to 3 g/day of marine-based ω–3 fatty acids is generally regarded as safe (GRAS). The FDA [87] has also approved a health claim for DHA and EPA in supplement form. Governmental regulatory bodies have issued statements concerning the potential for hemorrhagic risk with intake of ω–3 LCPUFAs >3 g/day [87; discussed in 84]. The antithrombotic and antihemostatic effects of ω–3 LCPUFAs operate within physiologic limits at intakes between 1.0 and 3.0 g/day [88–92]; at these levels, hemorrhagic risk is not considered a major issue, and most studies using higher amounts, up to 6 g/day, have not reported any adverse effects.

DHA Is an Essential Structural Component of Retinal Membranes

Biophysical and biochemical properties of DHA affect membrane function by altering permeability, fluidity, thickness, lipid phase properties, and the activation of membrane-bound proteins [93, 94]. DHA-rich membranes impart properties to outer segments that impact the dynamic of cellular communication processes [67, 95–99]. The

stereochemical structure of DHA allows efficient conformational change of the transmembrane protein rhodopsin, in response to the capture of light that initiates visual sensation. LCPUFA-rich membranes exhibit less rigid global properties than those concentrated in cholesterol or saturated fatty acids, since multiple unsaturated bonds and long carbon chains in LCPUFAs do not permit dense packing of the hydrophobic fatty acid components. A more fluid membrane enables faster response to light stimulation. For DHA, the position of the first unsaturated bond at the ω–3 (between Δ20 and Δ19) carbon yields gains in efficiency for membrane dynamics over those observed in an otherwise structurally identical fatty acid with the first double bond at the ω–6 carbon [100].

Biochemical characteristics of DHA provide biologic credibility of its concentration in the metabolically active retinal outer segment. The highly unsaturated nature of DHA makes it highly susceptible to oxidation in the metabolically active photoreceptor. The evolutionary significance of concentrating DHA in the photoreceptor may be that membrane-bound DHA is a primary source of lipid-derived signaling molecules that modulate intercellular communication and autocrine signaling from the plasma membrane. These processes have the capacity to influence the nuclear control of gene expression [101–105]. While esterified AA is more efficiently released from membrane stores than DHA [54], retinal astrocytes can provide a readily mobilized source of DHA for such purposes [106].

DHA Tissue Status Is Associated with Alterations in Retinal and Visual Function

Litman et al. [67, 96, 98] investigated mechanisms by which membrane fatty acid composition may affect phototransduction. Phototransduction is the process through which the retina processes light energy and converts it to a cellular signal. In a dark-adapted state, retinal photoreceptors maintain a depolarizing 'dark current' that is mediated by the effect of high cytosolic concentrations of 3′,5′-cyclic guanosine monophosphate (cGMP) that open Na^+/Ca^{2+} channels. Phototransduction starts with the capture of a photon by rhodopsin. Rhodopsin is subsequently transformed to metarhodopsin II (M(II)). M(II) binds to and activates the α-subunit of the trimeric G-protein transducin. The M(II)-transducin complex binds to and activates tetrameric cGMP phosphodiesterase (PDE) through extraction of one of inhibitory γ-subunit. Activated PDE hydrolyzes cGMP to GMP, which produces a hyperpolarized membrane due to dissociation of cGMP from Na^+/Ca^{2+} ion channels. The hyperpolarized state of the photoreceptor yields a graded decrease of glutamate release on horizontal and bipolar cells. Bipolar cells form synapses with retinal ganglion cells; axons of the retinal ganglion cells form the optic nerve that connects to brain centers that work in visual sensation and perception. The process is stopped when rhodopsin is phosphorylated by rhodopsin kinase and then bound with visual arrestin; this process inhibits formation of the M(II)-transducin complex.

Litman and Mitchell [67] demonstrated that M(II) formation to an activated membrane-bound receptor state was higher in DHA-containing model membrane

systems than in those containing AA and cholesterol. DHA enhances production of M(II) and activation of the M(II)-transducin complex is more than 2-fold greater in DHA-containing systems than it is in those concentrated with saturated and monounsaturated fatty acid species [54]. These findings support the concept that interaction in M(II)-transducin coupling is enhanced (essentially rendered more efficient) in DHA-rich membranes. Similar relationships were observed for PDE activity [96].

Actions and Associations of ω–3 LCPUFAs on Processes Implicated in AMD Pathogenesis

A number of metabolic and environmental factors and processes activate molecules associated with abnormal angiogenesis, proliferative neovascularization, excessive vascular endothelial permeability, immunoregulatory dysfunction, alterations in physiologic redox balance, and neuronal/RPE cell degeneration observed in AMD. As mentioned above, key factors and processes associated with AMD pathogeneis include ischemia, light exposure, oxidative stress, apoptosis, inflammation, neuroactive cell signaling molecules, and age-related developmental processes. These factors and processes operate in a complex system, share common modulators, and yield common outcomes. It is essential to note that in addition to affecting molecules associated with the pathogenesis of retinal disease, AMD-associated factors and processes are capable of activating: (1) PLA_2, leading to release of unesterified LCPUFAs from phospholipid membranes, and (2) COXs and LOXs that drive eicosanoid synthesis. As the concentration and composition of ω–3 LCPUFAs stored in phospholipid membranes is modifiable by and dependent upon dietary intake, the balance of free LCPUFAs and their metabolites is thus affected after activation of PLA_2, COX, and LOX. It is also essential to consider the role of factors capable of altering the concentrations of free LCPUFAs and the activity of key fatty acid cleavage and biosynthetic enzymes when investigating potential actions of ω–3 LCPUFAs in the retina. As such, we first review basic concepts related to PLA_2, COX, and LOX in the context of AMD-associated metabolic and environmental exposures. Table 2 contains references to the evidence base on the relationship of these AMD-associated factors and PLA_2, COX, and LOX.

PLA_2 in LCPUFA Hydrolysis. PLA_2s catalyze hydrolysis of fatty acids from the ester bond at the *sn-2* position of phospholipids. Hydrolysis yields free LCPUFAs and lysophospholipids. Approximately 20 groups of PLA_2s have been identified; PLA_2s are generally classified into cytosolic ($cPLA_2$), secretory ($sPLA_2$), and calcium-independent ($iPLA_2$) isoforms [111]. $cPLA_2$s are high molecular weight and cleave AA preferentially; Ca^{2+}-dependent and -independent forms exist. Intracellular $sPLA_2$s are low molecular weight; these enzymes do not show specificity for particular fatty acids. $cPLA_2\alpha$ has an *N*-terminal calcium-dependent phospholipid domain that may permit post-translational regulation by Ca^{2+} or phosphorylation via mitogen-activated protein kinase (MAPK) and PKC [129–133]. Extracellular signal-regulated kinases (ERKs) act in communication between $cPLA_2\alpha$ and $sPLA_2$s [134, 135]. DHA has been

Table 2. AMD-associated processes usually increase enzyme expression or activity of PLA_2, COX, and LOX

Disease-associated factor	Enzyme		
	PLA_2	COX	LOX
Ischemia [3]	↑ PLA_2 activity [107, 108]	↑ COX activity [109, 110]	↑ LOX activity [111]
Light exposure [112]	↑20:4ω–6 release [113]	↑ COX-2 production [114]	↑ LTB_4 production [115]
Redox balance [6]	ROS ↑ PLA_2 activity [116]	ROS ↑ COX-2 mRNA [117, 118]	ROS ↑ 5-LOX activity [119]
Cell cycle [3]	↑ [116]	Necrosis ↑ COX-2 mRNA [120]	
Inflammation [121]	↑ PLA_2 activity [122]	PAF ↑ COX-2 mRNA [123–125]	5-LOX ↓ with Rx [126]
Aging [2]	↑ [127]	↑COX-2 in senescent cells [128]	

PLA_2 = Phospholipase A_2, COX = cyclooxygenase, LOX = lipoxygenase, Redox = oxidation-reduction; ROS = reactive oxygen species; PAF = platelet-activating factor; ↑ = increases; ↓ = decreases.

shown to decrease PLA_2 activity in nerve growth cones of nerve growth factor-differentiated PC12 cells, with a predominant effect on $sPLA_2$ in calcium-independent pathways [136].

COX in Eicosanoid Biosynthesis. COX (prostaglandin endoperoxide synthase) is a protein complex that converts 20-carbon LCPUFA substrates from ω–6 (AA) and ω–3 (EPA) families to G-prostaglandin endoperoxides. Figure 1 presents a basic structure of eicosanoid metabolism. This process involves hydrogen subtraction (at carbon 11) and subsequent addition of 2 molecules of oxygen. A hydroperoxidase subsequently uses glutathione to convert the G-prostaglandins to H-prostaglandins. FitzGerald [137] reviews basic aspects of COX production, structure, and actions of COX metabolites. The constitutive form of COX (COX-1) exists mainly in the gastric mucosa, kidney and platelets, and operates primarily in the role of hemostatic regulation. The inducible form (COX-2) is expressed constitutively in the central nervous system, is activated by cytokines and mitogens, and acts in formation of prostaglandins in inflammatory response. Ringbom et al. [138] have demonstrated that DHA and EPA are effective in inhibiting COX-1- and COX-2-catalyzed prostaglandin biosynthesis with an in vitro assay. COX-2 showed higher potency. Corey et al. [139] discuss potential for DHA to operate as a competitive inhibitor of COX. COXs are activated in response to PLA_2 activation and free LCPUFA concentration.

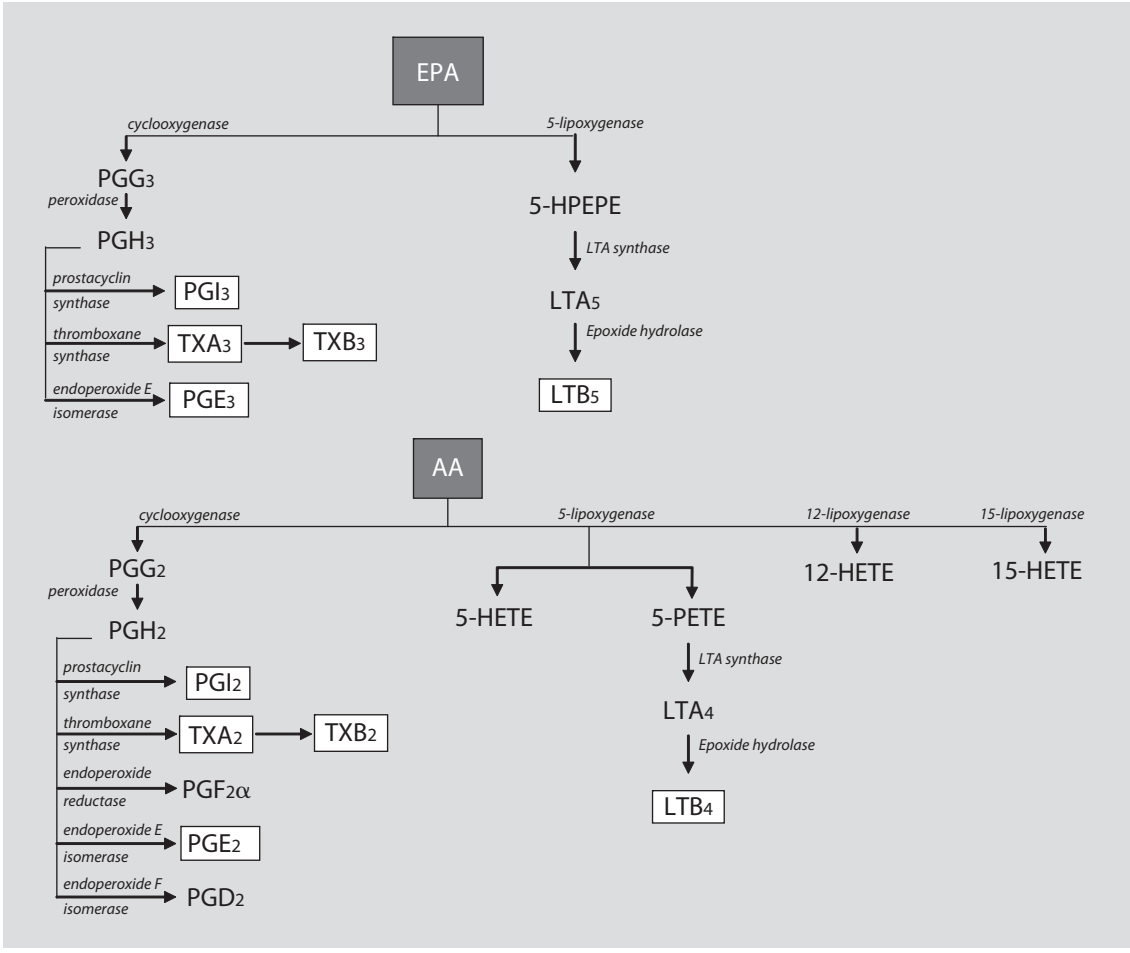

Fig. 1. Overview of eicosanoid biosynthetic pathways. AA = Arachidonic acid (20:4ω–6); EPA = eicosapentaenoic acid (20:5ω–3); HETE = hydroxyeicosatetraenoic acid; HPETE = hydroperoxyeicosatetraenoic acid; LT = leukotriene; PG = prostaglandin; TX = thromboxane.

