OMEGA-3 FATTY ACID STATUS IN PATIENTS DIAGNOSED WITH USHER SYNDROME: A DESCRIPTIVE STUDY OF RED BLOOD CELL (RBC) DOCOSAHEXAENOIC ACID (DHA) LEVELS IN USHER SUBTYPES

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DIANNA K. HUGHBANKS-WHEATON, B.S., M.S.

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To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Dianna K. Hughbanks-Wheaton entitled "Omega-3 Fatty Acid Status in Patients Diagnosed With Usher Syndrome: A Descriptive Study of Red Blood Cell (RBC) Docosahexaenoic Acid (DHA) Levels in Usher Subtypes." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Health Studies.

Kristin Wiginton
Kristin Wiginton, Ph.D., Major Professor

We have read this dissertation and recommend its acceptance:

Anna Love

Jody Domen-Early

Department Chair

Accepted:
Jennifer Martin

Dean of the Graduate School

DEDICATION

This dissertation is dedicated to my loving and supportive family. My parents, Roger and Betty Hughbanks, instilled in me a strong value system including the compassionate desire to help others. You have celebrated each milestone and attended each graduation on my quest for higher education. Little do you realize that the values you cultivated are the very ones that provide the foundation and the passion for my scientific pursuit.

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ABSTRACT

DIANNA K. HUGHBANKS-WHEATON

OMEGA-3 FATTY ACID STATUS IN PATIENTS DIAGNOSED WITH USHER SYNDROME: A DESCRIPTIVE STUDY OF RED BLOOD CELL (RBC) DOCOSAHEXAENOIC ACID (DHA) LEVELS IN USHER SUBTYPES

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Usher syndrome is a genetic disease that includes visual impairment, due to progressive retinal degeneration, as well as congenital hearing loss. Night blindness is frequently the first ocular symptom and can occur within the first decade of life. Severe tunnel vision will typically lead to legal blindness by the second or third decade. Reduced blood levels of the long-chain omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) have been reported in patients with Usher syndrome. DHA is found at its highest concentration in the human body in rod and cone photoreceptors suggesting a potential functional role in the phototransduction process of vision. Numerous studies have found defects in retinal and visual function of both animal models and humans deficient in omega-3 fatty acids including DHA. The current study used a retrospective, cross-sectional design to assess if blood levels of DHA (RBC-DHA) in patients with Usher syndrome were significantly different from normal. A secondary purpose was to determine if differences in RBC-DHA were evident between clinical and genotypic subgroups of Usher syndrome. The relationship between dietary intake and blood fatty acid status was explored and compared between the normal and Usher study cohorts.

Study participants were comprised of a convenience sample of normal controls (n = 60) and patients with Usher syndrome (n = 114) recruited from multi-state and nationwide patient registries. The present study demonstrated that the Usher syndrome cohort had lower mean RBC-DHA levels compared to normal (3.70% vs. 4.09%; p = .017). However, significant differences were not observed in DHA levels between clinical or genotypic subgroups. Calculated daily dietary DHA intake was not significantly different between the normal and Usher cohorts. Significant relationships were observed between DHA intake and RBC-DHA levels among both the normal and Usher cohorts (r = .470 and r = .433, respectively; $p < .001$). This study represents the first comprehensive comparison of blood DHA levels between each of the Usher clinical and genotypically-defined subtypes. These results contribute to our understanding of the omega-3 fatty acid status among patients with Usher syndrome and the association of dietary intake of DHA from omega-3 rich food sources.

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CHAPTER I

INTRODUCTION

Usher syndrome is a genetic disease that includes visual impairment, due to a progressive retinal degeneration, as well as congenital hearing loss. In the United States, the overall prevalence of this autosomal recessive disease has been reported as 4.4/100,000 (Boughman, Vernon, & Shaver, 1983). Three distinct clinical subtypes, USH I, USH II, and USH III, can be distinguished by age of onset and disease severity with the USH I subtype being the most severe. Prevalence is approximately 40%, 60%, and 1% for subtypes I, II, and III, respectively. According to Online Inheritance in Man [OMIM], there are eleven genes believed to cause Usher syndrome; six are associated with type I (2007a), four for type II (2007b), and one for type III (2007c) of which nine have been genetically confirmed and well-characterized. Thus, molecular testing currently allows the unequivocal identification of nine genetic subtypes (i.e., genotypes) within the three clinical forms.

Disease onset is early in life. Hearing loss is prelingual in USH I and USH II (OMIM, 2007a) and progressive in USH III (OMIM, 2007c). Night blindness is frequently the first ocular symptom and can occur within the first decade of life. Severe tunnel vision will typically lead to legal blindness by the second or third decade. Blood levels of the long-chain omega-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) in patients with Usher syndrome have been reported to be reduced from normal

by several investigators (Bazan, Scott, Reddy, & Pelias, 1986; Maude, Anderson, & Anderson, 1998); however, blood DHA levels have not been determined in the specific genetically-defined subtypes.

DHA is found at its highest concentration in the human body in rod and cone photoreceptors (Fliesler & Anderson, 1983). The elevated levels in retinal tissue suggest a potential functional role associated with the phototransduction process of vision. Indeed, numerous studies have found defects in retinal and visual function of both animal models and humans deficient in omega-3 fatty acids (including DHA). Compared to those with normal vision, 75% of male patients with one of the most severe inherited retinal diseases, namely X-linked retinitis pigmentosa, have 30 to 40% reductions in red blood cell (RBC) DHA levels and this correlates with reduced retinal function in these patients (Hoffman & Birch, 1995).

There is some expectation that each of the Usher syndrome subtypes may present with differing DHA metabolism. For example, Usher subtypes may exhibit different blood DHA levels and respond to DHA supplementation differently. Therefore, an understanding of the possible differences in DHA status between genetic types would be critical in the concept and design of a clinical trial of DHA supplementation in Usher syndrome.

Purpose of the Study

Although blood fatty acid levels have been fairly well-studied in retinitis pigmentosa (reviewed by McColl & Converse, 1995; Hoffman, 2000), few studies have

focused specifically on Usher syndrome, an allied retinal disorder. Usher patients have occasionally been included in research studies intermingled within the autosomal recessive category of retinitis pigmentosa. Four studies (Bazan, Scott, Reddy, & Pelias, 1986; Connor, Weleber, DeFrancesco, Lin, & Wolf, 1997; Maude, Anderson, & Anderson, 1998; Williams, Horrocks, Leguire, & Shannon, 1989) have notably studied blood fatty acids in Usher syndrome, each with some measure of success and limitation.

This study was designed to address previous deficits of small sample size, undifferentiated clinical subtypes, absence of Usher III patient data, and lack of a dietary assessment to evaluate intake of omega-3 rich food sources. Through the use of a larger, well-defined cohort of patients with Usher syndrome, the study was intended to address the intuitively conflicting outcomes of the Connor et al. (1997) sperm-DHA analysis and Maude et al. (1998) blood-DHA analysis in patients with Usher type I or II. Recent molecular genetic advances allow further clarification of Usher syndrome according to genotype; thus, this study was able to conduct sub-group analysis of blood lipid levels by both clinical and major genotypic subtype.

The current study evaluated blood fatty acid levels (RBC-DHA as % total fatty acid) and dietary intake in a retrospective analysis of a large patient cohort ($n>100$). Data obtained from the study both clarifies and extends the empirical knowledge of the omega-3 fatty acid status in patients with Usher syndrome. Ultimately, these results will assist in determining the applicability and design of a nutritional supplementation trial in Usher syndrome to retard retinal degeneration.

Theoretical Foundations

This study was grounded in the philosophical framework of logical positivism, relying on deductive reasoning and objective, quantitative measures (DePoy & Gitlin, 2005). Theoretical foundations for viewing the phenomenon under study were the Mendelian model of single-gene disorders (Beaudet, Scriver, Sly, & Valle, 1995) and the fluid mosaic model of lipid bilayer membranes (Singer & Nicholson, 1972). Single-gene disorders are a group of diseases caused by mutation in a single gene occurring with a frequency of 1 in 100 live births; Usher syndrome is one such example. While the single gene defect may account for the overt phenotype in Usher, the fluid mosaic model elucidates the biological significance of alterations in lipid biochemistry.

Research Hypotheses

This study assessed the following null hypotheses; significance was determined at $p \leq .05$:

HO1: There will be no significant difference between blood levels of DHA (RBC-DHA percent of total fatty acids) in patients with Usher syndrome compared to normal controls.

HO2: There will be no significant difference in DHA blood levels between the clinical subtypes of Usher syndrome.

HO3: There will be no significant difference in DHA blood levels between the major known genotypes of Usher syndrome.

HO4: There will be no significant difference between the calculated milligrams of dietary DHA consumed/day in patients with Usher syndrome compared to normal controls.

HO5: There will be no significant relationship between the calculated milligrams of dietary DHA and the blood-DHA levels in normal controls.

HO6: There will be no significant relationship between the calculated milligrams of dietary DHA and the blood-DHA levels in patients with Usher syndrome.

HO7: There will be no significant difference between the strength of relationship of the calculated milligrams of dietary DHA to blood-DHA levels for the normal control group compared to the Usher syndrome group.

Delimitations

This study involved the retrospective analysis of de-identified data obtained through the conduct of a research study with the following inclusion and exclusion criteria:

1. Usher syndrome patients were eligible for the study if participating in the Retina Foundation of the Southwest genetic registry (i.e., Southwest Eye Registry, [SER]), or the Boys Town National Research Hospital [BTNRH] genetic database.
2. Normal control participants were eligible for recruitment if designated as "normal" or a "normal family member" in either the SER or BTNRH database.

3. Exclusion criteria for patients and normal control participants included non-traditional dietary habits (i.e. vegetarian), systemic disease (i.e., diabetes, cardiovascular disease), excessive fish consumption (containing DHA), or nutritional supplementation with fish oil or DHA.

Limitations

This study was subject to the following limitations:

1. All participants voluntarily participated in the genetic registry(s) and subsequently, consented to participate in this study. Therefore, they may reflect an eager, motivated sub-population.
2. Participants were obtained as a convenience sample. Although both registries draw from multi-state or nationwide patient populations (SER and BRNRH, respectively), registry participants may not fully represent the breadth of Usher patients in the United States.
3. This study was exploratory in nature involving a rare disease with numerous clinical and genotypic subtypes, thus the resulting size of some subgroups may have affected the power of statistical comparisons.
4. The study design included a self-report questionnaire introducing sources of potential error.
5. The dietary questionnaire was developed prior to the commercial availability of DHA-fortified foods, thus it may not have fully indexed the daily dietary intake of DHA-rich foods.

6. Diffusion of innovation regarding the potential benefit of DHA in slowing retinal degeneration in retinitis pigmentosa might have influenced the dietary behavior of Usher patients leading to alteration of dietary intake via increased DHA-rich food sources or nutritional supplements.

Assumptions

1. Participants read and comprehended English well enough to understand study instructions and follow directions appropriately.
2. Participants responded truthfully and completely to the self-report questionnaire.

Definition of Terms

Autosomal Recessive- A non-sex-linked trait determined by information coded on both copies of a gene, one gene copy is inherited from each parent. Thus, an autosomal recessive disease is caused by mutations in both copies of the responsible gene.

Clinical Subtypes- Usher type I, type II, and type III as determined by clinical presentation (e.g., severity of hearing loss, vestibular dysfunction).

Docosahexaenoic Acid (DHA)- A long-chain, omega-3, polyunsaturated fatty acid comprised of 22 carbon atoms with 6 double bonds (i.e., 22:6ω3).

Genotype- The underlying genetic coding in an individual's DNA. In disease, it is the ascribed genetic causation (e.g., specific gene, gene defect). At the outset of this study six genotypes were identifiable for Usher syndrome (i.e., IB, IC, ID, IF, IIA, & IIIA), although nine are now genetically verifiable (above listing, plus IG, IIC and IID).

Normal, Control Participants- Individuals were normal if designated as "normal" or "normal family member" in either recruitment database, wherein normal was operationalized as the absence of a known genetic eye disease.

Omega-3 Rich Food- Food sources that are relatively high in the omega-3 series of long-chain polyunsaturated fats. For this study, the focus was specifically on food sources rich in DHA and EPA (i.e., eicosapentaenoic acid), for example salmon, mackerel and tuna.

Retinitis Pigmentosa- A degenerative retinal disease caused by progressive photoreceptor cell death due to underlying heritable gene mutations. Clinical presentation is typified by night blindness and progressive peripheral visual field loss (i.e., tunnel vision). Inheritance can be autosomal (i.e., dominant, recessive), sex-linked (i.e., x-linked), or part of a genetic syndrome.

Usher Syndrome- An autosomal recessive genetic disorder that involves both deafness and progressive vision loss due to retinal degeneration (i.e., retinitis pigmentosa).

Usher Syndrome Patients- Individuals (male/female) diagnosed with Usher syndrome based on clinical presentation; diagnosis routinely occurs as late teen to early adult, thus most known patients are adults.

Importance of the Study

The study was designed to clarify and extend current knowledge of the omega-3 fatty acid status in patients with Usher syndrome. Currently, there is no cure for Usher syndrome and treatment options are very limited for this debilitating disease (e.g., cochlear implants, Vitamin A supplementation). Ultimately, the results of this study will be used to illuminate the applicability and design of a nutritional intervention trial to retard progression of visual loss like that currently underway in the X-linked form of retinitis pigmentosa (U.S. National Institutes of Health [USNIH], 2007a).

CHAPTER II

LITERATURE REVIEW

The first known clinical description of Usher syndrome was recorded in 1858 by noted German ophthalmologist Albrecht von Graefe in his case report of a deaf, mute male presenting with retinal degeneration whose family history included two similarly affected siblings (von Grafe, 1858). Dr. von Graefe's student, Richard Liebreich later elucidated the heritable nature of the deaf-blindness in a study of deafness among the Jewish population in Berlin (Liebreich, 1861). Liebreich's study was instrumental in demonstrating that the two traits were inherited together and were more likely to occur in children of consanguinious parents thus, providing the first suggestion of a syndrome with an autosomal recessive pattern of transmission. Over fifty years later, Dr. Charles Usher, an ophthalmologist in London published a manuscript describing the combined pathology of deafness and visual problems further confirming the heritability of the condition in a cohort of 69 cases (Usher, 1914).

Clinical Description

Usher syndrome is typically diagnosed based on the hallmarks of coincident hearing loss and retinal degeneration. There are three distinct clinical subtypes, USH I, USH II, and USH III which vary by age of onset and disease severity with the USH I subtype being the most severe. Approximate prevalence of Usher subtypes USH I and USH II in the U.S. and Northern European population are estimated to be 40% and 60%,

respectively (Petit 2001). USH III is relatively uncommon in the U.S. population, but has been described as the most common subtype in Finland, accounting for 40% of cases (Pakarinen, Karjalainen, Simola, Laippala, & Kaitalo, 1995), likely due to the effect of a founder mutation.

Establishment of Clinical Criteria for Diagnosis

The recognition that Usher syndrome is clinically heterogeneous has evolved over time expanding and contracting as researchers honed their ability to differentiate the disorder. The earliest descriptions eluded to a singular disorder (von Graefe, 1858; Liebreich, 1861; Usher, 1914) followed by recognition of two subtypes distinguishable by severity of hearing loss and age of observed vision loss (Fishman, Kumar, Joseph, Torok, & Anderson, 1983; Forsius, Erikkson, Nuutila, Vainio-Mattila, & Krause, 1971), and also a third progressive subtype (Gorlin, Tilsner, Feinstein, & Duvall, 1979; Karjalainen, Vartianen, Terasvirta, Karja, & Kaariainen, 1985). An expert panel of researchers convened as the Usher Syndrome Consortium in 1992 to establish the clinical criteria recommended for diagnosis of Usher syndrome and categorization to clinical subtypes (Smith, et al., 1994). The Consortium's recommendations were intended to guide standardization of diagnosis and lend uniformity to subsequent publications.

Differentiation of Clinical Subtypes

Onset of symptoms associated with Usher syndrome typically begins early in life. Bilateral, sensorineural hearing impairment is prelingual, and non-progressive in USH I and USH II. Type I patients exhibit severe to profound congenital deafness that is often

associated with impaired speech development, vestibular defects that impair and/or delay achievement of motor development in children, and juvenile-onset retinitis pigmentosa (RP) (Fishman et al., 1983; Möller et al., 1989). Type II patients exhibit mild to moderate congenital deafness that can be severe at higher frequencies, normal vestibular function, and later-onset RP. Type III patients exhibit post-lingual, progressive deafness, adult-onset RP, and variable vestibular responses (Karjalainen, et al., 1985; Pakarinen, Tuppurainen, Laippala, Mantyjarvi, & Puhakka, 1995). Night blindness is often the first ocular symptom and can occur as early as the first decade as in type I or the second to third decade as in type II and type III (Fishman et al., 1983). Progressive constriction of the visual field due to retinal degeneration leads to severe tunnel vision that will eventually lead to legal blindness, often by the second or third decade.

The three subtypes are thus clinically distinguishable based on severity of hearing loss, vestibular involvement, age of onset, and progression of hearing loss. Audiograms are utilized to document the extent of hearing loss. Type I patients are typically profoundly deaf while type II patients may be "hard of hearing" and demonstrate a characteristic audiogram with mild impairment at lower frequencies and severe loss at higher frequencies (Kimberling & Möller, 1995). Vestibular dysfunction is a crucial component to the differentiation between Usher types I and II. Sophisticated vestibular studies, such as rotary chair, electrooculography (EOG), or electronystagmogram (ENG) are utilized to diagnose vestibular areflexia. Electrophysiology studies utilizing the electroretinogram (ERG) are used to diagnose the retinal dysfunction due to retinitis

pigmentosa. The ERG can reliably provide an early diagnosis of RP often before subjective visual function abnormalities are detected or retinal changes (i.e., bone spicule, retinal pigment epithelium atrophy, and blood vessel attenuation) are apparent on fundoscopic examination (Berson, 1993).

Mendelian Inheritance

Gregor Mendel is widely credited with the distinction of the "Father of Genetics" due to his landmark work (Mendel, 1866) studying the transmission of heritable characteristics in plants providing the concepts of dominant and recessive traits. The discovery of the chromosomal basis of inheritance is attributed to Thomas Hunt Morgan (Morgan, Sturtevant, Muller, & Bridges 1915) based on his study of mutations in *Drosophilia melanogaster*. In 1966, Victor McKusick catalogued all the known genes and genetic disorders and published "Mendelian Inheritance in Man" which is currently available online (i.e., Online Mendelian Inheritance in Man; OMIM) or in its 12th printed edition (McKusick, 1998). Comparison of the original and current editions illustrates the explosion of medical genetic knowledge over the past 40 years, as 1,600 entries were initially reported compared now to over 17,000 (McKusick, 2007).