LOX in Eicosanoid Biosynthesis. 5-LOX converts AA or EPA to hydroperoxides (hydroperoxyeicosatetraenoic acids, HPETE); this process involves removal of hydrogen at carbon 7 and insertion of molecular oxygen at carbon 5. HPETE is essential for leukotriene biosynthesis; it may also be reduced to hydroxyeicosatetraenoic acid (HETE). Activation of 5-LOX is modulated by Ca^{2+}, ATP, and 5-LOX-activating protein. 5-LOX metabolites act in immunoregulation within the inflammatory response [reviewed in 140]. LOXs are activated in response to PLA_2 activation and free LCPUFA concentration. 12- and 15-LOX are other LOX enzymes that catalyze conversion of EPA and AA to other potent compounds of physiological significance.

Ischemia, O_2 Delivery, and Hemodynamics in AMD. Retinal ischemia and concomitant hemodynamic change is associated with AMD [141, 142]. Lipid-based modulators of retinal ischemia operate as paracrine and autocrine effectors within blood and

on vascular membranes to alter blood flow and O_2 regulation. LCPUFAs are precursors to vasoregulatory thromboxanes (TX) and prostaglandins (PG) affecting blood flow [143], vasomotility, platelet activity, and platelet aggregation [88–91]. As precursors to lipid mediators and endogenous ligands to nuclear transcription factors, ω–3 LCPUFAs are associated with beneficial or non-damaging cellular response in vascular systems subjected to physiologic stresses that alter blood flow, oxygen delivery [70, 143–148] and lipoprotein metabolism [50, 149, 150].

Chronic Light Exposure and AMD. Under normal physiological conditions, retinal photic damage is unlikely to occur, despite the high density of photosensitive compounds (chromophores) and the chronic nature of light exposure. Findings on sunlight exposure and AMD are equivocal [151], however photic damage is difficult to measure in free-living humans. Boulton et al. [112] review plausible processes that may operate in retinal diseases associated with an overstimulation of photoreceptors; in some cases, models of chronic light exposure may offer important insight to relationships between photoreceptor activity and AMD. Cellular response to chronic light exposure (light adaptation) alters rhodopsin and membrane fatty acid concentration. As a result of this regulation the healthy retina exhibits a stable capacity for photon capture that is independent of stimulus intensity. In this way cells maintain sensitive response to light without sustaining injury from high metabolic demand. DHA may impact this process, as IRBP, a key transport protein involved in photopigment regeneration, contains a high-affinity DHA-binding site [152, 153]. Light exposure leads to transformation of 11-*cis*-retinaldehyde to all-*trans*-retinaldehyde (a chromophore with a peak absorption spectrum in the range of high-energy short-wavelength light associated with photic damage) and all-*trans*-retinol (a by-product of phototransduction with membranolytic characteristics) [112]. The cycle of rhodopsin regeneration from all-*trans*-retinaldehyde to 11-*cis*-retinaldehyde and reduction of all-*trans*-retinol is implicated in photoreceptor survival. IRBP transports regenerated 11-*cis*-retinaldehyde from the RPE to the photoreceptor. Chen et al. [152] reported that DHA rapidly and specifically displaced 11-*cis*-retinaldehyde from IRBP of bovine samples and suggest that the gradient of DHA between RPE (3.5% of total lipids as DHA) and photoreceptor cells (20% of total lipids as DHA) would enable a swap of 11-*cis*-retinaldehyde for DHA as IRBP approaches the RPE. All-*trans*-retinol exhibits a high affinity to IRBP. As the protein comes in contact with the DHA-rich photoreceptor, the 11-*cis*-retinoid may be released, allowing DHA to bind in its place. All-*trans*-retinol also exhibits a high affinity to the receptor in and may thus bind to the complex as it approaches the outer segments in transit to the RPE.

In the healthy retina, light adaptation is related to reduction of oxidative stress, as it leads to a decrease in photoreceptor oxygen consumption [142]. Organisciak et al. [154] provide evidence to suggest that light damage and photopigment concentration affect oxidative processes since heme oxygenase, an oxygen-sensitive stress protein, is upregulated by retinal photic injury and rhodopsin loss.

Retinal Energy Production, Regulation, Metabolism, Reduction-Oxidation Balance and AMD. Beatty et al. [6] discuss how natural characteristics of the retina affect cellular oxidation-reduction (redox) balance to favor oxidation. These are: the volume of O_2 consumption required to support the metabolic needs of the photoreceptors, the high concentration of photosensitizing compounds in the photoreceptors and RPE, the high concentration of PUFAs in photoreceptors, and active phagocytosis of photoreceptor outer segments by the RPE. The metabolically active neural retina supports energy requirements with ATP produced from oxygen and nutrient-based substrates (fatty acids and glucose, pyruvate, and lactate) within photoreceptor inner segment mitochondria. As a means of supporting the metabolic needs of the cell, photoreceptor inner segments are densely packed with these with organelles.

Mitochondria are the major site of ROS generation and gains in energy processing efficiency are believed to lower production rates and volume of these compounds. ROS can damage bases in nucleic acids, amino acid side chains in proteins, and unsaturated bonds in fatty acids. Mitochondria are the main site for superoxide anion ($O_2^{-\bullet}$) generation; because it is highly reactive, superoxide is unlikely to exist far from mitochondria-dense cytosolic regions. The superoxide anion is produced via addition of an electron to molecular oxygen; it may react with nitric oxide (NO) to form peroxynitrate ($ONOO^-$). Superoxide may also be converted to hydrogen peroxide. Hydrogen peroxide has a long enough half-life to allow it to travel to the nuclear domain; hydrogen peroxide oxidizes –SH groups of resident proteins and reacts with divalent metal catalysts (released from injury of hemolysis), and via single electron transfer, yields highly reactive hydroxyl radicals. The hydroxyl radical is the most ROS in the body. The half-life of the hydroxyl radical is short ($\sim 10^{-8}$ s), but it has a relatively high oxidation potential. The hydroxyl radical can be formed in the nucleus and produce covalent cross-linking of nucleic acid bases. The hydroxyl radical reacts with esterified membrane lipids to yield lipid radicals. Lipid radicals combine with oxygen to yield highly reactive lipid peroxyl and –hydroperoxyl radicals. Lipid peroxyl and –hydroperoxyl radicals exist mainly in biological membranes rich in PUFA. Phospholipid bilayers of cell membranes, when concentrated with PUFAs, are rich sources of electrons used to reduce ROS. Balazy and Nigam [127] review the multiple aspects of lipid peroxidation. Free radicals extract hydrogen from unsaturated bonds of membrane lipids, yielding lipid peroxyl radicals and lipid peroxides. Adjacent fatty acids are subsequently oxidized in the attempt to reduce the peroxyl radical to a stable compound.

DHA may be important as a structural or signaling molecule (or precursor for a molecule) in mitochondrial function. DHA-supplemented primary photoreceptor cultures exhibit no change in proportion of cells maintaining mitochondrial membrane potential after exposure to oxidative stress from paraquat (a superoxide anion generator), while 50% of cells in unsupplemented cultures cannot sustain function [41]. A 20% higher proportion of cells with functional mitochondria are observed in the same model system when comparing DHA- and DHA-free cultures exposed to ambient air [42]. Brenna and Diau [155] report that brain DHA concentration

increases directly as a function metabolic of rate in vervet monkeys and suggest that DHA may act as an essential structural element in bioenergetic processes.

A number of model systems have demonstrated increased efficiency of ATP production and energy use within mitochondrial membranes of cardiac tissue in animals with higher levels of phospholipid ω–3 LCPUFAs [156]. After ischemic challenge, recovery of cardiac mitochondrial function in rats fed a fish oil diet was better than that observed in a group consuming an ω–3 LCPUFA-free diet [157]. These results suggest that ω–3 LCPUFAs enhance processes of energy metabolism with minimal cost of energy substrate expenditure. It is currently unknown whether ω–3 LCPUFAs may influence efficiency of energy production within the retina.

Age-Related Developmental Processes and Chronic Environmental Exposures in AMD. Structure and metabolic efficiency of the retina changes across developmental periods. These events may be driven by systemic development processes or exposures and we offer information in this context. Pieri [158] notes the kinetics of aging are impacted by multiple causes and presents biochemical evidence to support the free radical theory of aging. This theory evolved from its original form (oxygen is a causal factor of aging) to the oxidative stress theory (age-related injury in cellular systems may result from an insufficient antioxidant potential and excessive oxidative stress that coexists with insufficient damage repair and detoxification activity). Yu and Yang [159] provide elements of the oxidative stress theory: (1) there is a diverse physiological origin of reactive species (oxygen, glucose, protein, DNA, NO, aldehyde); (2) there is value in assessing the balance of oxidants to antioxidant defense systems – defense processes and factors include cellular compartmentalization, DNA repair, antioxidant enzymes, antioxidant vitamins, other compounds with antioxidant properties, and (3) there are various types of reactions between ROS and other bioactive molecules – these include damage, damage repair, detoxification. Beatty et al. [6] suggest changes in oxidant load, elastin, collagen, and mitochondrial and nuclear DNA as putative pathogenic factors associated with age-related oxidative retinal damage. These authors observe that exogenous and endogenous retinal defense systems are compromised with advancing age.

Mitochondrial structure and function are affected with age [160]; these changes may lead to increased production of ROS. In addition to the potential effects of age-related cumulative oxygen load, there is a concomitant alteration in tissue status of aqueous and lipid-soluble vitamins with antioxidant properties [reviewed in 112]. Mitochondrial aging involves membrane composition and fluidity alterations that are purported to negatively affect biophysical response in bioenergetics. Study of this topic is germane to issues discussed throughout this work because the bulk of free radicals in retina are derived from mitochondrial respiration. Aging membranes become less fluid and fluidity is determined largely in part by membrane lipid concentration and composition (phospholipid species, fatty acid acyl chain length, number of double bonds, position of the first double bond from the methyl terminal, phospholipids-to-cholesterol ratio). Mitochondria produce ROS that target membrane lipids. The

role of mitochondria in AMD is discussed in the section above and has been a central focus in our research.

The biochemical nature of DHA and AA and the selective tissue distribution of these compounds to metabolically active retinal tissue are paradoxical, as it would appear to facilitate formation of lipid radicals, lipid peroxyl radicals, and lipid peroxides. The importance of LCPUFAs in the retina is indicated by the efficient conservation and use of these 'easily oxidized' lipids in areas highly susceptible to oxidative stress and under conditions that facilitate production of ROS [47]. There is evidence to suggest that foveal regions exposed to highest intensity light have lower concentration of LCPUFAs [161]; in age-related (chronic) retinal disease the fovea is often spared until late stages of disease.

In vitro studies generally report reactive LCPUFA peroxidation in response to energy or oxygen exposure, implicating these compounds as 'suicide antioxidants.' In contrast, most in vivo studies have reported relationships in the direction of benefit. Muggli [162] reviews studies reporting relationships of ω–3 LCPUFA or fish intake with ROS-mediated events, effects on ROS biomarkers, and effects on antioxidant defense systems. Free radical-induced hemolysis [163] and in vitro LDL oxidation [164] were both reduced in samples from people on fish oil diets. Urinary F2-isoprostanes are in vivo markers of lipid peroxidation and oxidant stress. Nonsmoking, treated-hypertensive, type 2 diabetic subjects consuming 4 g/day of purified EPA and DHA had lower levels of this biomarker than a matched comparison group consuming an olive oil supplement [165]. Fish oil exposure is also associated with reduction in superoxide anion generation in human samples [166, 167]. In some cases, in vivo oxidation of LDL was not altered as a function of LCPUFA intake [168–170]; in others it was decreased [171]. In elderly subjects, ω–3 LCPUFA intake at low doses (180 mg/day) was associated with decreases in oxidative stress within platelets [88]. At higher doses (50 μmol/l) DHA operated as a pro-oxidant [172].

An in vitro study on human cells reported an age- and area-related susceptibility to peroxidation, with higher posterior pole oxidation within tissue from the oldest subjects [173]. The oxidative damage of peripheral retina did not vary with age. Rotstein et al. [40] applied an in vitro model of oxidative stress on pure rat retina neurons to elucidate a mechanism by which DHA may operate as a neuroprotective factor. After primary retinal cells were exposed to an environmental oxidant that generates the superoxide anion, they were observed to die by apoptosis; loss of mitochondrial membrane integrity was seen as a key factor in this event. Addition of DHA to the cultures protected photoreceptors from oxidative stress-induced apoptosis. Authors speculate that DHA operates to preserve mitochondrial membrane structure and function by reducing Bax and increasing Bcl-2 expression. In rats, lower DHA tissue status is associated with lower susceptibility to light damage from acute exposure of 700–800 lx followed by 90 min of darkness [174]. After exposure to intense green light using intermittent or hyperthermic light treatments, rats fed a depleted ω–3 diet exhibited better structural outcomes than rats fed a linolenic

acid-enriched diet from flaxseed [175]. A series of intriguing studies on the role of CYP450 EPA-derived epoxides in regulation of vascular systems is now emerging to suggest that oxygenated ω–3 LCPUFAs may have positive physiologic significance [176].