Usher syndrome was clearly recognized as an autosomal recessive disorder early in the 20th century (Usher, 1914). Autosomal recessive diseases are part of a family of single-gene or monogenic disorders that follow inheritance described by Mendelian genetics (i.e., dominant, recessive, x-linked) (Beaudet, Scriver, Sly, & Valle, 1995). Single-gene disorders are caused by changes (i.e., mutations) in the DNA of one gene.

Genes encode proteins that are vital to biochemical processes and serve as major structural components. Mutations or aberration in DNA sequence may result in impaired production or function of the associated protein. If normal function of the protein is impaired, it may manifest as a disease. Single-gene disorders occur with a frequency of 1 in 100 live births and more than 4,500 different disorders are currently known (Beaudet et al., 1995).

Population Genetics

In the United States, the overall prevalence of Usher syndrome has been reported as 4.4/100,000 (Boughman et al., 1983). Population studies throughout Europe have estimated the prevalence as 3.0/100,000 in Scandinavia (Hallgren, 1959), 3.6/100,000 in Norway (Grondahl, 1987), 4.2/100,000 in Spain (Espinós, Millán, Beneyto, & Nájera, 1998), 5.0/100,000 in Denmark (Rosenberg, Haim, Hauch, & Parving, 1997), and 6.2/100,000 in Germany (Spandau & Rohrschneider, 2002). Based on Boughman's estimated prevalence rate for the U.S., the extrapolated number of individuals affected with Usher syndrome numbers greater than13,000 based on the July 1, 2007 population census estimates (U.S. Census Bureau, n.d.). However, this study may have underestimated the prevalence due to bias toward ascertainment of the more severely affected individuals; the authors themselves caution that their estimate should be interpreted as conservative (Boughman et al., 1983). The Foundation Fighting Blindness (2008) estimates that 20,000 individuals in the U.S. have Usher syndrome whereas The Boys Town National Center for the Study and Treatment of Usher Syndrome, estimates

as many as 30,000 to 40,000 Americans may be affected (Boys Town National Research Hospital, n.d.).

Reports have varied regarding the proportionate distribution among the Usher syndrome clinical subtypes, particularly regarding Usher type I and II. Rosenberg et al. (1997) and Spandau and Rohrschneider (2002) both found greater prevalence for the type II presentation among separate, relatively large patient populations (i.e., > 100) illustrated by type I to type II ratios of 2:3 and 1:3, respectively. In contrast, Tamayo and colleagues (Tamayo et al., 1991) studying a smaller cohort (i.e., < 50) in Columbia found a distribution of 70% type I, 26% type II, and 4% type III, although their overall prevalence of 3.2/100,000 aligned well with the other studies.

Genetic Heterogeneity

Usher syndrome is genetically heterogeneous such that numerous genes are responsible for causing this disease. According to OMIM, there are eleven genes currently believed to cause Usher syndrome; six are associated with type I (i.e., IB-IG) (2007a), four for type II (i.e., IIA, B, C, D) (2007b), and one for type III (i.e., IIIA) (2007c) of which nine have been genetically confirmed and well-characterized. The two remaining genes, accounting for genotypes IE and IIB have been mapped to locations on chromosome 21q (Chaïb et al., 1997) and 3p (Hmani et al., 1999). An additional genotype, IA, was postulated and mapped in a French kindred (Kaplan et al., 1992), but it was later determined that individuals that mapped to the IA locus actually had mutations in the IB gene (Gerber et al., 2006).

Two recent studies, Ouyang et al. (2005) and Roux et al. (2006) have attempted to determine the frequency and distribution of Usher type I gene mutations. The following ranges represent the compiled results from the two studies: 39-55% IB, 6-7% IC, 19-35% ID, 11-19% IF, 0-7% IG. There is a paucity of information regarding the frequency and distribution of Usher II gene mutations. According to W. Kimberling (personal communication, July 15, 2008), the distribution of the type II genotypes can be estimated as 80% IIA, 5% IIC, 5% IID, and 10% novel. Although the overriding phenotype of Usher syndrome is very similar across the subtypes, the underlying gene defects are in genes that encode very different proteins.

Usher IB. The Usher IB genotype was mapped to chromosome 11q by Kimberling et al. (1992) and later determined to be caused by mutations in the *myosin VIIa* gene (i.e., *MYO7A*) (Weil et al., 1995). A subsequent study of 189 independent Usher I patients identified 23 mutations in *MYO7A* segregating with the disease status thereby, further substantiating the causative nature of mutations in this gene (Weston et al., 1996). Gibson et al. (1995) identified mutations in the mouse homolog to *MYO7A* in the shaker-1 mouse. The shaker-1 mouse is considered a natural model for USHIB as it exhibits deafness and vestibular perturbation due to dysfunction and progressive degeneration of the orgin of Corti in the inner ear, although retinal degeneration is absent.

MYO7A belongs to a family of unconventional myosins that do not assemble into filaments as other myosin proteins. Weil and colleagues (1996) demonstrated that *MYO7A* is expressed in photoreceptor cells and the pigment epithelium of the retina as

well as in embryonic cochlear and vestibular neuroepithelia. Co-localization experiments (Wolfrum, Liu, Schmitt, Udovichenko, & Williams, 1998) indicate that MYO7A always segregates with cilia and is concentrated in the connecting cilium of rod and cone photoreceptors (Liu, Vansant, Udovichenko, Wolfrum, & Williams, 1997) indicating a possible role in maintaining axonemal structures. Udovichenko, Gibbs, and Williams (2002) demonstrated that MYO7A functions as an actin-based motor protein capable of moving along the actin filament at a rate of 190 nm s^{-1}.

Usher IC. Usher type IC was first described in a French-Acadian population in Louisiana (Smith, et al., 1992) and is often referred to as the Acadian variety. Smith and colleagues mapped the *USHIC* gene to the short arm of chromosome 11 and the locus was further refined to a 2-3 cM interval by Keats, Nouri, Pelias, Deininger, and Litt (1994). Verpy et al. (2000), using a cDNA library derived from mouse inner ear sensory cells identified the *USHIC* gene encoding harmonin and identified a variety of causative mutations (i.e., splice site, frameshift, variable number tandem repeat expansion) in Usher syndrome patients. They further demonstrated that in the inner ear only the sensory hair cells expressed harmonin. It should be noted that hair cells are the sensory receptors of both the auditory and vestibular system existing in the cochlea of the inner ear.

The function of harmonin is not fully understood but the protein contains three PDZ domains. These protein-interaction domains are known to help organize and hold together protein complexes and are a common motif for scaffolding proteins. Adato et al. (2002) demonstrated that harmonin integrates the five known USHI proteins into a

network. Subsequently, Reiners et al. (2005) extended this finding to include interaction between harmonin and products of three of the USHII genes (i.e., IIA, IIC, & candidate IIB). Reiners et al. demonstrated that the USHII proteins (i.e., USH2A, VLGR1, & NBC3) were co-expressed with harmonin in the synapses of both retinal photoreceptors and inner ear hair cells. The protein interaction activities between USHI and USHII gene products provide the first evidence for molecular linkage between Usher type I and II pathophysiology.

Usher ID. The Usher ID locus was localized to a 15 cM region of chromosome 10q using homozygosity mapping in a consanguineous family (Wayne et al., 1996). Boltz et al. (2001) later identified the gene as *CDH23* encoding cadherin 23 using a positional candidate approach and demonstrated that a family linked to the ID locus had mutations in the *CDH23* gene. Similarly, Bork et al. (2001) using seven consanguineous families, independently identified *CDH23* as the cause of type ID Usher. Interestingly, Bork also demonstrated that *CDH23* defects are a cause of non-syndromic hearing loss by identifying mutations in five families with autosomal recessive deafness 12 (i.e., DFNB12). Evidence for a digenic form of Usher type I has been suggested by Zheng et al. (2005) caused by mutation in the genes responsible for ID and IF (i.e., *CDH23* and *PCDH15*).

Cadherin 23, bearing a single transmembrane domain and 27 cadherin repeats, belongs to the superfamily of cadherin genes that are involved in cell adhesion, migration, and compaction as components of adherens junctions (Boltz et al., 2001).

Siemens et al. (2002) demonstrated interaction between cadherin 23 and the scaffolding protein harmonin proposing that the proteins form a transmembrane complex that connects stereocilia into a bundle. Studying hair bundle differentiation in mouse and rats, Boeda et al. (2002) illustrated that harmonin anchors cadherin 23 to stereocilia microfilaments and also interacts with myosin VIIA in a manner that conveys harmonin along the actin core of the developing stereocilia. According to Adato et al. (2005), mouse models mutant for myosin VIIA, harmonin, cadherin 23, protocadherin 15, and sans all exhibit disorganization of their hair bundles. Together these results suggest that these proteins are critical to the proper organization and cohesion of the stereocilia.

Usher IF. The locus for type IF was linked to a 15 cM region on chromosome 10 using homozygosity mapping in a consanguineous family with two members affected with Usher type I (Wayne et al., 1997). Ahmed and colleagues (Ahmed et al., 2001) later refined the locus to 10q21-22 that is homologous to a region in mouse chromosome 10 that harbors the *pcdh15* gene encoding protocadherin. Ahmed screened two families with Usher IF and demonstrated two homozygous truncating mutations in the *PCDH15* gene. Ames waltzer mutant mice carry an autosomal recessive mutation in protocadherin resulting in deafness and vestibular dysfunction (Alagramam, Murcia et al., 2001), further supporting the disease-causing nature of mutations in this gene. Subsequently, Alagramam, Yuan et al. (2001) screened for and described mutations in *PCDH15* for two families with phenotypes consistent with type IF.

The *PCDH15* gene sequence is predicted to encode a protein with 11 cadherin repeats, one transmembrane domain, and a cytoplasmic domain with two proline-rich regions (Alagramam, Yuan et al., 2001). Using immunohistochemistry and reverse transcriptase PCR (i.e., RT-PCR) techniques, Alagramam demonstrated *PCHD15* expression in inner and outer synaptic layers and nerve fiber layers of human adult and fetal retinas as well as in fetal cochlea. Also using immunohistochemistry, Ahmed et al. (2003) localized protocadherin to inner hair cell stereocilia and retinal photoreceptors. At its apical surface, each hair cell contains a bundle of stereocilia that by deflection senses sound waves and movement transducing these mechanical forces into electrochemical signals (Kazmierczak et al., 2007). Kazmierczak et al., using rodent hair cells, recently demonstrated that cadherin 23 and protocaderin 15 interact to form tip-link filaments that connect the stereocilia and are thought to gate the mechanoelectrical transduction channel.

Usher IG. The Usher IG locus was mapped to a 23 cM region on chromosome 17q24-25 using genome-wide screening in a consanguineous family with three affected children (Mustapha et al., 2002). The margins of the mapped locus were subsequently reduced to a 2.6 Mb interval and a candidate gene approach was used to identify the *SANS* gene (Weil et al., 2003). Weil and colleagues identified two different frameshift mutations in the *SANS* gene accounting for the USH type I phenotype in two families. The Jackson shaker mouse, a mouse line that carries a recessive deafness phenotype, was shown by Kikkawa et al. (2003) to have mutations in the *sans* gene. Mice mutant for *sans*

exhibit deafness, abnormal behavior (i.e., circling, head-tossing), and degeneration of the inner ear neuroepithelia.

Weil et al. (2003) predicted the SANS protein to contain three ankyrin-like domains, a sterile alpha motif (i.e., SAM domain) and a PDZ-binding domain. By using co-transfection experiments, Weil demonstrated that SANS associates with harmonin. Adato et al. (2005) later documented interaction between SANS and harmonin as well as SANS and MYO7A. In contrast to the other four USHI proteins, SANS was localized to the apical region of hair cell bodies underneath the cuticular plate with no SANS labeling detected within stereocilia. Mice mutant for *sans* exhibit disorganized stereocilia (Kikkawa et al., 2003) underscoring the importance of this protein to development and maintenance of the stereocilia bundles. These findings led Adato (2005) to suggest that SANS is involved in the proper trafficking of USHI proteins en route to the stereocilia.

Usher IIA. The Usher IIA gene (i.e., *USH2A*) was localized to a 2.1 cM region on chromosome 1q41 (Kimberling et al., 1995). Eudy et al. (1998) further refined the locus and identified a 21 exon gene within the critical region bearing biologically significant mutations in three Usher syndrome patients. Subsequently, Weston et al. (2000), screening 57 independent Usher type IIA probands, identified at least one *USH2A* mutation in 65% of the cohort. Weston further reported that a single mutation, 2299delG, accounts for 16% of the mutant alleles. More recently, van Wijk et al. (2004), identified 51 novel exons at the 3' end of the *USH2A* gene and demonstrated that this region accounted for mutations missed by screening only the first 21 exons in 5 of 12

individuals in a cohort of USH2A patients in which only a single *USH2A* mutation could previously be identified. Aller (2006), screening the new exons identified by van Wijk and an additional exon identified by Adato et al. (2005) similarly found that these new exons accounted for 44% of previously undetected mutations in USH2A patients.

The *USH2A* gene encodes the usherin protein. Based on the original 21 exons, the gene was predicted to produce a protein 1,551 amino acids in length (Eudy et al., 1998). However, multiple isoforms of usherin are transcribed by means of alternative splicing, the largest protein product is composed of over 5,000 amino acids (van Wijk et al., 2004). The usherin protein possesses laminin epidermal growth factor and fibronectin type III domains (Eudy et al., 1998) which are common motifs of basal lamina and extracellular matrixes. The long isoform bears additional functional domains including a transmembrane region and an intracellular domain with a PDZ-binding motif (van Wijk et al., 2004). Using RT-PCR techniques, Eudy et al. (1998) demonstrated that USH2A is expressed in human fetal cochlea, eye, brain, and kidney. Similarly, van Wijk et al. (2004) found that usherin was most highly expressed in fetal cochlea and eye and the adult neural retina. Liu et al. (2007) subsequently demonstrated that usherin localized to the apical inner segment recess that wraps around the connecting cilia that link the inner and outer segment of mammalian photoreceptors and is transiently associated with the hair bundles of the cochlea during postnatal development. Thus, suggesting that usherin plays an important role in long-term maintenance (i.e., structure, signaling) and development in the photoreceptors and sensory hair cells, respectively.

Usher IIC. The IIC locus was confined to a 20-cM region on chromosome 5q using two large families with Usher type II that were shown to be unlinked to the *USH2A* gene (Pieke-Dahl et al., 2000). Weston, Luijendijk, Humphrey, Möller, and Kimberling (2004), explored the very large G protein-couple receptor-1 (i.e., *VLGR1*) gene within the 5q14.3-q21.1 USH2C locus as a candidate gene based on its reported protein structure motifs and expression in cochlea and retina. Weston et al. identified mutations in *VLGR1* for five of the ten independent probands with Usher type IIC and accounted for mutations in two sporadic cases in a cohort of 152 USHII patients. Four different *VLGR1* mutations were identified among the screened patients, none of these mutations were observed among a cohort of 190 control samples further implicating the pathogenicity of mutations in this gene.

The *VLGR1* gene encodes the largest known cell surface receptor (McMillian, Kayes-Wandover, Richardson, & White, 2002). It is found as three alternative transcripts in humans VLGR1a, VLGR1b, VLGR1c, with the 1b transcript accounting for the largest form of the protein. VLGR1b is comprised of 90 exons and greater than 600 kilobases leading to a 6307 amino acid protein. All mutations identified among the Usher IIC patients are found in the VLGR1b isoform (Weston et al, 2004). McMillian and colleagues (2002) report that the protein contains multiple calcium exchanger beta repeats reminiscent of regulatory domains of sodium-calcium exchanger proteins capable of mediating cell aggregation. McMillian et al. further demonstrated that high-level expression of VLGR1 is found in the developing central nervous system and eye using *in*

situ hybridization in mouse embryos. The specific activity of this protein and how it contributes to Usher syndrome is yet to be determined.

Usher IID. The Usher type IID gene was recently identified using a candidate gene approach. Ebermann et al. (2007), using an Usher II family unlinked to known loci, screened the *WHRN* gene for mutations and identified compound heterozygosity for two pathogenic mutations. Mutations in the *WHRN* gene located at 9q32-34 have previously been identified as a cause of the DFNB31 form of autosomal recessive nonsyndromic deafness (Mburu et al., 2003). Mburu et al. demonstrated that defects in whirlin, the product of the *WHRN* gene, underlies deafness in the whirler mouse mutant.

The *WHRN* gene is transcribed as a short or long protein isoform derived from an 8- or 12-exon gene sequence (Mburu et al., 2003). The short protein isoform includes one PDZ domain and one proline-rich domain compared to the long isoform that includes two additional PDZ domains. Kikkawa et al. (2005) demonstrated that the whirlin protein localizes to the tips of hair cell stereocilia and its expression is particularly dynamic during stereocilia growth. Kikkawa et al. concluded that whirlin is involved in stereocilia elongation and actin polymerization. Belyantseva et al. (2005) and Delprat et al. (2005) demonstrated interaction between whirlin and myosin XVa, a motor protein, via the third whirlin PDZ domain thereby delivering whirlin to the tips of stereocilia and effecting differential elongation. The mutations in the reported Usher IID family were found to affect the long isoform of the protein, leading Ebermann et al. (2007) to suggest that

mutations in exons 1-6 may be more detrimental to the retina and cause Usher syndrome, whereas mutations that affect the C-terminus lead to nonsyndromic deafness.

Usher IIIA. The Usher IIIA gene locus was linked to 5-cM region of chromosome 3q (Sankila et al., 1995) with the Finnish mutation region being further narrowed to1-cM (Joensuu et al., 1996) and again to 250 kb (Joensuu et al., 2001). Joensuu et al. (2001) identified a candidate gene within the refined locus that encoded a predicted 120-amino acid protein and demonstrated mutations in a cohort of Finnish Usher III patients, such that 52 patients exhibited a homozygous termination mutation (i.e., Y100X) and four individuals derived from two families were compound heterozygotes (i.e., Y100X and M44K). An Italian family, previously described by Gasparini, De Fazio, Croce, Stanziale, and Zelante (1998), was found to have a homozygous 3-bp deletion (Joensuu et al., 2001).