Cellular Differentiation and Survival. DHA acts as a trophic molecule in photoreceptor development, differentiation, and growth. It works in part by increasing opsin expression and apical process differentiation [37, 38, 177]. The proportion DHA-fed cells expressing opsin was significantly higher than in those from a DHA-free culture. Opsin combines with 11-*cis*-retinal to form rhodopsin; the relevance of this issue for retinal health is that expression of the opsin gene may be required for assembly of photoreceptor disc membranes [178]. Although DHA may not act in determination of photoreceptor fate, it was shown to enhance differentiation in cone-rod homeobox (Crx)-expressing cells [43]. Crx in photoreceptor progenitors is necessary for expression of transcripts associated with photoreceptor maturation.

DHA or its metabolities prolong retinal cell survival and prevent apoptosis in model systems of photoreceptors [37–40, 177] and RPE cells [7, 9, 10, 14, 46]. The proportion photoreceptors supplemented with DHA that survived for 11 days in vitro was approximately twice that observed from cultures existing on DHA-free media. Measures of apoptosis (fragmented photoreceptor nuclei) suggested a protective effect of DHA at post-plating days 7 and 11. In primary cultures of rat neural retina cells, DHA acted as a protective agent against oxidant- and ceramide-induced cell death by upregulating expression of the anti-apoptotic factor, Bcl-2 [179]. Ceramide is a lipid-based mediator of apoptosis; DHA acts via ceramide glycosylation to limit endogenous levels of this molecule [179]. In photoreceptor cultures DHA was also shown to act on the ERK/MAPK pathway to prevent apoptosis [42]. DHA-derived neuroprotectin 1 acts as a potent cytokine- and oxidant-induced factor preventing apoptosis in RPE cell cultures [8]. In these systems NPD1 decreased proapoptotic proteins Bax and Bad, upregulated the anti-apoptotic Bcl-2 and Bcl-x(L) expression, inhibited oxidative-stress-induced caspase-3 activation [7, 14]. Detailed coverage on the role of NPD1 in the retina and its relationship with neurotrophins exists elsewhere in this issue of *WRND*.

Inflammation and Lipid-Based Molecules. Calder et al. [61, 180, 181] provide a number of excellent reviews on the modulatory role of ω–3 LCPUFAs in immune and inflammatory processes. We have reviewed the putative relationship of LCPUFAs with retinal inflammation in detail elsewhere [13]. Inflammation, the immediate biologic response to injury or infection, is the result of increased capillary permeability and blood flow. Increased capillary permeability enables regulatory proteins (antibodies, complement, and cytokines) and leukocytes (monocytes, macrophages, natural killer (NK) lymphocytes, and granulocytes) to pass from the bloodstream across the vascular endothelial wall. Integration of this innate immune response with an acquired one subsequently occurs as activated macrophages and monocytes present antigen to cytotoxic (CD8+) and helper (CD4+) T lymphocytes.

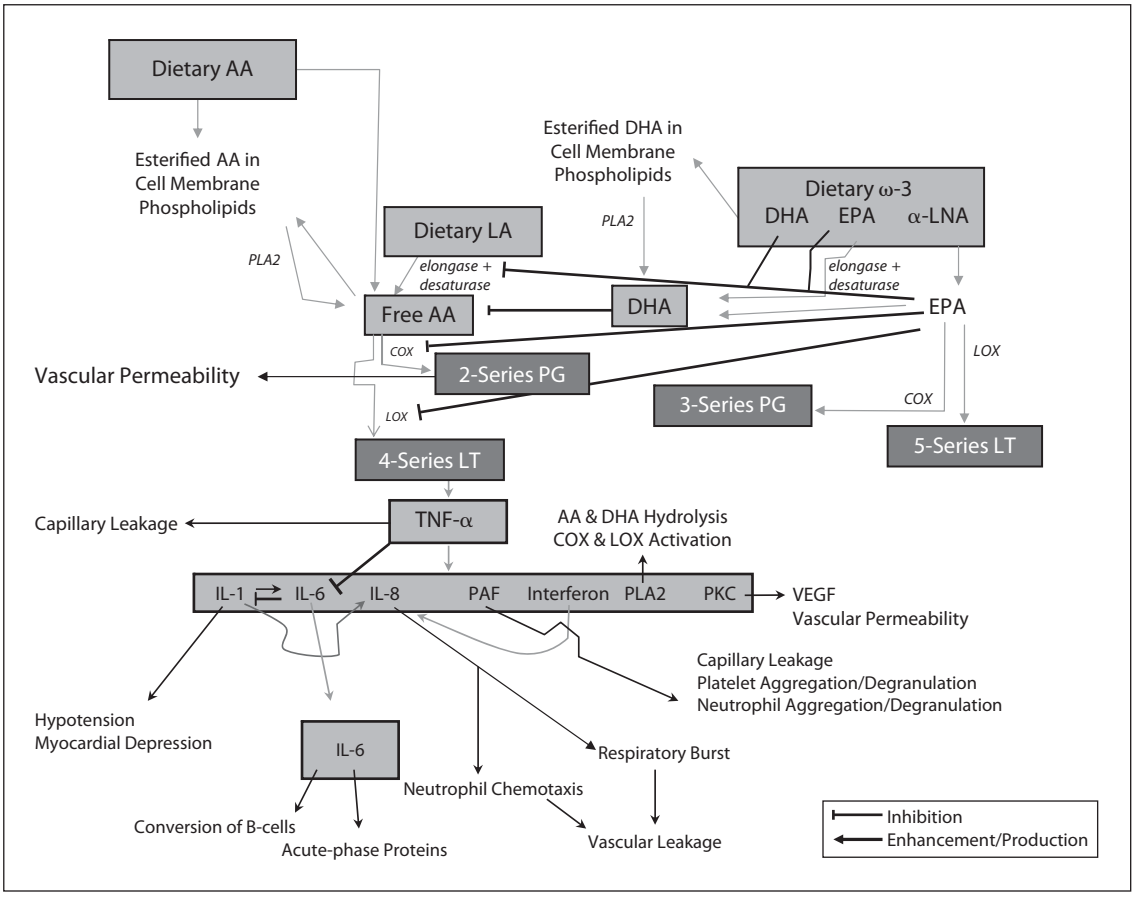

Fig. 2. Impact of ω–3 LCPUFAs on inflammatory mediators derived from arachidonic acid. AA = Arachidonic acid (20:4ω–6); COX = cyclooxygenase; DHA = docosahexaenoic acid (22:6ω–3); EPA = eicosapentaenoic acid (20:5ω–3); HETE = hydroxyeicosatetraenoic acid; HPETE = hydroperoxyeicosatetraenoic acid; IFN = interferon; Ig = immunoglobulin; IL = interleukin; LOX = lipoxygenase; LT = leukotriene; PAF = platelet-activating factor; PKC = protein kinase C; PLA_2 = phospholipase A_2; PG = prostaglandin; TX = thromboxane.

Helper T lymphocytes express CD4+ receptors that recognize peptide fragments on cell surfaces that are bound in the class II major histocompatibility complex (MHCII). These peptides are derived from extracellular pathogens that have been phagocytosed by macrophages or endocytosed by antigen-presenting cells (macrophages, dendritic cells, B lymphocytes). Inflammation activates PLA_2, COX, and LOX. An emerging evidence base suggests that inflammatory processes [121] as well as genes affecting and affected by such processes [5] are implicated processes and factors in AMD pathogenesis, and ROP. ω–3 LCPUFAs act as the precursors to a number of bioactive lipid-based immuno- and inflammatory eicosanoids, resolvins, and neuroprotectins [7–10, 14, 182]. Metabolic pathways of eicosanoid biosynthesis are presented in figure 1.

ω–3 LCPUFAs modulate production of AA-derived eicosanoids and this is important for a number of reasons. First, LTB_4 is associated with TNF-α production [183]. TNF-α mediates production of a number of potent pro-inflammatory and immunoregulatory cytokines [180]. Also, ω–3 LCPUFA-derived eicosanoids may operate directly on factors in the immune system or via a number soluble mediators, the inflammatory phospholipids platelet-activating factor (PAF), NO, and tyrosine and serine/threonine kinases. While AA-derived eicosanoids play different roles in the inflammatory process, they are all associated with vascular leakage. PGE_2 decreases T-cell proliferation, lymphocyte migration, and secretion of IL-1 and IL-2. PGI_2 blocks leukocyte aggregation, T-cell proliferation, and lymphocyte migration and secretion of IL-1 and IL-2. TXA_2 increases lymphocyte proliferation. LTB_4 increases leukocyte chemotaxis and aggregation, T-cell proliferation, and the release of TNF-α, IFN-γ, IL-1, and IL-2. Figure 2 presents one scenario on the putative effect of ω–3 LCPUFAs and their metabolites on AA metabolites (and associated compounds) implicated in inflammatory processes.

AA-based COX and LOX metabolites are extremely potent at low concentrations and act as autocoids through G-protein-mediated signaling pathways to increase cAMP levels at the site of biosynthesis. PGE_2, PGI_2, TXA_2, and LTB_4 are the most potent AA-derived angiogenic eicosanoids. PGE_2 increases vasodilation and enhances vasodilatory effects of bradykinin and histamine. PGI_2 induces vasodilation and cAMP production and inhibits leukocyte and platelet aggregation – thus the tissue system in which this molecule acts is an important consideration when speculating about its role in health and disease. TXA_2 induces platelet aggregation and vasoconstriction and LTB_4 induces vascular permeability. As capillary integrity is broached, vascular leakage and recruitment of immune system cells occurs.

The immune system cells are capable of producing pathogenic inflammatory mediators and angiogenic growth factors. The existing instability of the capillary basement membrane then permits out-migration of activated vascular endothelial cells seen in abnormal angiogenesis. AA metabolites of the COX pathway induce vascular endothelial cell migration and tube formation [184]. The AA-derived LOX product 12-HETE promotes tumor angiogenesis through induction of mitogenic activity and migration in microvascular endothelial cells [185, 186]. 12-HETE also induces cell surface integrin $\alpha_v\beta_3$ expression [187, 188] in cell cultures from rodent models [187, 188]. AA-derived leukotrienes affect the production of TNF-α [189], a potent cytokine activating numerous cascades that modulate systemic inflammation and apoptosis. ω–3 LCPUFAs alter both substrate and enzymes involved in the production of immuno- and vasoregulatory eicosanoids.

Calder [61, 180] proposes mechanisms by which ω–3 LCPUFAs may reduce the effect of AA-derived angiogenic eicosanoids – these are: (1) ω–6-based eicosanoid precursor (AA) displacement. This action alters AA availability for catalysis in membrane phospholipids. (2) PLA_2 inhibition. PLA_2 is the enzyme necessary to release the precursor for metabolism. (3) Biosynthetic eicosanoid synthase and peroxidase

quenching. These enzymes are used for series-2 prostanoid and series-4 leukotriene production. (4) Decreased 3-series (PG and TX) and the 5-series (LT) eicosanoid production. ω–3 LCPUFAs act as the precursors to these molecules. 3- and 5-series eicosanoids are in most cases less potent than AA-based eicosanoids of the 2- and 4-series. In many cases the ω–3 LCPUFA metabolites have been linked to protective actions in model cell systems.

AA-derived LTB_4 affects the production of TNF-α [189]. TNF-α is detectable in retinal vascular tissue and extracellular matrix of fibrovascular tissue in people with vasoproliferative diabetic retinopathy (PDR) [190]. A TNF-α gene polymorphism in the MCH of people with non-insulin-dependent diabetes mellitus (NIDDM) is associated with PDR and vitreous levels of soluble TNF-α receptors were higher in eyes with PDR than in eyes of healthy people without diabetes [191]; these results were interpreted to represent a form of genetic susceptibility [192]. People with insulin-dependent diabetes mellitus (IDDM) and PDR have higher concentrations of soluble TNF-α receptors than both (1) people with IDDM who are PDR-free, and (2) healthy controls [193]. TNF-α production and expression are also modified in a rodent model ischemia-induced retinopathy affecting retinal vasculature [194] – while ischemia may play less of a part in AMD pathogenesis than in that of PDR, it is reasonable to suspect that the shared neovascular component may be modulated by similar factors and processes.

A large body of evidence implicates ω–3 LCPUFAs in alteration of the innate and acquired immune systems and the inflammatory response within neural and vasucular tissue. Model studies on human cell lines incubated with ω–3 LCPUFAs demonstrated decreased: (1) monocyte cell surface antigen presentation [195] and TNF-α and IL-1β expression [196]; (2) neutrophil superoxide presentation [167]; (3) NK lymphocyte activation [197, 198], and (4) lymphocyte proliferation [197, 199–204], antigen expression [205], and IL-2 production [197, 199]. De Caterina et al. [206] have added DHA to adult saphenous vein endothelial cell cultures activated by cytokines to observe reduced expression of IL-6 and IL-8.