The *USH3A* gene encodes the clarin-1 protein (Adato et al., 2002). Multiple isoforms of varied transcript length and structure have been described (Adato et al., 2002; Fields et al, 2002; Joensuu et al, 2001). The full-length transcript encodes a protein of 232 amino acids with four transmembrane domains (Adato et al., 2002). Using PCR amplification of cDNA, Adato and colleagues demonstrated expression in human tissue including retina, skeletal muscle, testis and olfactory epithelium as well as localization to inner and outer cochlear hair cells and spiral ganglion cells using mouse derived transcripts. To date, little is known about the function of clarin-1. It belongs to a large hyperfamily of small integral membrane glycoproteins. Sequence homology comparisons

show a limited similarity to stargazin, a cerebellar synapse protein, leading Adato et al. to suggest that clarin-1 may play a role in hair cell and photoreceptor cell synapses.

Treatment for Usher Syndrome

There is no cure for Usher syndrome and treatment options to reduce or compensate for disease symptoms are limited. The National Institute for Deafness and Other Communication Disorders (NIDCD, 2008) recommends a variety of approaches to cope with Usher syndrome including hearing aids/assistive listening devices, auditory training, Braille instruction, low vision services, and orientation/mobility training. Multiple approaches may be used to maximize the compensation and the specific intervention will depend on characteristics of the individual such as age and severity of symptoms.

Cochlear Implants

Cochlear implants have been shown efficacious for treating bilateral severe-to-profound sensorineural hearing loss in children with early onset deafness as indexed by improved speech/language development and sound discrimination as summarized by Papsin and Gordon (2007). A recent position statement by the Joint Committee on Infant Hearing [JCIH] states that in accordance with FDA guidelines for children 12 months and older with profound bilateral sensorineural loss and 24 months and older with severe bilateral loss "cochlear implantation should be given careful consideration for any child who seems to receive limited benefit from a trial with appropriately fitted hearing aids" (JCIH, 2007).

Due to the differential level of hearing loss between the Usher syndrome clinical subtypes, cochlear implants are most applicable to Usher type I, as it is more typically associated with severe-to-profound deafness. Pennings et al. (2006) conducted a retrospective study of 14 Usher type I patients with cochlear implants and observed improved audiologic performance. Pennings et al. further demonstrated that among their cohort implantation at an earlier age was associated with better performance. A comparative study assessing quality of life between Usher I patients with (n = 14) and without (n = 14) cochlear implants illustrated that cochlear implants improved the ability of patients to live independently, although the effect was observed most strongly for hearing-related quality of life issues (Damen, Pennings, Snik, & Mylanus, 2006).

Vitamin A Supplementation

Berson et al. (1993) conducted a randomized, double-blind clinical trial of vitamin A and vitamin E supplementation to determine if visual decline due to retinitis pigmentosa could be attenuated. The cohort (n = 601; 18-49 years) was divided among four treatment groups receiving either 1) 15,000 IU/day vitamin A, 2) 15,000 IU/day vitamin A + 400 IU/day vitamin E, 3) trace amounts of vitamin A + vitamin E, or 4) 400 IU/day vitamin E for the duration of the 4-6 year trial. The study concluded that individuals taking 15,000 IU vitamin A/day on average had about a 20 percent slower annual decline of retinal function as indexed by electroretinography. Conversely, vitamin E supplementation at 400 IU/day was suggested to have an adverse effect on the course of RP. Of interest, the authors reported that 12% of the cohort had partial hearing loss in

addition to RP and defined these individuals as Usher syndrome type II. Thus, the clinical recommendation that patients diagnosed with RP take 15,000 IU vitamin A (i.e., retinyl palmitate) also extends to Usher type II.

Supplementation with vitamin A at this level is a concern as daily intake greater than 25,000 IU over the long-term can be toxic and cause side effects including liver disease (Hathcock et al., 1990; Geubel, DeGalocsy, Alves, Rahier, & Dive, 1991). Hathcock et al. caution that children and pregnant women represent particularly vulnerable groups for toxicity; 1,500 IU/kg/day has been observed to cause adverse effects in children and maternal intakes of 25,000 IU/day have been associated with birth defects. A review by Semba (2002) concludes that vitamin A supplementation at 15,000 IU/day is safe in healthy adult men and non-pregnant women without otherwise excessive dietary intake of vitamin A from food sources. However, Semba underscores the importance of annual liver enzyme and triglyceride assays for those who follow this supplementation regime.

Gene Therapy

Gene-based approaches for treatment of RP are under investigation and include gene replacement therapy, gene suppression via ribozymes or siRNA, neuroprotection via growth factors (e.g., ciliary neurotrophic factor [CNTF], glial-derived neurotrophic factor [GDNF], and brain fibroblast neurotrophic factor [bFGF]), and neuroprotection via antiapoptotic factors (e.g., bcl-2) (Hamel, 2006). Hashimoto et al. (2007) recently reported promising gene replacement therapy in a mouse model for Usher type IB using a

lentiviral vector. Hashimoto et al. observations include the *in vivo* restitution of wild-type levels of MYO7A in cultured retinal pigment epithelium [RPE] cells and retina, the restoration of the apical location of melanosomes in RPE cells, and the correction of opsin accumulation in the photoreceptor connecting cilium. These findings demonstrate that gene replacement of *MYO7A* can mediate correction of cellular perturbations in the retina. Rebibo-Sabbah, Nudelman, Ahmed, Baasov, and Ben-Yosef (2007) also recently reported the outcome of a gene therapy approach for Usher I. In contrast to gene replacement, Rebibo-Sabbah et al. explored the use of aminoglycosides to suppress nonsense mutations in *PCDH15*, the gene responsible for Usher IF. Rebibo-Sabbah and colleagues successfully demonstrated suppression both *in vitro* and *ex vivo* and reduced the observed cytotoxicity of commercial aminoglycosides by creating a new aminoglycoside-derivative, NB30.

The use of growth factors, specifically CNTF, as neuroprotective agents to preserve retinal integrity has progressed to human clinical trials. A Phase I trial exploring intraocular encapsulated CNTF implants demonstrated both safety and utility of this mode of administration in 10 patients with retinal degeneration (Sieving et al., 2006). Two Phase II/III clinical trials are currently underway investigating the encapsulated CNTF technology in patients with early stage (USNIH, 2007b) and late stage (USNIH, 2007c) retinitis pigmentosa. The CNTF trials involve implanting a capsule containing human RPE cells into the ocular vitreous. The RPE cells were transfected to produce CNTF and excrete it via diffusion through the capsule membrane into the vitreous. Both a

high-dose and low-dose treatment are being evaluated for efficacy of vision preservation. The eligibility criteria for both trials allowed the inclusion of patients with Usher type II and III.

Biological Significance of DHA

Docosahexaenoic acid is a long-chain, omega-3 polyunsaturated fatty acid. It is available directly from dietary sources or synthesized by the liver from precursor fatty acids. With six double bonds, DHA is the most unsaturated fatty acid present in biological systems. Typically, DHA is present in membranes throughout the human body at levels of 1 to 5% of total fatty acids; however, higher levels of 9%, 20% and 35% are found in the neural cortex, the retina, and rod photoreceptor outer segments, respectively (Fliesler & Anderson, 1983; Futterman, Downer, & Hendrickson, 1971; Martínez, 1992a). The high levels of DHA observed in retinal tissues are suggestive of biological significance.

DHA As a Membrane Component

The functional role of DHA in the retina is still under investigation. One explanation of its significance centers on the fluid mosaic model (Singer & Nicholson, 1972) of lipid bilayer membranes. The highly unsaturated nature of DHA may contribute to enhanced membrane fluidity thereby optimizing visual transduction (Dratz & Deese, 1986). Support for this can be found in membrane fluidity studies (Stubbs & Smith, 1984), the tight association of the photo-pigment rhodopsin to DHA in cell membranes (Rodriguez-deTurco, Jackson, Parkins, & Gordon, 2000), and the rate of G protein-

coupled signaling as measured by the formation of the activated rhodopsin-transducin complex (Mitchell, Niu, & Litman, 2001; Niu, Mitchell, & Litman, 2001).

Mitchell (1998) demonstrated that enrichment of the lipid membrane with DHA modified physiological and biophysical actions ranging from enzyme and receptor function to nutrient transport systems. Rotstein, Aveldano, Barrantes, and Politi (1996) found that DHA promoted differentiation (i.e., ospin expression and apical process formation) of cultured rat photoreceptors and concluded that DHA was required for retinal photoreceptor survival. Kim and colleagues (Kim, Akbar, Lau, & Edsall, 2000) later reported a reduction of apoptotic cell death in mouse neuronal cells exposed to DHA as both DNA fragmentation and caspase-3 activity were attenuated.

DHA and Influence on Transcriptional Activity

Docosahexaenoic acid has also been implicated in the regulation of transcription in mouse brain acting as a ligand for the retinoid X receptor (de Urquiza et al., 2000). The retinoid X receptor belongs to a superfamily of nuclear receptors (i.e., ligand-activated transcription factors) that bind Vitamin A metabolites. Nuclear receptors in general are responsible for regulating a wide range of biological process such as development, metabolism, and reproduction. A follow-up study (Lengqvist et al., 2004) demonstrated that DHA is a potent retinoid X receptor ligand and induces robust activation at even low micromolar concentrations. Rojas and colleagues (Rojas, Martinez, Flores, Hoffman, & Uauy, 2003) demonstrated that DHA altered transcription of a diverse variety of genes

including those associated with neurogenesis, neurotransmission, and lipid metabolism in cultured human fetal retinal explants.

Eicosanoid and Docosanoid Mediators

The omega-3 and omega-6 fatty acids represent two major classes of polyunsaturated fatty acids, in mammals these pathways are metabolically and functionally distinct (Lands, 1992). Linoleic acid (18:2) and arachidonic acid (20:4) are key omega-6 fatty acids. In vertebrates, linoleic acid is an essential fatty acid and thus, must be consumed in the diet. Arachidonic acid may be ingested or biosynthesized through a series of desaturation and elongation reactions from the shorter chain linoleic acid. Key nutritional fatty acids of the omega-3 pathway are α-linolenic acid (18:3), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6); eicosapentaenoic and docosahexaenoic acids may be obtained from the diet or biosynthesized. However, the shorter chain omega-3 precursor, α-linolenic acid, is an essential fatty acid.

Arachidonic acid and eicosapentaenoic acid are physiologically significant as precursors for bioactive eicosanoid compounds such as, prostaglandins, prostacyclin, thomboxanes and leukotrienes (Lands, 1992). The activity of omega-6 derived prostaglandin and leukotriene mediators are primarily pro-inflammatory, whereas omega-3 derived eicosanoids are mostly anti-inflammatory (Calder, 2001, 2006). Both biochemical and physiologic competition exist for arachidonic and docosahexaenoic acid as well as for their biologic mediators demanding that a balance of both fatty acids and mediators be maintained (Lands, 1992). An excess of omega-6 fatty acids shifts the

balance toward omega-6 mediated events that over a long-term culminate in pathology such as thrombosis (heart attacks and ischemic strokes) and immune-inflammatory disease (arthritis, lupus, asthma) (Lands, 1992; Simopoulos, 1999).

Long-chain omega-3 polyunsaturated fatty acids may act both directly and indirectly to decrease the production of inflammatory eicosanoids, cytokines, reactive oxygen species, and expression of adhesion molecules (Calder, 2006). Indirect action may involve altering the transcriptional activation of inflammatory genes. Direct action may involve inhibiting the production of omega-6 mediators by replacing arachidonic acid with an omega-3 eicosanoid substrate. In concert or alternatively, eicosapentaenoic acid and docosahexaenoic acid may produce a group of novel mediators that possess both anti-inflammatory and protective properties, namely, resolvins, docosatrienes, and neuroprotectins (Serhan, 2005).

Resolvins. Serhan et al. (2002) identified a novel family of bioactive docosanoids synthesized via the cyclooxygenase-2 [COX-2] pathway in the presence of aspirin. These compounds were first observed in inflammatory dorsal pouch exudates during the resolution phase in mice treated with aspirin and docosahexaenoic acid. Experiments showed that exudates, vascular cells, leuckocytes, and neural cells treated with aspirin were capable of endogenous conversion of docosahexaenoic acid to a series of 17*R*-hydroxy-docosanoids (i.e., resolvins). Serhan further demonstrated that these compounds inhibited tumor necrosis factor α-induced [TNF α] cytokine expression in microglial cells

at nanonmolar concentrations. The term resolvin was coined to describe these chemical mediators of the pro-resolution of inflammation.

The omega-3 derived resolvins can be classified into two series (Serhan, 2005). Those derived from precursor eicosapentaenoic acid give rise to the 18*R* E-series (i.e., Resolvin E1-E2) whereas precursor docosahexaenoic acid gives rise to the 17*R/S* D-series (i.e., Resolvin D1-D6). According to Serhan (2005), both E and D class resolvins are potent mediators of anti-inflammation. A comparison of mice injected with 100ng of either D1 or E1 resolvin resulted in 50% and 75-80% inhibition of polymorphonuclear [PMN] leukocyte infiltration, respectively. No difference was oberved in bioactivity between the D-series *S* or asprin-induced *R* epimers.

Docosatrienes. Compounds with conjugated triene structures that are derived from docosahexaenoic acid are named docosatrienes. Hong, Gronert, Devchand, Moussignac, and Serhan (2003) identified a novel 17*S*-hydroxy docosanoid via a high-performance liquid chromatography and tandem mass spectrometry lipidomic analysis of whole blood, leukocytes, brain, and glial cells. The predominant bioactive docosatriene observed by Hong was 10,17*S*-docosatriene. Assessed immunoregulatory bioactivity included reduced leukocyte infiltration and reduced cytokine production. Hong demonstrated that *in vivo* treatment of acute inflammation (i.e., murine peritonitis) with the isolated novel docosatriene was a potent systemic inhibitor of PMN infiltration as evidenced by a 42% reduction of PMN in the peritoneal exudates, compared to a 40% reduction induced by indomethacin, a non-steroidal anti-inflammatory drug. A follow-up

study (Marcheselli et al., 2003) later demonstrated that 10,17*S*-docosatriene also possessed neuroprotective activity.

Neuroprotectins. Using a murine model to study ischemic stroke, Marcheselli et al. (2003) demonstrated that 10,17*S*-docosatriene inhibited leukocyte infiltration, NFκB, and COX-2 induction. Similarly, cell culture experiments treating human neural progenitor cells with interleukin 1-β demonstrated that 10,17*S*-docosatriene down-regulated NFκB (i.e., nuclear factor-kappa B, a transcription factor sensitive to cell stress) to a level below that of unstimulated cells in a concentration-dependent manner and decreased COX-2 expression. Lipid peroxidation, leukocyte infiltration and pro-inflammatory gene expression each contribute to stroke damage. Thus, this novel endogenous docosanoid countered leukocyte-mediated injury as well as inhibited the induction of pro-inflammatory genes equating to potent neuroprotection.

Additional neuroprotective behavior was subsequently described in studies of human retinal pigment epithelial cells [RPE] (Mukherjee, Marcheselli, Serhan, & Bazan, 2004) and aging human neural progenitor cells (Lukiw et al., 2005). Mukherjee et al. demonstrated that cultured human RPE cells synthesized 10,17*S*-docosatriene. Culturing cells in media enriched with DHA enhanced the endogenous production of 10,17*S*-docosatriene and when treated with H_2O_2 and TNFα to induce oxidative stress, apoptotic cell death was reduced as indexed by fewer Hoechst-positive cells. Mukherjee et al. observed up-regulation of anti-apoptotic proteins (i.e., Bcl-2 and Bcl-x_L), down-regulation of pro-apoptotic expression (i.e., Bax and Bad), and inhibition of caspase-3

activation. Based on these and previously described neuroprotective properties, 10,17*S*-docosatriene was named neuroprotectin D1 (i.e., NPD1).

Lukiw et al. (2005) also observed that DHA served as a precursor for NPD1 as supplemental DHA enhanced the endogenous production of NPD1 and was associated with decreased amyloid-β secretion in aging human neuronal cell cultures subjected to cytokine-induced (i.e., IL-1 β) oxidative stress. Interestingly, Lukiw and colleagues also assessed DHA and NPD1 concentrations in the postmortem brain of moderate-stage Alzheimer disease patients compared to age-matched controls and determined that DHA and NPD1 was reduced in some regions of the Alzheimer brain. For example, the hippocampal region was modestly decreased in DHA (two-fold reduction), but exhibited dramatically reduced levels of NPD1 (i.e., twenty-fold reduction). The reductions in NPD1 were in excess of that expected by the observed decrease in DHA precursor or due to the estimated loss of neurons in the diseased brain perhaps indicating an excessive level of oxidative stress sufficient to overwhelm the neuroprotective capability of NPD1.

Mukherjee, Chawla, Loayza, and Bazan (2007) recently summarized the multifunctional role of NPD1 in directing cell fate and the associated implications for aging and disease. In studies of RPE and aging neural cells, signaling mediated by cell-damaging events initiate the generation of NPD1 and other members (e.g., Bcl-2, neutrophins) of a cell fate-regulatory pathway whose interactive function appear to cooperatively redirect cell fate toward preservation. Mukherjee et al. concluded that

"activation of NPD1 biosynthesis, NPD1 analogs, or dietary regimens may be useful for exploring new preventive/therapeutic strategies for neurogenerative diseases" (p. 237).

DHA, Visual Function, and Neural Development

Several of the earliest studies to investigate the relationship of DHA to vision were in dietary fatty acid deprivation studies of rats (Benolken, Anderson, & Wheeler, 1973; Wheeler, Benolken, & Anderson, 1975) and non-human primates (Neuringer, Connor, Lin, Barstad, & Luck, 1986; Neuringer, Connor, Van Petten, & Barstad, 1984). These studies demonstrated that omega-3 deprived animals had poorer retinal function as indexed by electroretinography (ERG). Similar findings have been observed in preterm human infants thus deprived of third trimester placental transfer of DHA. Studies of preterm infants randomized to either commercial formula (DHA absent) or DHA-enriched formula found reduced (poorer) ERG responses (D.G. Birch, E. E. Birch, Hoffman, & Uauy, 1992; Uauy, Birch, Birch, Tyson, & Hoffman, 1990) and visual acuity (E. E. Birch, D. G. Birch, Hoffman, & Uauy, 1992) in the commercial formula fed infants. Observed benefits of preterm supplementation, subsequently brought into question the potential need for added DHA in the term infant diet.