Animal feeding studies demonstrate differences in immune system function in comparison of animals receiving ω–3 LCPUFA-rich diets to those consuming ω–3 LCPUFA-free diets; in these studies animals on ω–3 LCPUFA-rich diets show decreased: (a) macrophage-based ROS production [207–210], cell surface antigen presentation [211, 212], TNF-α expression [183, 213–215], IL-1β expression [183, 213–215], IL-6 expression [183, 213–215], and IFN-γ receptor expression [216]; (b) monocyte IL-1β and TNF-α expression [217]; (c) NK cell activation [218–221]; (d) cytotoxic T-lymphocyte activation [222], and (e) lymphocyte proliferation [218, 222–232] and production of IL-2 and IFN-γ [233].

Human feeding studies demonstrate similar results at high doses of ω–3 LCPUFAs. Human studies also emphasize the importance of considering the balance of ω–3/ω–6 LCPUFAs. Three [234–236] of four studies demonstrate reduced monocyte chemotaxis in populations consuming ω–3 LCPUFA-rich diets. Subjects in the study that

did not demonstrate a difference between dietary groups [237] consumed a relatively lower amount of ω–3 LCPUFAs. Monocyte surface antigen expression, NK lymphocyte activation [238] and lymphocyte proliferation [239, 240] decreased in people consuming high levels of ω–3 LCPUFAs. The relationship between intake of ω–3 LCPUFAs and production of pro-inflammatory cytokines is not clear from studies in human populations. Approximately half [236, 239, 241, 242] of the existing studies [237, 240, 243–245] show an inhibitory effect of ω–3 LCPUFA feeding on TNF-α expression. Four [202, 236, 239, 241, 242] feeding studies demonstrated an inhibitory effect on IL-1β expression while others did not [237, 240, 243–246]. An inhibitory effect on IL-6 expression was reported from two [239, 247] of four [237, 244] studies. All feeding studies examining the effect of ω–3 LCPUFAs on IL-2 production and IFN-γ production demonstrated an inhibitory effect in people consuming ω–3 LCPUFAs [239, 242].

In addition to their effects on AA-eicosanoid metabolism, ω–3 LCPUFAs may influence vascular function and inflammatory response via modulation of intracellular signaling pathways and transcription factor activation [102, 103]. DHA and EPA act as natural ligands to a number of nuclear hormone receptors that affect transcriptional activities. Among these are PPAR [105] and RXR [104]. The activated PPAR-γ/RXR heterodimer regulates genes modulating induction of inflammatory signaling pathways (NF-κB, MAP kinase pathways, IL-2 secretion). DHA binds to specific DNA motifs on *cis*-regulatory elements in promoter regions of target genes. DHA binding impacts activation of the PPAR and RXR receptors that subsequently operate as transcription factors [248]. α, β, and γ isoforms of PPAR receptors are affected by DHA [248–250]. DHA can act directly in transcription, as it is concentrated in PS, a negatively charged aminophospholipid known to activate protein kinases involved in gene expression [54]. DHA may also operate at the posttranscriptional level to induce changes of phosphorylation events in native mRNA processing, mRNA transport and stabilization, and mRNA degradation rates [178]. These pathways modulate production of inflammatory mediators. The activated PPAR-γ/RXR complex also leads to cell proliferation, production of adhesion molecules (VCAM-1, ICAM-1, E- and P-selectins) and modulation of endothelial-leukocyte adhesion pathways in vascular tissue [251]. Jump [252] and De Caterina and Madonna [57] provide review of the role and actions of dietary PUFAs in regulation of gene transcription.

Increased levels of inflammatory mediators are associated with AMD [121]. How may proliferative retinopathies be affected by inflammation? A number of retinal diseases characterized partially by an inflammatory component involve leukocyte migration and adhesion within the retinal vasculature; this is preceded by induction of inflammatory (TNF-α, VCAM-1) and redox-sensitive genes (NF-κB). TNF-α plays a dominant role in modulating endothelial adhesion molecules. Ischemia- and inflammation-induced activation of COX-2 regulates the production of VEGF. Both VEGF and TNF-α upregulate ICAM-1 via NF-κB and serine/threonine kinase Akt-PI3 kinase-eNOS signaling pathways [253]. PKC may be an important factor in

Table 3. Vasoregulatory or vasotrophic factors affected by LCPUFAs, eicosanoids, COX, or LOX

Molecule		Reference
Angiogenin	Angiogenin → PLA_2	262
Angiopoietin (Ang)-2	DHA ↓ Ang-2	263
Angiotensin II	EPA and DHA ↑ response to Ang II in diabetes	264
Fibroblast growth factors: acidic/basic	EPA ↑ cell migration in response to bFGF	265 (no effect), 266
Follistatin	PGE_2 ↑ follistatin-related gene (FLRG) mRNA	267
Granulocyte colony-stimulating factor (G-CSF)	COX-2 inhibitor ↓ G-CSF mRNA	268
Interleukin-8 (IL-8)	DHA ↓ endothelial IL-8 expression	206
Leptin	EPA and DHA ↓ leptin secretion	269, 270
Platelet-derived growth factor-BB	EPA ⊣ PDGF-induced mitogenesis	271–273
Transforming growth factor-β	EPA ↑ TGF-β secretion	272, 274
Tumor necrosis factor-α	EPA and DHA ↓ TNF-α mRNA	275–278
Vascular endothelial growth factor	EPA ↓ VEGF and ⊣ VEGF receptor expression	265, 279–284

→ = Activates; ↑ increases; ⊣ = suppresses; ↓ decreases.

retinal vascular pathology; PKC activates PLA_2 [254]. Recall that PLA_2 is responsible for releasing esterified AA as a substrate for COX- and LOX-based eicosanoid production. These processes are associated with blood-retinal barrier degradation and manifested in increased vascular permeability. Extant data from model systems suggests relationships between angiogenesis and enzymes synthesizing eicosanoids from LCPUFAs. Nude mice with human breast cancer xenografts and fed ω–3 LCPUFA-rich diets showed displacement of AA from tumor cell membranes, a reduction in PGE_2 and 12-HETE [255, 256], loss of COX-2 mRNA expression [257], and reduction in COX-2- and COX-1-immunoreactive protein [258]. Both EPA and DHA inhibit COX-2 and COX-1 induction of PG biosynthesis [138]. There may be a link between this pathway, growth factor expression, and angiogenesis as the selective COX-2 inhibitor celecoxib (SC-58635) is capable of regulating production of VEGF and VEGF receptors in animal models of retinal neovascularization [259, 260]. This agent is also effective in suppressing FGF-2-induced corneal angiogenesis in rats [261].

Inflammation, Vascular Pathology, and Proteins Impacting and Impacted by Lipid-Based Molecules. A number of immunoregulatory cytokines, angiogenic growth factors, and their signaling pathways are affected by and affect LCPUFAs, eicosanoids, and PLA_2, COX, and LOX activities (table 3). We review VEGF, TGF-β, TNF-α, angiogenin, angiopoietin-1, FGF, follistatin, G-CSF, IL-8, leptin, and PDGF-BB. Cytokines are regulatory proteins that function as chemical messengers between natural and acquired immune systems and within the acquired immune system. Cytokines are also ligands for cell surface receptors that induce cellular growth, development, and activity related to vascular remodeling. They can potently induce capillary leakage and leukocyte migration. The LOX-5-catalyzed AA-metabolite (LTB_4) directly increases the production of the pro-inflammatory cytokine TNF-α [189]. Through its effect on this factor, LTB_4 indirectly enhances production other pro-inflammatory cytokines (IL-1, IL-2, IL-8, IL-6, IFN-γ) and the inflammatory lysophospholipid PAF. Figure 2 illustrates these relationships.

Angiogenic growth factors operate directly or indirectly on endothelial cells. Direct activity occurs via endothelial cell-surface receptors and induces endothelial cell migration and proliferation. Macrophage- and mast cell-derived polypeptide growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) operate as such. Indirect mechanisms act via chemotactic induction in macrophages that subsequently secrete VEGF and bFGF. This is the case of the eicosanoids reviewed above.

VEGF is a 45-kDa glycoprotein produced in the neural retina by astrocytes. It has specificity to several tyrosine kinase receptors (Flk-1, Flt-1) and has been identified as a central factor involved in retinal vasoproliferation [285]. VEGF is a key molecule in induction of: endothelial cell migration and proliferation, microvascular permeability, endothelial cell release of metalloproteinases and interstitial collagenases, and endothelial cell tube formation [286]. VEGF stimulates capillary meshwork formation in vivo [283] and has thus been implicated as a proangiogenic factor in ischemia-induced proliferative retinopathies [287–289]. Insulin-like growth factor (IGF-1) modulates VEGF-induced activation of MAPK. MAPK is involved in endothelial cell proliferation. Low levels of IGF-1 inhibit VEGF-induced activation of protein kinase B (Akt). The Akt/PI-3 kinase system modulates processes controlling vascular endothelial cell activation and survival, as well as leukocyte migration and adhesion.

In some cases, LCPUFAs affect activation and expression of VEGF-specific tyrosine kinase receptors. EPA affected VEGF-induced proliferation of bovine carotid artery endothelial (BAE) cells by inhibition of the fetal liver tyrosine kinase 1 (Flk-1) receptor, a factor essential for endothelial cell differentiation and proliferation. Neither DHA nor AA affected this system. The *fms*-like tyrosine kinase receptor (Flt-1), a factor involved in vessel construction [283], was not affected by EPA, DHA, or AA. bFGF-induced endothelial cell proliferation was not inhibited by EPA, DHA, or AA. However, EPA inhibited VEGF-induced activation of

MAPK. EPA downregulated Flk-1 receptor expression in a dose-dependent manner and upregulated Flt-1 receptor expression [283]. It also inhibited endothelial cell tube formation in vitro [290]. EPA-treated endothelial cells had low MAPK activity relative to cells that were not incubated with EPA. The mechanism of VEGF receptor downregulation may occur at the tyrosine kinase NF-κB site as EPA treatment caused suppression of NF-κB activation. NF-κB is a nuclear transcription factor that upregulates COX-2 expression, NO synthase, and intracellular adhesion molecule (ICAM) [291].

ω–3 LCPUFAs may also influence activation of IGF-1 pathways necessary to mediate effects of VEGF. In a study on burn patients (burns reduce IGF-1 levels) applying a nutritional intervention with a 15% fat diet with fish oil (50% of total dietary lipids from fish oil vs. 15 and 30% fat without fish oil), subjects returned to normal serum IGF-1 concentrations by 30 days post-intervention. Subjects in the no fish oil groups were consistently lower on IGF-1 and did not approach normal values within the first month of the study [292]. IGF-1 and VEGF interact in retinal neovascularization via the Akt/PI-3 kinase signaling pathway, a serine/threonine kinase activated system. DHA-enriched Neuro 2A cells survived a staurosporine-induced apoptotic signal through inhibition of normally observed decreases in Akt phosphorylation and activity [293]. Akt activity is essential for vascular endothelial cell survival [294].

Role of LCPUFAs in Structure and Function of Vascular Retina

The evidence base reviewed until this point suggests that ω–3 LCPUFAs have the capacity to modulate production and activities of a number of factors and processes implicated in retinal neovascularization, inflammation of retinal vasculature, and alterations in retinal capillary ultrastructure. Vascular pathology in AMD is localized predominantly within the choriocapillaris. Increased microvascular density is usually accompanied by alterations in the integrity of the capillary walls. Choroidal permeability may lead to abnormal vascular remodeling, capillary leakage, inflammation, and thrombosis. These processes are manifested in vitreous hemorrhage, fibrovascular scarring, mechanical stress, and subsequent retinal detachment. Table 4 contains an overview of potential points in a neovascular cascade at which ω–3 LCPUFAs may exert a meaningful impact. Table 5 contains similar information in the context of a number of etiologic factors for AMD.

The following section contains an application of knowledge base discussed above within the framework of a genome-wide association study on functionally relevant gene sets and pathways. We started with a general approach to inquiry on the role of lipid-associated compounds in AMD for the purpose of conducting an unbiased descriptive analysis that was optimized for discovery of novel relationships. Evidence converged to allow inference on a number of biologically credible explanations of our findings. It is our hope that this work will be extended in replicate samples.

Relationships of Advanced AMD with Lipid-Associated Gene Variants in the Age-Related Eye Disease Study

The Age-Related Eye Disease Study (AREDS) was a US-based, multi-center, 12-year prospective project examining the clinical course of AMD and age-related cataract. Analyses of AREDS data on dietary ω–3 LCPUFA-AMD relationships yielded inverse (protective) associations of reported intake with prevalent neovascular AMD [12] and progression to geographic atrophy [26] (a neurodegenerative form of the disease). AREDS genetic samples were used in one of the first successful applications of microarray technology to identify susceptibility variants in a complex disease [318].