Since the preterm infant studies, numerous randomized, controlled clinical trials have been conducted to assess the functional benefits of DHA supplementation in term infants with mixed results. Two recent reviews have assessed the reported studies for benefits (Eilander, Hundscheid, Osendarp, Transler, & Zock, 2007; Simmer, Patole, & Rao, 2008). Eilander et al. reviewed the results from nine randomized trials in term

infants and observed that four trials reported beneficial effects of DHA supplementation on visual development (e.g., visual acuity, stereoacuity). Eilander and colleagues noted the studies that observed benefits were supplementing at higher doses of DHA (i.e., 0.36% DHA) and using sensitive electrophysiological testing. The Simmer et al. Cochrane analysis reviewed 14 randomized trials and concluded that both visual function and neurodevelopmental outcomes were inconsistent. Three of nine studies conducting visual function throughout the first three years of life observed a benefit of DHA supplementation. Of the eight studies using the Bayley Scales of Infant Development, only one study reported a benefit. Comparison across all the reported term DHA supplementation trials is confounded by differing levels of DHA supplementation, duration of supplementation, and functional measures. The mixed results observed among the studies may suggest that the level of DHA supplementation is highly relevant as those studies supplementing with the higher levels of DHA consistently found benefits.

Fatty Acid Studies in Retinal Disease

Fatty Acid Status in Retinitis Pigmentosa

With the exception of visual impairments, patients with RP are without systemic disease and commonly considered healthy, yet reports of low blood DHA levels among this population are common. A study by Hoffman, Uauy, and Birch (1993) found marked differences in the RBC content of long-chain polyunsaturates between patients with the autosomal dominant form of RP (adRP) and normally-sighted controls. Although DHA per se was not significantly different (12% decrease), total ω3 long-chain polyunsaturates

were reduced in the adRP patients ($p = .009$). In consideration of the genetic diversity of the population, patients with low DHA values were determined to have significant abnormalities in their ω3 fatty acid elongation and desaturation biosynthetic pathways compared to patients with high DHA values. Evidence for a relationship between DHA levels and severity of rod loss was found across all patients. The ratio of dark-adapted rod to cone ERG amplitude, which varies less with age than b-wave amplitude alone, was found to be significantly correlated ($p = .036$) with DHA in RBCs of adRP patients.

McColl and Converse (1995) and more recently Hoffman (2000) reviewed studies of fatty acids and retinal disease. Ten of fourteen studies cited by Hoffman in RP patients reported decreased blood-DHA levels. Of the six studies that reported RBC-DHA, five found reductions ranging from 12-38% compared to unaffected control participants. Hoffman and Birch (1995) reported the most striking reductions (i.e., 38%) in 18 patients with the X-linked form of RP such that RBC-DHA levels on average were 2.5% of total fatty acids in xlRP compared to 4.0% among controls. This study also demonstrated a positive correlation between RBC-DHA content and cone ERG amplitude, such that patients with higher RBC-DHA also had better cone function. Even more interesting was that b-wave implicit times, which did not vary with age, were more prolonged in patients with low levels of RBC-DHA. Reduced RBC-DHA concentrations associated with diminished cone ERG function were also found in about 70% of xlRP heterozygote carriers (Hoffman, Wheaton, Locke, & Birch, 1998).

To ascertain whether a defect in the DHA biosynthesis pathway accounted for low blood levels of DHA in xlRP patients, Hoffman, DeMar, Heird, Birch and Anderson (2001) conducted a clinical trial using oral administration of a stable isotope precursor. DHA biosynthesis was assessed in five severely affected patients and five age-matched normally sighted controls by quantifying conversion of [U-^{13}C]-α -linolenic acid (α-LNA) to ^{13}C-DHA in blood samples by gas chromatography/mass spectroscopy analysis. The mean peak of isotopic enrichment in DHA of xlRP patients was one-half that of controls and the peak incorporation time was significantly delayed ($p = .03$). The product-to-precursor ratios of ^{13}C-DHA to ^{13}C-18:3ω3 and that of ^{13}C-20:5ω3 to ^{13}C-20:4ω3 (Δ^5-desaturase mediated conversion) were significantly lower in patients compared to controls ($p = .03$ and .05, respectively). The estimated biosynthetic rates for numerous pathway intermediates beyond 20:5ω3 were also lower supporting downregulation of Δ^5-desaturase in xlRP. Increased rates of fatty acid oxidation or other routes of catabolism were not higher among patients. Thus, despite individual variation among both patients and controls, Hoffman et al. concluded that the data were consistent with a lower rate of Δ^5-desaturation suggesting that decreased biosynthesis of DHA may contribute to lower blood levels of DHA in patients with xlRP.

Fatty Acid Status in Usher Syndrome

Few studies have focused specifically on Usher syndrome, an allied retinal disorder. As an autosomal recessive disease composed of both deafness and RP, Usher

patients have been intermingled in some previous studies among the recessive sub-group of RP. Four studies (Bazan, Scott, Reddy, & Pelias, 1986; Connor, Weleber, DeFrancesco, Lin, & Wolf, 1997; Maude, Anderson, & Anderson, 1998; Williams, Horrocks, Leguire, & Shannon, 1989) notably evaluated blood fatty acids in Usher syndrome.

Bazan et al. (1986) described analysis of plasma phospholipids in 15 Usher syndrome patients from four families thought descended from the same ancestral parents. DHA was determined to be 64% of normal; however, the patients' clinical subtypes were not assessed. In a cohort of RP patients, Williams et al. (1989) reported that the levels of both DHA and the omega-6 fatty acid, arachidonic acid of seven Usher patients were lower than normals. However, neither mass/percentage fatty acid values nor clinical subtype characterization were given.

Connor et al. (1997) conducted a study in 26 patients with RP to evaluate fatty acid composition in both RBCs and sperm. Sperm were selected as a supplemental index as their cellular membranes, like retina and brain, are known to be highly enriched in DHA. Additionally, both retina and sperm have axonemal structures found in the photoreceptors and flagellum (Hunter, Fishman, Mehta, & Kretzer, 1986) as do developing cochlear hair cells (Steyger, Furness, Hackney, & Richardson, 1989). Six patients within the Connor et al. cohort were determined to be Usher II and two were Usher I. RBC-DHA levels were reported to be 78% of normal in the heterogeneous grouping of RP patients ($n = 26$). However, no RBC-DHA values were given for the

Usher subtypes. Sperm-DHA levels were the lowest in Usher II at 24% of normal, whereas the remaining cohort had levels 67% of normal.

Subsequently, Maude et al. (1998) reported fatty acid levels in plasma and RBC phospholipids from patients with Usher I ($n = 35$) and Usher II ($n = 30$) compared to normal controls ($n = 54$). In contrast to the Connor et al. (1997) findings in sperm, Maude et al. reports that plasma and RBC-DHA were no different between Usher II and controls. However, reductions were reported for Usher I such that DHA was deceased by 20% and 37% in plasma and RBC. Interestingly, Maude also reported significant elevations of palmitic acid (i.e., 16:0) and 18:1ω7 in type I Usher patients, as well as a reduction in arachidonic acid, a major omega-6 fatty acid (i.e., 20:4ω6).

Gaps, inconsistencies, and flaws in current knowledge are due to the small number of studies, small sample size, data sets from patients with indeterminate clinical subtypes, absence of Usher III patient data, and lack of a dietary assessment to evaluate intake of omega-3 rich food sources. Furthermore, the Connor et al. (1997) and Maude et al. (1998) studies report intuitively conflicting outcomes in the assessment of sperm and blood. Genetic advances now allow genotypic classification of Usher syndrome, thus improving the ability to subtype patients.

Clinical Intervention Trials Supplementing with DHA

Evidence for health benefits associated with eating an omega-3 rich diet or boosting intake with supplements is plentiful. A brief search of the literature revealed an ample volume of information regarding both cardiovascular and immunoregulatory

benefits. According to Simopoulos (2002), coronary heart disease and a variety of inflammatory and autoimmune diseases are characterized by high levels of interleukin 1, a proinflammatory cytokine. The knowledge that DHA and eicosapentaenoic acid have biologically potent anti-inflammatory activity (Serhan, 2005), has lead to clinical supplementation trials assessing their usefulness in the management of such diseases. Simopolous reported a diverse array of clinical trials assessing fish oil in the treatment of coronary disease, rheumatoid arthritis, Crohn's disease, ulcerative colitis, psoriasis, lupus, multiple sclerosis, and migraine headaches concluding that among the placebo-controlled trials of chronic inflammatory disease there were significant benefits such as decreased disease activity and lowered use of anti-inflammatory medications. DHA supplementation trials among diseases with a retinal degeneration phenotype have also been conducted and are assessed in the paragraphs to follow.

Peroxisomal Diseases

Blood fatty acid abnormalities have been reported for Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease; three diseases due to peroxisome dysfunction (Moser, Jones, Raymond, & Moser, 1999). Peroxisomal disorders are severe congenital diseases characterized by deterioration of the central nervous system, psychomotor retardation, retinopathy, liver disease, and early death (Martínez et al., 2000). Martínez (1989) was the first to show that DHA was profoundly diminished in Zellweger disease in a variety of body tissues; specifically, RBCs, forebrain, liver, and kidney of a 3-month old infant decedent compared to six age-

matched controls. There was also an increase in very long-chain fatty acids (i.e., 26:0 and 26:1) and a great reduction in plasmalogens that are classically associated with this disease. Evidence of low DHA levels was subsequently substantiated in additional patients with severe peroxisomal disorders (Martinez, 1992b). As DHA is a critical component of membranes of the central nervous system and brain, several trials to investigate the efficacy of nutritional supplementation to attenuate the severity of these disorders were untaken.