The single marker elucidated in our original genome-wide association study has led to a set of important findings on inflammatory and immunoregulatory processes in AMD pathogenesis. The polygenic nature of AMD and the variable expressivity in the disease also warrants study on joint action of functionally-related genes encoding elements of metabolic or signaling pathways. In the context of our ω–3 LCPUFA-AMD relationships, we felt that it would be informative to examine the putative impact of sequence variants in genes encoding structures affecting and affected by lipid-based molecules as they relate to AMD. We used data from 508 AREDS participants examined in a genome-wide association study with a 100K microarray. Joint action models predicted a 99-fold increased susceptibility of advanced AMD from 11 variants of 11 inositol-related genes (odds ratio = 99.2, $p \leq 1.0 \times 10^{-17}$). Comparison of these findings against p value distributions from 1,500 analyses on randomly selected sets of single nucleotide polymorphisms (SNPs) (obtained from our complete panel of 96,774) indicated that these results were not likely due to random sampling error; in no instance did any of the 1,500 test models yield a p value $<1.2 \times 10^{-9}$. These novel results may provide meaningful insight into the role of molecular genetics in modifying nutrient-AMD relationships.

Methods

Study Population

All data in this work are from elderly self-identified white, non-Hispanic US residents participating in the AREDS. Details on the design and sample demographics of the study exist in extant publications [319]. Our final experimental population contained 368 people with advanced AMD and 141 people who had no clinical signs of AMD across the entire course of the study.

Outcomes

Masked professional graders at the University of Wisconsin Fundus Photography Reading Center ascertained phenotype annually across a 12-year period from stereoscopic color fundus images using a standardized and validated protocol. All photographs were taken with a standardized method by certified photographers. AREDS

Table 4. Potential influence of ω–3 LCPUFAs at key points in the neovascular progression

Step in neovascular cascade	Putative action of ω–3 LCPUFAs
Growth factors bind on endothelial cells	VEGF Flk-1 expression inhibited in EPA-treated bovine carotid artery endothelial (BAE) cells. Flk-1 expression increased [283]
Activated endothelial cells send signals to the nucleus for production of signaling molecules and enzymes	EPA selectively inhibits VEGF-induced, but not bFGF-induced activation of MAPK [283]. Members of the ERK-MAPK family respond to proliferative and mitogenic stimuli, regulate changes in transcription, and are associated with cellular differentiation and proliferation. c-Jun amino-terminal kinase (JNK) and p38 MAPK pathways may be activated by exposures associated with retinal disease; these include hypoxia, ultraviolet light, inflammatory cytokines, osmotic shock, or environmental stress [295]. MAPKs are important mediators in endothelial barrier function [296]
Enzymatic BM digestion	Incubation of human vascular ECs with EPA protects against gap junctional intercellular communication injury after hypoxia/reperfusion challenge. Effect mediated by inhibition of tyrosine kinase activation [297]. EPA inhibits urokinase-type plasminogen (uPA) activator activity [298]. uPA catalyzes conversion of plasminogen to plasmin. Plasmin is a serine proteinase involved in conversion of fibrin to soluble forms. uPA implicated in retinal neovascularization and may affect cell associated proteolytic activity [299]
EC division and migration through BM	ω–3 LCPUFAs prevent serotonin-induced EC proliferation [300]. Bovine aortic endothelial cells, treated with 0–5 μg/ml EPA for 48 h, displayed dose-dependent suppression to VEGF-induced proliferation [283]. EPA inhibited cell growth in a dose-dependent manner in bovine carotid ECs cultured between collagen gels [290]. Prostacyclin (PGI_2) is the main prostanoid in most vascular systems. PGI_2 regulates vascular EC proliferation. DHA and EPA incubation led to changes in fatty acid composition of membrane phospholipids of human umbilical vein endothelial cells (HUVEC) co-incubated with allogeneic peripheral blood lymphocytes and resulted in reduced basal PGI_2 production [302]
Adhesion molecules or integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$) pull blood vessel sprouts forward.	Incubation of human adult saphenous vein endothelial cell cultures with DHA reduced endothelial expression of VCAM-1, E-selectin, ICAM-1, IL-6 and IL-8 after challenge with IL-1, IL-4, TNF-α, or bacterial endotoxin [206]. Primary HUVECs activated with IL-1β produce ICAM-1, E-selectin and VCAM-1 transcripts. Both EPA and DHA attenuate induction of these adhesion molecules after challenge with IL-1β [303]
Matrix metalloproteinases (MMP) dissolve tissue in front of the sprouting vessel tip. As the vessel extends, the tissue is remolded around it	MMPs are a family of neutral zinc endopeptidases secreted as pro-enzymes in extracellular areas. MMPs modulate aspects of angiogenesis, inflammation, and affect tissue remodeling through: degradation of specific extracellular matrix components; destruction of proteinase inhibitors, cell surface proteins, cytokines; and activation or release of signaling molecules and proteinases. Expression of MMPs is induced by cytokines, growth factors, and reactive oxygen species. Endogenous

Table 4. Continued

Step in neovascular cascade	Putative action of ω–3 LCPUFAs
	tissue inhibitors of metalloproteinases (TIMPs) bind non-covalently to zinc-dependent active site of MMPs to regulate their activity. MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B) operate against type IV collagens and laminins in vascular endothelial basement membranes. MMP-2 binds on vascular EC surfaces to $\alpha_v\beta_3$ integrin during capillary tube formation. MMP-9 is constitutively produced in the retinal ganglion cell layer. These MMPs exist in the interphotoreceptor matrix and are implicated in pathogenesis of retinopathy of prematurity and proliferative diabetic retinopathy [304] MMP-2 and MMP-9 expression was elevated in C557Bl/J6 mice with ischemia-induced retinal neovascularization, relative to animals reared under normal conditions [305]. TIMP-1 (MMP-9 inhibitor) and TIMP-2 (MMP-2 inhibitor) were decreased within the same animals, indicating that these extracellular proteinases are important in retinal angiogenesis. The mechanism by which MMPs are expressed occurs via soluble mediators TNF-α and VEGF (not via the hypoxic insult introduced in the ischemic challenge) [306]. Serum MMP-2 and -9 activity decreased in female CD-VAF rats fed DHA-containing diets with various compositions of EFAs and LCPUFAs, relative to a comparison group fed DHA-free diets [307]. CDF_1 and BALB/c mice receiving diets enriched in DHA had lower MMP-9 activity in tumor tissue extract than animals fed an LCPUFA-free diet. MMP-9 activities were also significantly lower in animals consuming DHA + oleic acid and DHA + LA, relative to controls fed a DHA-free diet. Gelatinolytic (MMP-2 and -9) activities were not different between controls and animals consuming oleic acid, LA, EPA, and EPA + DHA enriched diets [308]. EPA treatment in nude mice prevented development of lung metastases from MDA-MB-435 human breast cancer cell mammary fat pad solid tumors. Incubation with yielded in a dose-related inhibition of cultured MDA-MB-435 cell 92-kDa type IV collagenase (MMP-9) mRNA expression [309]
ECs sprout from tubes	Bovine carotid artery ECs treated for 2 days with EPA showed dose-dependent inhibition of tube formation. AA increased tube formation, and docosahexaenoic acid had no effect [290]. DPA (22:5ω–3) suppressed tube-forming activity in endothelial cells induced by vascular endothelial growth factor [279]. ECs cultured in collagen gel and treated with EPA displayed a dose-dependent suppression of tube formation, VEGF-induced proliferation, and activation of p42/p44 MAP kinase [265]

BM = Basement membrane, EC = endothelial cell, PDR = proliferative diabetic retinopathy, ROP = retinopathy of prematurity.

Table 5. Etiologic factors for AMD and putative actions of ω–3 LCPUFAs

Factor/process	Putative pathogenic mode of action	Putative action of ω–3 LCPUFAs
Neovascularization		
VEGF	VEGF → vascular endothelial cell proliferation VEGF → vascular endothelial cell migration VEGF → vascular endothelial cell survival VEGF → vascular endothelial cell permeability VEGF → uPA + tPA → ECM degradation VEGF → MMP → ECM degradation VEGF ↔ NO → vascular hyperpermeability VEGF → ICAM-1	EPA ⊣ VEGF KDR [265] ω–3 LCPUFA ⊣ VEGF KDR [279] EPA ⊣ VEGF Flk-1 [283] EPA ⊣ urokinase activity [298] DHA ⊣ MMP-2 [307] DHA ⊣ ICAM-1 [206] DHA ⊣ VCAM [206]
Angiopoietins	Ang1 → pericyte recruitment → vascular integrity Ang2 ⊣ Ang1 Ang2 + VEGF → neovascularization	DHA ⊣ Ang-2 [310]
Nitric oxide	NO → integrin $\alpha_v\beta_3$ → endothelial cell migration NO → integrin $\alpha_v\beta_3$ → endothelial cell different VEGF ↔ NO → vascular hyperpermeability VEGF → eNOS → NO → VEGF eNOS → vaso-obliteration eNOS → vitreous neovascularization iNOS ⊣ VEGF receptor expression	DHA → iNOS [311,312]
Extracellular matrix	Integrins + ECM proteins → endothelial migration TNF-α → $\alpha_v\beta_3$ → endothelial cell migration MMP → ECM degradation TIMP-3 ⊣ VEGF-ind. endothelial cell migration	DHA ⊣ TNF-α [196] DHA ⊣ MMP-2 [307] ω–3 LCPUFA → TIMP [313]
Oxidative stress		
Reactive O_2 intermediates	Membrane lipid peroxidation Mitochondrial DNA damage H_2O_2 → p53/p21 → apoptosis H_2O_2 ⊣ Bcl-2 ⊣ apoptosis tBHP → caspase → mito. damage → apoptosis H_2O_2 → VEGF in RPE → apoptosis	Fish oil → mitochondrial function [157] Fish oil → ATP metabolism [314] DHA → Bcl-2 [40] DHA ⊣ caspase-3 [315] DHA → Akt/PI3 pathway [293]
Hypoxia	Hypoxia → VEGF in RPE Hypoxia → Ang2	See VEGF and Ang sections of table
Lipofuscin/A2E	Short wavelength light-induced RPE apoptosis A2E⊣ photoreceptor lysosomal degradation Physical disruption of RPE cytoarchitecture	DHA → IRBP [152]

Table 5. Continued

Factor/process	Putative pathogenic mode of action	Putative action of ω–3 LCPUFAs
AGE/RAGE	AGE → RPE aging genes AGE → VEGF in RPE	ω–3 LCPUFA ⊣ AGE [316]
Hemo-hydrodynamic Δ		
Bruch's membrane	Thickening ⊣ diffusion ⊣ choroidal perfusion	DHA → IRBP [152] DHA ⊣collagen aggregation [147]
Choriocapillaris	↓ diameter ⊣ choroidal perfusion → transport ↓ density ⊣choroidal perfusion → RPE transport	DHA → vascular compliance [143]
Neutral fat deposits	Modification of diffusion characteristics of BM	DHA → IRBP [152]
RPE senescence		
Age	AGE ⊣ gene expression lysosomal enzymes AGE shortened chromosomal telomeres	ω–3 LCPUFA ⊣ AGE [316]
β-Galactosidase	↓ proteolytic activity → RPE deposits	ω–3 LCPUFA → galactosidase [317]

The conceptual structure of this table was partially adapted from Ambati et al. [3]. AGE = Advanced glycation end products; Ang = angiopoietin; bFGF = basic fibroblast growth factor; BM = Bruch's membrane; eNOS = endothelial nitric oxide synthase; ECM = extracellular matrix; H_2O_2 = hydrogen peroxide; iNOS = inducible nitric oxide synthase; ICAM = intracellular adhesion molecule; IRBP = interphotoreceptor-binding protein; KDR = VEGF receptor-2, kinase insert domain-containing receptor; MMP = matrix metalloproteinase; mitochond. fct = mitochondrial function; NO = nitric oxide; PEDF = pigment epithelium derived factor; ROI = reactive O_2 intermediates; RAGE = receptor for advanced glycation end products; RPE = retinal pigment epithelium; SWL = near-UV light; TIMP = tissue inhibitor of matrix metalloproteinase; tPA = tissue-type plasminogen activator; uPA = urokinase-type plasminogen activator; VEGF = vascular endothelial growth factor; Δ = change; ↓ = reduction; ⊣ = decreased expression, inhibition or deactivation; → = factor or process leads to outcome.

Report 1 [319] contains details on the process of outcome ascertainment. Advanced AMD cases meet the following criteria: (1) presence in either eye of geographic atrophy or neovascular AMD defined as photocoagulation or other treatment for choroidal neovascularization (based on clinical center reports), or (2) photographic documentation of any of the following: non-drusenoid retinal pigment epithelial detachment, serous or hemorrhagic retinal detachment, hemorrhage under the retina or RPE, and/or subretinal fibrosis either at baseline or during the course of the study.

Our AMD-free group had good vision in both eyes and no existing eye disease. Controls were selected to be the oldest AREDS participants with DNA available who had a drusen area of <5 small drusen (<63 μm diameter) and no pigmentary abnormalities in both eyes at all visits for which fundus photographs were gradable. Both eyes had visual acuity of 20/32 or better measured by a standard protocol, ocular media was clear enough for good quality fundus photographs, and there was absence

of any ocular disorder that might obscure assessment of either AMD or lens opacities. The AMD-free group has three distinguishing characteristics that make it a robust and appropriate comparison group: (1) phenotype was determined annually over a 12-year period with a standardized protocol by multiple professional graders who were masked to phenotypic information from previous years – adjudication with a standardized protocol occurred when discrepancies emerged; (2) criteria for AMD-free classification (≤5 drusen of ≤63 μm in both eyes for the entire 12-year period) is stringent relative to those applied in previous association studies for AMD; (3) the age of the AREDS AMD-free group is in the range that AMD prevalence increases ~3 times (from ~4% in those aged 74–79 years to ~12% in those aged ≥80 years) in population-based studies.

The mean age (±SE) of controls in was 76.9 ± 0.38 years; the value for the advanced AMD group was 79.7 ± 0.26 years. Half of those in the AMD-free group were women, while the corresponding value for the advanced AMD group was 58%. 5% of people in the AMD-free group were current smokers; the value for the advanced AMD group was 11%.

Bioinformatics and Statistical Modeling

Our bioinformatic and statistical approach is described in figure 3. In phase 1 we employed an empirical process to identify candidate gene sets and pathways associated with advanced AMD (steps 1–6). The first issue was to apply a valid definition of the lipid-associated genes from which we would interrogate relevant variants (step 1). The Lipid Metabolites and Pathways (LIPIDMAPS) Research Group maintains a proteomic database of 2,919 human transcripts related to lipid-associated enzymatic activity, metabolic processes, and signaling pathways described in the Kyoto Encyclopedia of Genes and Genomes (www.genome.ad.jp/kegg) and Gene Ontology (www.geneontology.org). We used transcript identifiers to extract information on the nature and positional coordinates of genes encoding these structures (step 2). Positional coordinates of gene regions in the range of ±10,000 base pairs from current gene boundaries were searched with utilities at The National Center for Biotechnology Information (NCBI) and Ensembl to define the universe of known variants from genes encoding LIPIDMAPS structures (step 3). We applied this information filter to microarray data from our 12-year natural history on AMD to extract SNPs for relationship with AMD (step 4). These variants were tested for association with advanced AMD in age-, sex-, and smoking-adjusted logistic regression models using a log-additive coding scheme (step 5). Results of these analyses were used to make inferences on candidate pathways and gene sets in the context of evidence on: (a) molecular genetics of AMD and (b) extant findings on processes both impacted by ω–3 LCPUFAs and implicated in health and disease of neural or vascular retina (step 6). Multiple single markers were associated with of inositol-based metabolic or signaling pathways.

In phase 2 we applied a knowledge-based approach to evaluate the predictive utility of our gene set in determining the likelihood of having advanced AMD (steps

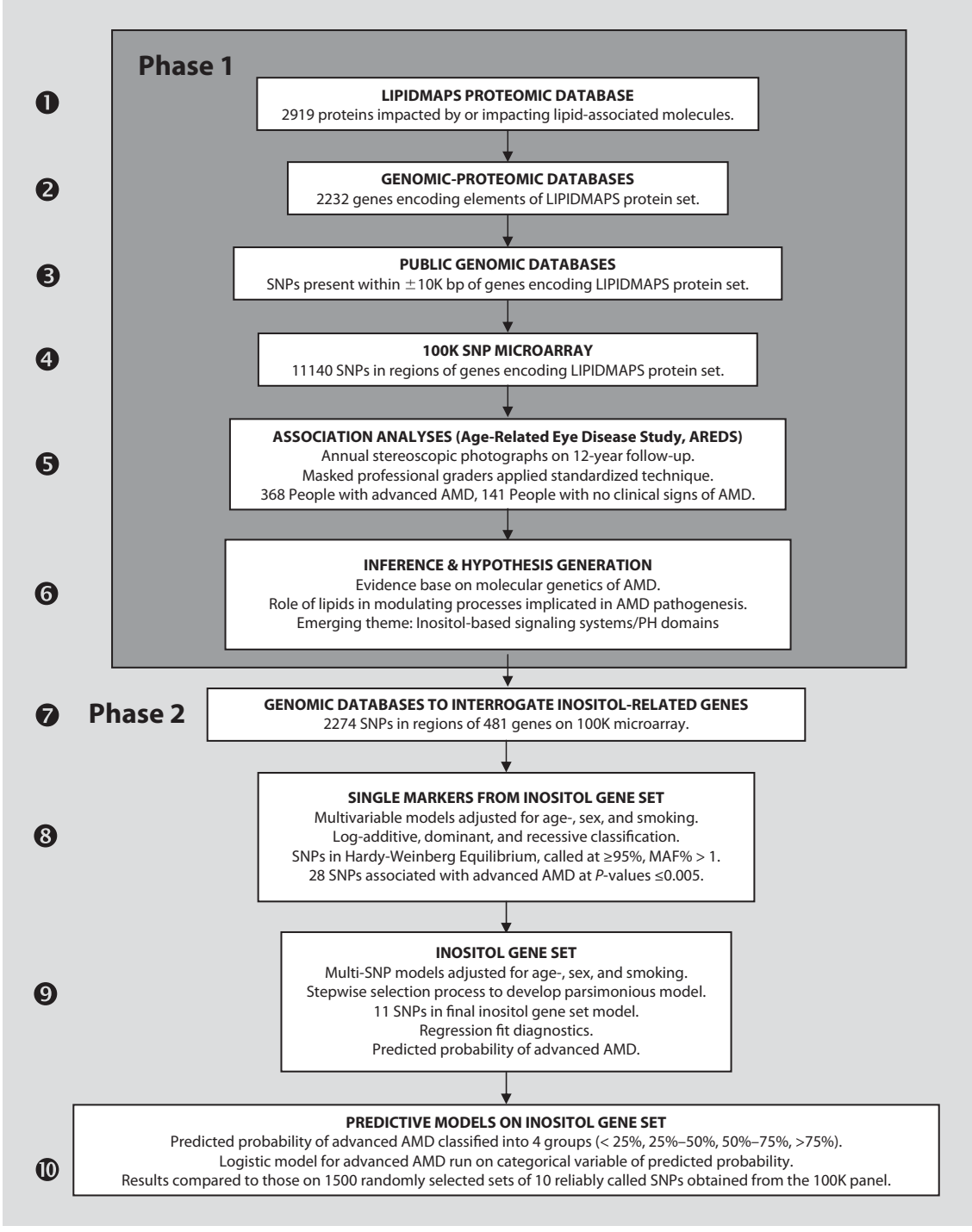

Fig. 3. Analytic method for identifying candidate gene set and specifying joint-action models.

7–10). Our analytic methods are based on those developed by Lesnick et al. [15] to examine joint actions of common gene variations within biologic pathways as predictors of disposition to complex disease. We used the NCBI search engine to obtain lists of all catalogued human genes associated with inositol (step 7). Positional coordinates of these genes ± 10,000 base pairs were obtained and used to filter SNPs in from our 100K microarray. Distributions of inositol-related SNPs in Hardy-Weinberg equilibrium, called reliably at ≥95%, and present in at least 1% of the total sample, were examined for log-additive, dominant, and recessive associations with advanced AMD in age-, sex-, and smoking-adjusted logistic regression models (step 8). We specified a candidate inositol gene set with single markers containing complete data for all participants and attaining two-sided p values ≤0.005. We then ran age-, sex-, and smoking-adjusted joint action logistic regression models with members of this set. A stepwise selection process was applied to develop parsimonious models and regression diagnostics with the Hosmer and Lemershow lackfit method were run to determine whether variance of the final model violated assumptions necessary for valid inference (step 9). Eleven SNPs persisted in the final model. We modeled these variants simultaneously on occurrence of advanced AMD to derive the predicted probability of having AMD for each participant (values may range naturally from 0 to 1) and classified these values into groups by increments of 0.25 (<0.25, 0.25–0.50, 0.50–0.75, and 0.75–1.00). These probability categories represented the primary independent variable of likelihood estimates in final predictive models of advanced AMD occurrence (step 10). The distribution of p *va*lues from 1,500 randomly selected sets of variants drawn from our total panel (96,774 SNPs) was used to evaluate significance of our findings.

Results

At the time of publication the LIPIDMAPS database contained 2,919 proteins. We identified 2,232 genes encoding elements of these structures. Our 100K microarray contains 11,140 variants in gene regions associated with the LIPIDMAPS constructs. After examining results from this set, a number of AMD-associated variants with diverse positions across the genome emerged for genes associated with the inositol signaling pathway. There were 2,274 variants in a NCBI-specified inositol gene set present on our microarray; of these, 28 that were in Hardy-Weinberg equilibrium, called reliably at ≥95%, and present in at least 1% of the total sample also yielded p values ≤0.005 (fig. 4). This candidate inositol gene set was used in joint action models.

Eleven common variants with complete data from 11 inositol-related genes persisted in the final model (table 6). This set yielded predictive probabilities that distinguished people with advanced AMD from their AMD-free peers (fig. 5). Relative to people with a predicted probability of advanced AMD <0.25 from our final model, the likelihood of having AMD among people with probabilities of >0.75 was increased 99-fold ($p = 1.0 \times 10^{-17}$, Model 1, table 7). The overall p value of the model is 3.1×10^{-21}. For each 25% increase in predicted probability there was a 3.5-fold increased

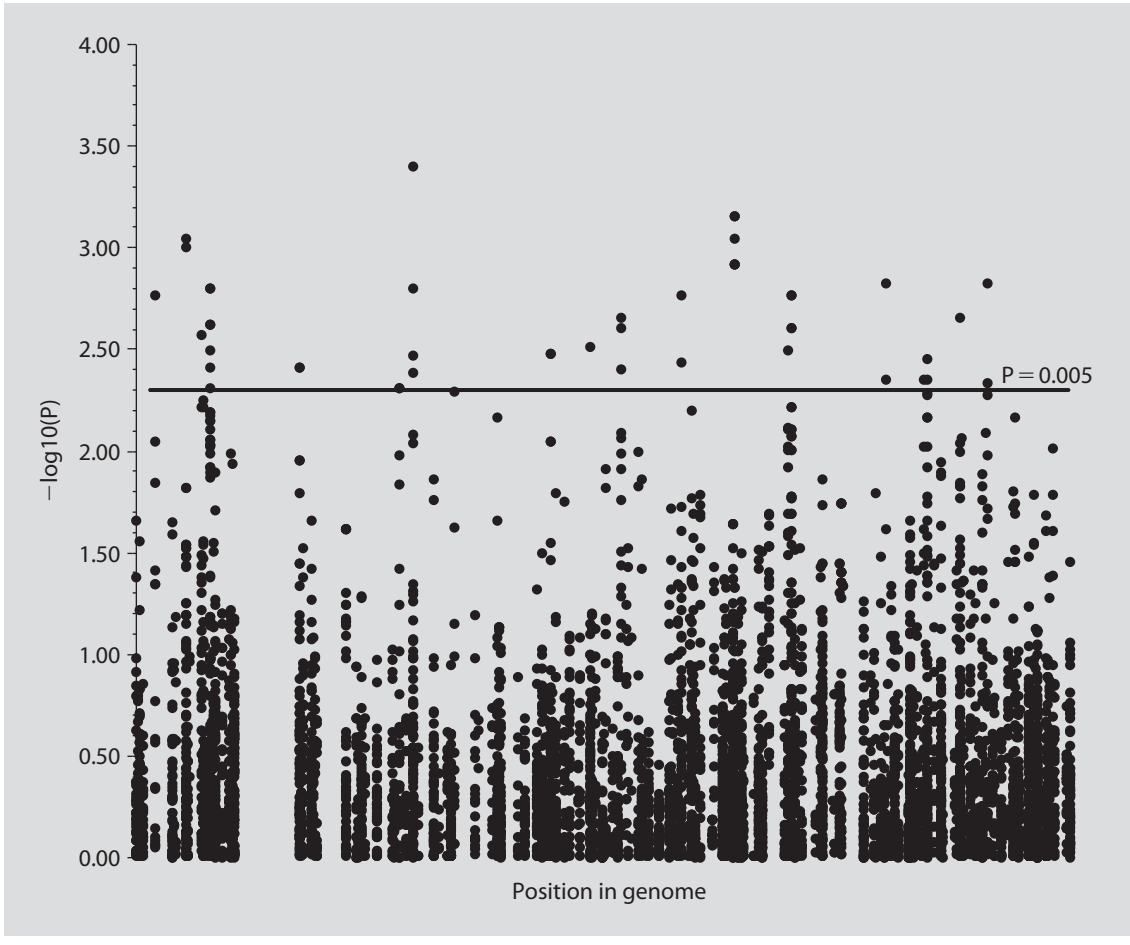

Fig. 4. Results from multivariable logistic regression analyses on SNPs identified as variants in genes encoding elements of inositol signaling pathways.

likelihood of having advanced AMD (odds ratio = 3.5, 95% confidence interval = 2.8–4.7). Comparison of our findings to those derived from p value distributions of 1,500 analyses on randomly selected sets of SNPs from our complete array panel indicated that results were not likely due to random sampling error. None of the 1,500 test models yielded a p value $<1.2 \times 10^{-9}$. In addition to analyses on variants with complete data, we applied our approach to a set with less than 10% missing data for single markers. The final analysis in this model contained 19 SNPs that were present in 339 people with advanced AMD and 128 AMD-free people (Model 2, table 7). Results did not change appreciably from the original model.

Discussion

These novel findings implicate molecular events driven by elements of inositol metabolism and signaling pathways in AMD pathogenesis. Our primary intent was

Table 6. Variants included in the final predictive model for inositol-related factors and processes

Variant	Gene		MAF%		
	HGNC symbol	Prox. exon (~bp)	no AMD	adv. AMD	p value
rs4146894[R]	PLEKHA1	<2,100	45.4	37.5	0.0001
rs2270960[R]	ITPR2	<50	46.5	37.4	0.0009
rs2038490[A]	PIGH	<100	28.0	17.3	0.0013
rs2575876[A]	ABCA1	<200	31.2	21.3	0.0022
rs1759752[R]	PPAP2B	<3,000	48.9	55.6	0.0028
rs2816955[A]	NR5A2	>5,000	40.4	49.7	0.0040
rs1424166[D]	CDH13	<2,000	49.3	55.8	0.0040
rs3784789[A]	CSK	>5,000	40.8	32.6	0.0055
rs3795451[R]	CDC42BPA	3′ UTR	46.8	44.4	0.0087
rs697852[A]	ITPKB	>5,000	23.0	16.2	0.0095
rs766366[D]	DGKB	>5,000	46.5	37.9	0.0110
rs1529819[A]	DEPDC2	<300	44.3	36.5	0.0116

The 'no AMD' (n = 141) group consisted of people who were AMD-free across the study. People in the advanced (Adv.) AMD group (n = 368) have neovascular AMD and/or geographic atrophy. p values are two-sided and from multivariable models including age at last photograph used to ascertain outcome, sex, and smoking (never, past, current). Superscripts in the 'Variant' column represent form of the variable applied in the final model: A = additive, D = dominant, R = recessive. Prox. exon = proximity to exon. ~bp = approximate number of base pairs. MAF = Minor allele frequency, PLEKHA1 = pleckstrin homology domain containing, family A (phosphoinositide-binding specific) member 1; ITPR2 = inositol 1,4,5-triphosphate receptor, type 2; PIGH = phosphatidylinositol glycan anchor biosynthesis, class H; ABCA1 = ATP-binding cassette, subfamily A (ABC1), member 1; PPAP2B = phosphatidic acid phosphatase type 2B; NR5A2 = nuclear receptor subfamily 5, group A, member 2; CDH13 = cadherin 13, H-cadherin; CSK = c-src tyrosine kinase; CDC42BPA = CDC42 binding protein kinase α (DMPK-like); ITPKB = inositol 1,4,5-trisphosphate 3-kinase B; DGKB = diacylglycerol kinase, β; DEPDC2 = DEP domain containing 2.

to determine whether the *joint action* of common gene variations within members a systematically identified lipid-associated gene set could predict predisposition to advanced AMD. It is important to emphasize that inferences are most appropriately applied to the inositol-associated *gene set* (and not the individual variants used for predictive modeling) since our microarray panel did not have complete coverage of all genes, and in some cases did not contain variants in (or in linkage disequilibrium with those in) coding regions. The magnitude of effect in the joint action model exceeded that of any single marker. In the absence of mechanistic validation studies we cannot yet make conclusive inferences; however, extant work on molecular genetics of AMD provides essential guidance in assessing the value of our findings.

We thus consider our results in the context of two biologically credible and related lines of evidence. The first is related to pleckstrin homology (PH) domain-containing

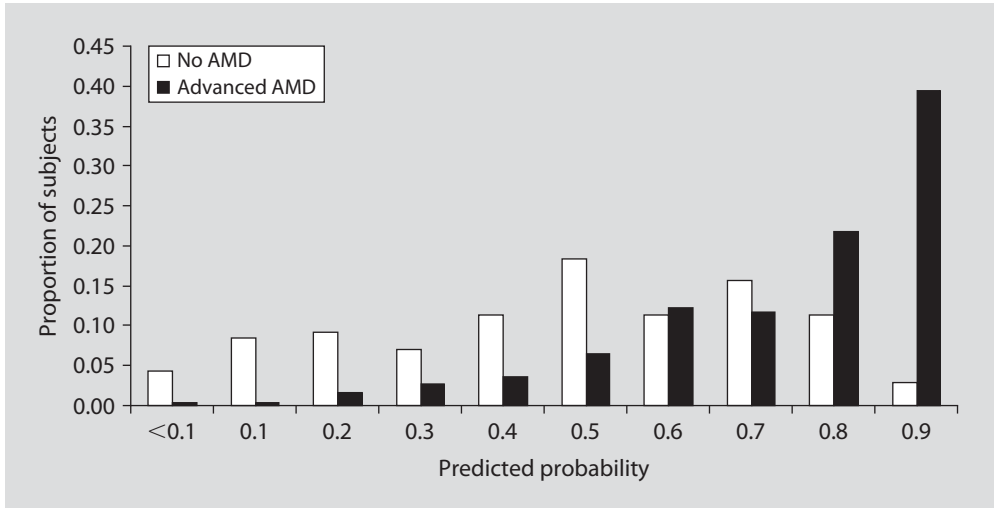

Fig. 5. Predicted probabilities of advanced AMD based upon the final joint action model of variants in inositol-related genes (cf. table 6). The explanatory value of the final model is strongest for the advanced AMD group, as approximately 7 in 10 people with AMD have at least a 70% predicted probability of having advanced AMD with the current set of variants (~40% of people with AMD have probability scores ≥0.90). In most instances, people in the no AMD group have lower probabilities than those with AMD. Within probability categories there is usually a clear separation between groups. Fit of the model may be improved (~20% of controls have predicted probabilities between 0.70 and 0.90) with data on additional SNPs.

effectors. A strong and often replicated susceptibility locus for advanced AMD exists on chromosome 10q26 [320–322]. PH domain-containing, family A (phosphoinositide binding-specific) member 1 (PLEKHA1) occupies this region and represents 1 of the 3 genes most strongly associated with AMD worldwide. The other two genes (LOC387715/age-related maculopathy susceptibility 2 (ARMS2) and HtrA serine peptidase 1 gene (HTRA1)) are proximally positioned on 10q26 and in linkage disequilibrium with PLEKHA1 [322]. Pleckstrin is a PKC substrate containing two PH domains that bind *phosphoinositides*; it is involved in plasma membrane-based signaling systems [323]. PH domains represent approximately 1 in 10 of all domain structures in the human proteome [324] and exist on a number of protein kinases, GTPases/GTP regulators, adaptors, and phospholipases central to physiologic function [324, 325]. Figure 6 includes examples of molecules containing PH domains along with phosphoinositide-binding specificity. The key points are that PH domains bind phosphoinositides and act in recruitment and translocation of host proteins to the plasma membrane so that macromolecules of PI3K, GTP, and Ras signaling systems may be assembled.

DHA modulates Akt signaling in neuronal survival by influencing events at the PH domain. DHA acts as an effect modifier in the process of PH-mediated membrane translocation for the Akt system in a model system of neuronal cell survival [326].

Table 7. Results of joint action models for inositol-related genes

Inositol-related genes	Comparison (probability group)		OR (95% CI)	p value
	A vs.	B		
Model 1				
Full data set	linear model	–	3.5 (2.8–4.7)	3.1×10^{-21}
11 markers	<0.25	0.25–0.50	10.5 (2.3–48.5)	0.64
	<0.25	0.50–0.75	19.8 (4.5–87.0)	0.04
	<0.25	0.75–1.00	99.2 (22.3–440.9)	1.0×10^{-17}
Model 2				
95% complete data	linear model	–	4.3 (3.3–5.7)	2.0×10^{-25}
19 markers	<0.25	0.25–0.50	7.8 (2.4–25.1)	0.26
	<0.25	0.50–0.75	13.4 (4.5–40.6)	0.26
	<0.25	0.75–1.00	114.9 (37.4–353.0)	5.5×10^{-25}

Odds ratios (OR) represent the increased likelihood of having advanced AMD relative to a one-step change in the scale of predicted probabilities ('linear model', e.g. moving from <0.25 to 0.25–0.50 or 0.25–0.50 to 0.50–0.75) or each predicted probability category relative to the <0.25 group (A vs. B). All p values are two-sided. Model 1 was run on SNPs with 100% base calls for all participants (listed in table 6: 'No AMD' group, n = 141; AMD group, n = 368). Model 2 was run on SNPs from Model 1 and others with ≥90% call frequency. Results for Model 2 are based on 128 people without AMD and 339 people with AMD.

This action has been linked to DHA enrichment of PS, a phospholipid present in appreciable quantities within the neural retina. Increases in PS concentration facilitate interaction of Akt's PH domain with the plasma membrane and thus allow a more efficient phosphorylation of the Akt. Akt activation in this system inhibits a key step in the apoptotic cascade and thereby promotes cell survival. How do these results relate to our own? PLEKHA1 binds specifically and with high affinity within the plasma membrane to phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), a bioactive lipid and the dephosphorylation product of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3). PtdIns(3,4,5)P3 is produced from a phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) substrate after class IB phosphoinositide 3-kinase (PI3Kγ) activation. PI3Kγ ligands have been associated with atrophic and vasoproliferative retinal pathology in model systems; these ligands include chemokines (IL-8, RANTES, macrophage inflammatory protein (MIP)-1a, MIP-2, MCP-1, Gro-α), pro-inflammatory lipids (PAF), leukotrienes (LTB_4), angiotensin II, and complement component C5. PtdIns(3,4)P2- and PtdIns(3,4,5)P3-pleckstrin complexes work within the PI3K pathway to regulate innate and adaptive immune cells, vascular smooth muscle cells,

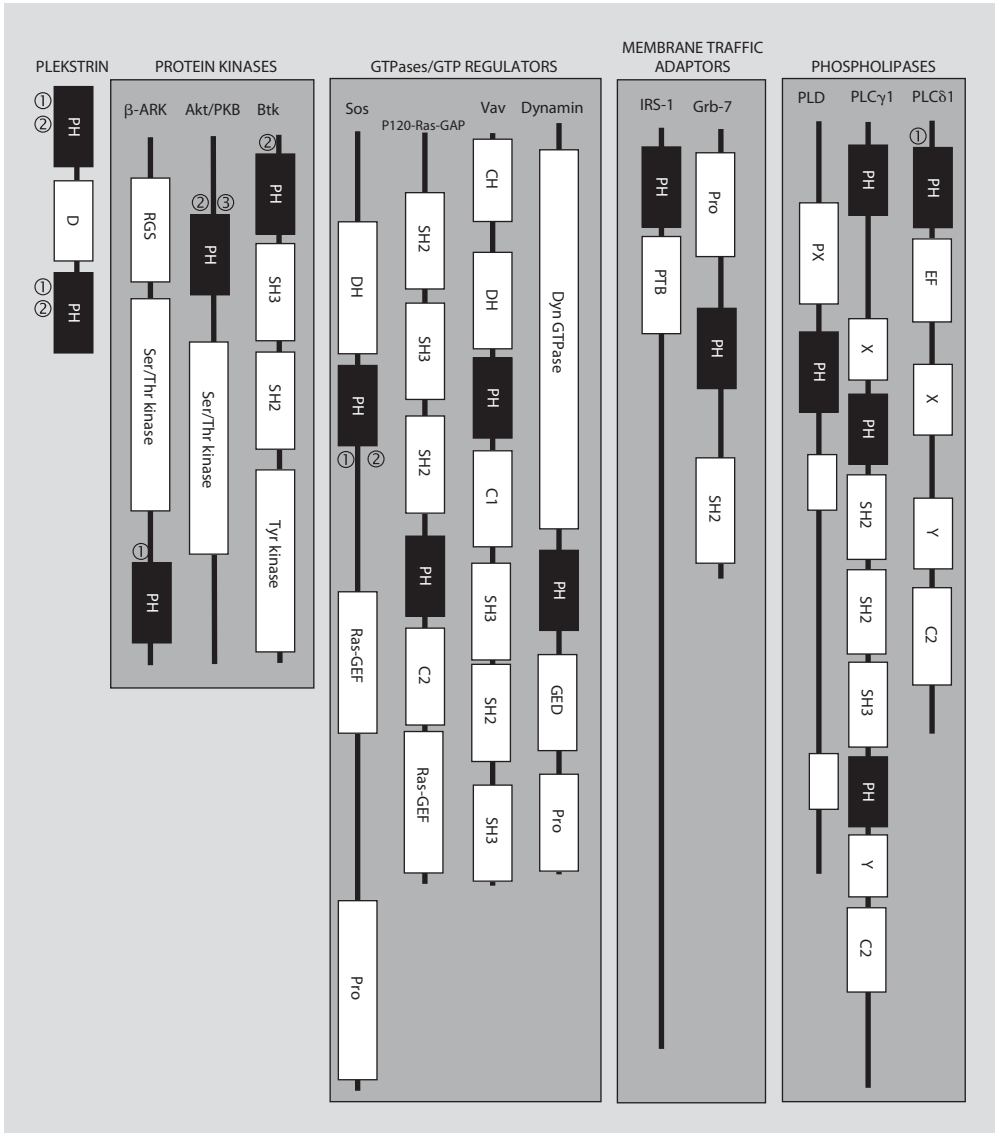

Fig. 6. Pleckstrin homology domains and phosphoinositide-binding sites adapted from Itoh and Takenawa [323]. Non-specific binding to PH domains from PtdIns3P and PtdIns4P. Specific binding according to the following symbols: ① = PtdIns(4,5)P2, ② = PtdIns(3,4,5)P3, ③ = PtdIns(3,4)P2. PH = Pleckstrin homology domain; D = disheveled, Egl-10, and pleckstrin; DH = Dbl homology; Dyn = dynamin; GEF = guanine nucleotide exchanging factor; GAP = GTPase-activating protein; CH = calponin homology; C1 = protein kinase C conserved region 1; C2 = protein kinase C conserved region 2; GED = GTPase effector domain; X = phospholipase C catalytic domain X; Y = phospholipase C catalytic domain Y; EF = EF hand; RGS = regulator of G-protein signaling; PL = phospholipase; Pro = proline-rich; PTB = phosphotyrosine binding; SH2 = Src homology 2; SH3 = Src homology 3; b-ARK = β-adhesion-related kinase; Btk = Bruton's tyrosine kinase; Sos = guanine nucleotide-exchange factor Son of sevenless; Vav = guanine-nucleotide exchange factor Vav.

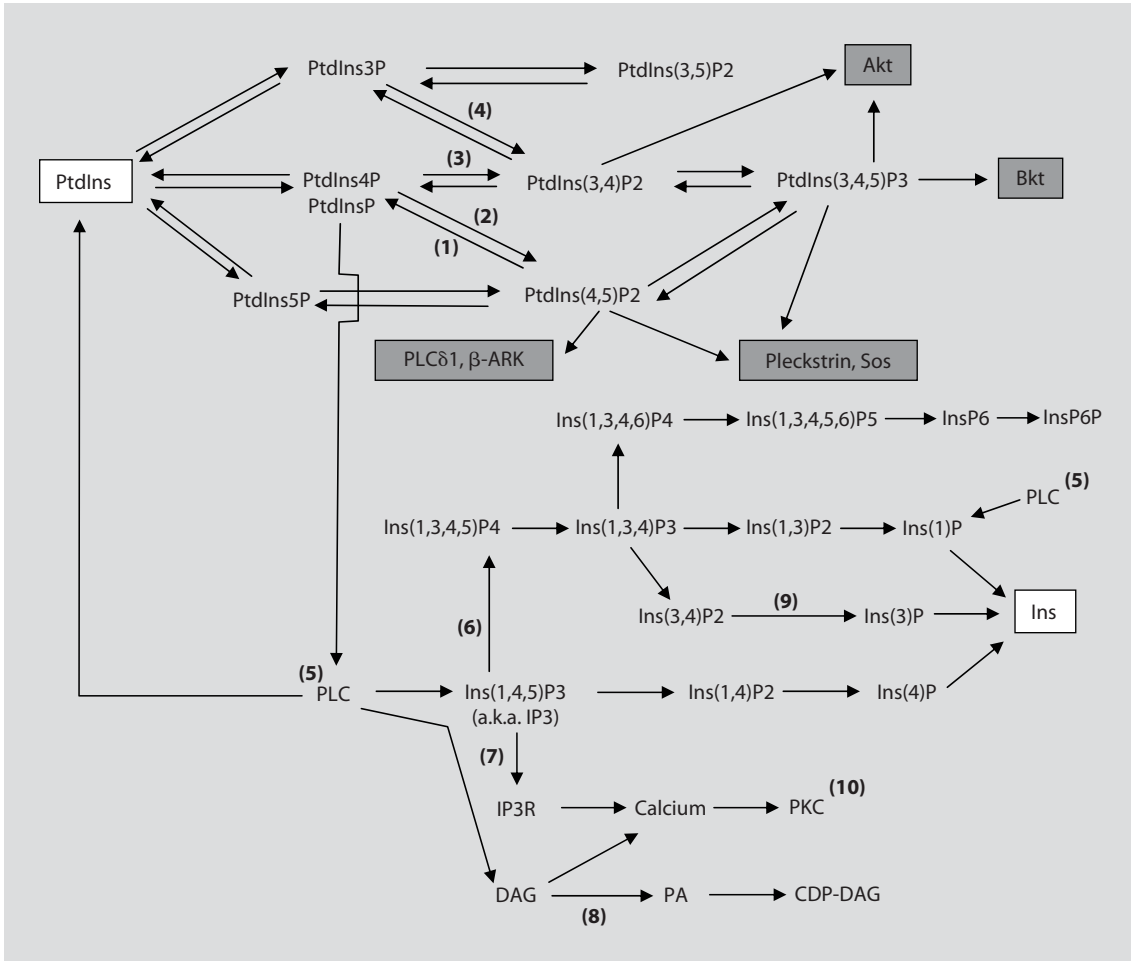

Fig. 7. Pathways are adapted from KEGG Pathway 04070. CPD-DAG = CDP-diacylglycerol; DAG = diacylglycerol; PA = phosphatidic acid; PtdIns = phosphatidylinositol; Ins = inositol. Numerals are used to represent roles of genes in this signaling pathway that contain variants associated with advanced age-related macular degeneration at p values ≤0.05 in age-, sex-, and smoking-adjusted logistic regression models. The HGNC symbols for genes containing AMD-associated variants are as follows: (1) INPP5E, SYNJ2; (2) PIP4K2A, PIP4K2B, PIP5K1A, PIP5K1C; (3) PIK3C2G; (4) INPP4B; (5) PLCB2, PLCE1, PLCG2; (6) ITPKB; (7) ITPR1, ITPR2; (8) DGKA, DGKB, DGKH, DGKI; (9) INPP4B; (10) PRKCA, PRKCB1. INPP5E = Inositol polyphosphate-5-phosphatase; SYNJ2 = synaptojanin 2, inositol phosphate 5′-phosphatase-2; PIP4K2A = phosphatidylinositol-5-phosphate 4-kinase, type II, α; PIP4K2B phosphatidylinositol-5-phosphate 4-kinase, type II, β; PIP5K1A = phosphatidylinositol-4-phosphate 5-kinase, type I, α; PIP5K1C = phosphatidylinositol-4-phosphate 5-kinase, type I, γ; PIK3C2G = phosphoinositide-3-kinase, class 2, γ polypeptide; INPP4B = inositol polyphosphate-4-phosphatase, type II; PLCB2 = phospholipase C, β 2; PLCE1 = phospholipase C, ε 1; PLCG2 = phospholipase C, γ 2 (phosphatidylinositol-specific); ITPKB = inositol 1,4,5-trisphosphate 3-kinase B; ITPR1 = inositol 1,4,5-triphosphate receptor, type 1; ITPR2 = inositol 1,4,5-triphosphate receptor, type 2; DGKA = diacylglycerol kinase, α; DGKB = diacylglycerol kinase, β; DGKH = diacylglycerol kinase, η; DGKI = diacylglycerol kinase, ι; INPP4B = inositol polyphosphate-4-phosphatase, type II; PRKCA = protein kinase C, α; PRKCB1 = protein kinase C, β 1.

and platelets partially through their interaction with PH domains on serine/threonine kinases in the protein kinase B (Akt) signaling pathway. Figure 7 plots information on gene variants associated with AMD within the framework of the PI signaling system (genes containing sequence variants associated with advanced AMD at p values ≤0.05 are represented in the diagram). The PH domains for Akt and pleckstrin share a specific affinity to PtdIns(3,4,5)P3. There are a number of points in figure 7, particularly (3) and (4), at which variants present in greater frequency among people with AMD may impact production of the precursor pool for the specific plekstrin ligand. These findings are intriguing as a number of research groups have emphasized the difficulty of making inferences about the independent effects of PLEKHA1, LOC387715/ARMS2, and HTRA1 because of the tendency for shared inheritance of haplotypes. Our findings support the importance of PLEKHA1 as part of an inositol-based system as there was a great diversity in the position of inositol-related genes specified in the final joint action model. The variant from our final model within the PLEKHA1 gene region yielded the strongest relationship of all 11 SNPs in the final model; but it is important to note, omitting this variable led to negligible change in the results. Table 8 contains annotations for the 20 genes represented in figure 7. These genes exist on 14 chromosomes. It is intriguing to observe genes with similar functions existing on different chromosomes were associated with our outcomes (diacylglycerol kinases on chromosomes 7 and 12, PKC isoforms on chromosomes 16 and 17, and phospholipase C isoforms on chromosomes 10, 15, and 16).

A second line of evidence supported by our findings involves the relationship of dietary lipids, phosphoinositides, and genetics with calcium signaling. Szado et al. [327] demonstrated that phosphorylation of inositol triphosphate (IP_3) receptors by the Akt system, prevented programmed cell death in a model cell system through inhibition of calcium release. This point is germane to our work, as ω–3 LCPUFAs have been shown to influence calcium homeostasis [328, 329]. Points (7) and (10) in figure 7 highlight the potential for disrupted inositol-modulated calcium signaling in people with advanced AMD.

Summary and Future Directions

Our general conclusions are that: (1) there is consistent evidence to suggest that ω–3 LCPUFAs may act in protective roles for AMD; (2) genome-wide association studies may be applied to efficiently elucidate novel pathway- and gene set-based associations with complex diseases like AMD, and (3) a knowledge-based approach to exposure ascertainment (in this case, identification and annotation of lipid-associated genes) is valuable in planning, implementation efforts necessary to make valid inference about complex systems. The essential need for replication of findings in an independent sample is now being planned.

Table 8. Annotations and positional coordinates of genes represented in the inositol-signaling system (cf. fig. 7)

Figure 7 group	Chr	Start bp	HGNC ID	Symbol	Gene name
(6)	1	224886016	6179	ITPKB	inositol 1,4,5-trisphosphate 3-kinase B
(2)	1	149437696	8994	PIP5K1A	phosphatidylinositol-4-phosphate 5-kinase, type I, α
(7)	3	4510136	6180	ITPR1	inositol 1,4,5-triphosphate receptor, type 1
(4) and (9)	4	143168636	6075	INPP4B	inositol polyphosphate-4-phosphatase, type II
(1)	6	158322907	11504	SYNJ2	synaptojanin, synaptic inositol 1,4,5-triphosphate 5 phosphatase, inositol phosphate 5′-phosphatase 2
(8)	7	14153770	2850	DGKB	diacylglycerol kinase, β
(8)	7	136724925	2855	DGKI	diacylglycerol kinase, ι
(1)	9	138442896	21474	INPP5E	inositol polyphosphate-5-phosphatase
(2)	10	22865829	8997	PIP4K2A	phosphatidylinositol 5 phosphate 4 kinase type 2 α
(5)	10	95743736	17175	PLCE1	phospholipase C, epsilon 1
(8)	12	54611213	2849	DGKA	diacylglycerol kinase, α
(7)	12	26379552	6181	ITPR2	inositol 1,4,5-triphosphate receptor, type 2
(3)	12	18305741	8973	PIK3C2G	phosphoinositide-3-kinase, class 2, γ polypeptide
(8)	13	41520889	2854	DGKH	diacylglycerol kinase, η
(5)	15	38367392	9055	PLCB2	phospholipase C, β 2 1 phosphatidylinositol 4,5-bisphosphate phosphodiesterase β
(5)	16	80370408	9066	PLCG2	phospholipase C, γ 2 (phosphatidylinositol-specific) 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase γ 1
(10)	16	23754823	9395	PRKCB1	protein kinase C, β 1
(2)	17	34175470	8998	PIP4K2B	phosphatidylinositol-5-phosphate 4-kinase, type II, β
(10)	17	61729216	9393	PRKCA	protein kinase C, α
(2)	19	3581182	8996	PIP5K1C	phosphatidylinositol-4-phosphate 5-kinase, type I, γ

Figure 7 contains labels in the far left column of this table to represent positions in the inositol pathway. Chr = Chromosome; bp = base pair.

New and emerging concepts related to the content of the work we present should focus on the interrelationship of LCPUFAs with genetic factors impacting neural and vascular structure and function. Dietary LCPUFAs operate as key structural and signaling molecules. They affect and are affected by key compounds with known vaso-, neuro-, and immunomodulatory actions. We have applied bioinformatic and statistical genetic techniques in an effort to examine lipid-dependent metabolism and signaling mechanisms as a step forward in guiding researchers in discovery of key pathways driving retinal response to developmental, demographic, environmental, and metabolic factors. To the extent that retinal tissue status of DHA is dependent upon and modifiable by diet, we may eventually arrive at some reasonable understanding of whether alterations in dietary or cellular lipid composition or modulation of lipid precursors, cleavage and biosynthetic enzymes, or metabolites will serve as an effective preventive intervention for retinal diseases.

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