Initial DHA supplementation (Martínez, Pineda, Vidal, Conill, & Martin, 1993) was conducted in two patients with neonatal adrenoleukodystrophy with promising results, namely, treatment with DHA ethyl ester raised RBC polyunsaturates to normal levels and one child exhibited neurological improvement. Moser, Jones, Raymond, and Moser (1999) supplemented a group of 12 patients with peroxisomal disease in an open study design using 100 mg/kg each of microencapsulated DHA and arachidonic acid added to food or formula. Moser et al. reported that supplementation resulted in normalization or moderate elevation of DHA and aracidonic acid in plasma and RBCs. Subsequently, Martinez et al. (2000) reported a treatment trial with 13 peroxisomal disorder patients (range = 5 months to 6 years old) supplemented with 100-500 mg of DHA ethyl ester per day demonstrating normalized RBC DHA levels within a few weeks of trial onset as well as increased plasmaologens and reductions in plasma very long-chain fatty acids. Supplementation also resulted in improvements in vision, liver function, muscle tone, and improvements in brain myelination. Ten participants were treated for a

range of eight months to six years; three participants succumbed to disease complications. Martinez and colleagues state that all patients experienced some clinical benefit with improvements in vision and liver function being the most consistent finding.

Retinitis Pigmentosa

XLRP. Hoffman et al. (2004) describe the outcome of a 4-year, Phase I, double-blind placebo-controlled clinical trial to evaluate the potential benefits of DHA supplementation in retarding the progressive loss of visual function in patients with xlRP. The trial goals were to elevate RBC lipid concentrations of DHA and determine, using ERG, whether variations in RBC-DHA of patients were related to the rate of disease progression. Forty-four patients (mean age = 16 years; range = 4-38 yr) with early-stage xlRP were randomized to groups receiving capsules containing DHA-enriched oil (400 mg DHA/day; $n = 23$) or a corn/soy oil placebo ($n = 21$). Blood samples were collected every six months for biosafety and fatty acid determination and visual function assessments (i.e., ERG, visual acuity, visual field perimetry, dark-adaptometry, and fundus photos) were conducted annually. The ERG cone response amplitudes to 31-hz flicker were the primary trial outcome measure. Hoffman et al. concluded that based on the cone ERG findings from the intent-to-treat protocol there was no benefit to DHA supplementation at the dosages utilized. The lack of a significant benefit may be attributable to numerous limitations of the study (small sample size, confounding variables such as patient ages, bodyweight, compliance, and dosage). However, a

supplemental analysis dividing the cohort according to age revealed that cone ERG loss was slowed in older xlRP patients while rod ERG loss was retarded in young patients.

Wheaton, Hoffman, Locke, Watkins, and Birch (2003) separately reported the biological safety aspects of long-term, low-dose DHA supplementation. The biological safety analysis included a battery of total (i.e., plasma, RBC) and phospholipid fraction fatty acid determinations, plasma vitamin A and E concentrations, plasma antioxidant capacity, alanine aminotransferase activity, lipoprotein cholesterol and triglyceride profiles, and whole blood platelet aggregation. Adverse events were reported on a "per incident" basis. Wheaton et al. determined that long-term DHA supplementation in xlRP patients at a dose of 400 mg/day elevated blood levels 2.5-fold and was associated with no identifiable safety risks in this 4-year Phase I clinical trial.

Mixed RP cohort. Berson et al. (2004a) conducted a 4-year, randomized, placebo-controlled, double-masked trial in 221 patients with retinitis pigmentosa due to various genetic inheritance patterns (e.g., dominant, recessive, isolate). The cohort was aged 18 to 55 years and 11% of patients reported partial hearing consistent with a diagnosis of Usher syndrome. All patients were supplemented with 15,000 IU of vitamin A (i.e., retinyl palmitate) and randomized to receive 1,200 mg/day DHA or control capsules. Visual function assessment and blood biosafety analyses were conducted annually throughout the trial. Dietary assessment of omega-3 intake was also assessed. The primary visual function outcome was the total point score for the 30-2 program of the Humphrey visual field analyzer. Secondary functional measures included the 30-hz ERG amplitude, visual

acuity, and additional visual field measures (i.e., 30-2 and 30/60-1 combined). Berson et al. concluded that there was no significant difference in the decline of visual function between the vitaminA/DHA group and the vitamin A/placebo group over the trial duration. There were no reported cases of toxicity.

A supplemental analysis of the study data divided the cohort according to prior vitamin A supplementation (Berson et al, 2004b). Seventy percent of patients were reported to have taken vitamin A at the trial dosage of 15,000 IU per day. The ocular decline of these patients was compared to patients that had not previously taken the vitamin A regime creating subgroups within the DHA and placebo cohorts. According to these comparisons, Berson and colleagues concluded that among patients not taking vitamin A prior to study entry, the vitamin A/DHA group had a slower decline in field sensitivity and ERG amplitude than the corresponding vitamin A/placebo group. This benefit was observed only over the first 2 years of the study. For those patients taking vitamin A for at least 2 years prior to the study onset, it was determined that a diet rich in omega-3 fatty acids slowed visual field sensitivity loss.

Usher Syndrome

The applicability of DHA supplementation in Usher syndrome is currently under evaluation. Previous studies of blood-DHA levels in RP and Usher syndrome, together with the more detailed results from the current study, will help guide decision making regarding the appropriateness and design of a clinical intervention trial. Although a nutritional trial is not meant to "cure" the disease, significant benefit to the patient may

be possible if useful vision is preserved for a greater length of time through simple, dietary manipulation and/or supplementation.

Conclusion

Studies of the bioactivity of DHA, functional assessment of DHA-deprived models, and correlation studies relating DHA to visual function in patients with retinal disease suggest a biologically valuable role for DHA in normal vision. Although the origin of degenerative retinal diseases is due to specific gene mutations, variability in disease severity among family members sharing the same gene mutation raise the possibility that secondary factors may play a role (Rosenberg, Schwahn, Feil, & Berger, 1999; Sharon et al., 2000). Such factors may be genetic, environmental, metabolic, or dietary and exert their influence in a multifactorial manner. For example, the microenvironment of the photoreceptors may contribute to the dysfunction in visual processing. As fatty acids are major membrane components that influence the mobilization and conformation shifts necessary for proteins and enzymes to function, deficiency in long-chain polyunsaturated fatty acids, such as DHA, that enhance membrane fluidity may compromise the efficiency of biomolecules vital to visual transduction. Clinical trials evaluating the safety (Wheaton, Hoffman, Locke, Watkins, & Birch, 2003) and benefit of DHA supplementation to slowing disease progression in RP have been conducted (Berson et al., 2004; Hoffman et al., 2004) and are in follow-up studies.

Small sample sizes, design inconsistencies, and omissions in previous studies of Usher syndrome suggest that a follow-up study would be beneficial to clarify earlier outcomes. Furthermore, the availability of genotypic data can be brought to bear to better characterize patients and evaluate whether blood-DHA differences exist between major identifiable genotypes. Thus, the current study was designed to clarify and extend current knowledge of the omega-3 fatty acid status in patients with Usher syndrome, and will subsequently assist in determining the merit of a nutritional intervention trial.

CHAPTER III

METHODOLOGY

This was a descriptive study using a retrospective, cross-sectional, quantitative design.

Population and Sampling Procedures

The de-identified dataset for this study was comprised of individuals who participated in a collaborative project to evaluate blood DHA levels in patients with Usher syndrome conducted between January 2004 and May 2008 at the Retina Foundation of the Southwest [RFSW]. The original study recruitment involved drawing a convenience sample of patients with Usher syndrome in the United States identified through genetic databases at BTNRH, Omaha, Nebraska and RFSW, Dallas, Texas. Both registries draw from multi-state or nationwide patient populations resulting in ascertainment of ~40 and ~1,300 Usher patients and at-risk family members from RFSW and BRNRH, respectively.

Eligible participants (see *Delimitations*) were approached for participation by letter of invitation. Recruitment emphasis was given to individuals who had been previously genotyped. The recruitment goal was twenty patients from each genotype known at the outset of the study (i.e., six genotypes). Additional genotypes were genetically characterized over the duration of study recruitment, thus Usher syndrome participant recruitment was expanded beyond the original proposal in order to include sufficient numbers of individuals from all available genotypes. A cohort of unaffected,

age-matched individuals (goal of $n = 60$) was also recruited from the BTNRH and RFSW databases. When available, an unaffected sibling was prioritized for recruitment as the control for the Usher participant.

Protection of Human Participants

The study was approved by the Institutional Review Board (IRB) of the University of Texas Southwestern Medical Center (Dallas, TX) as part of a continuing renewal of "Retinal Pathophysiology in Infants and Adults" (Appendix A). Participants were informed of the nature of the study and written consent was obtained in all cases. Exempt status approval was obtained from the Texas Woman's University's IRB (Appendix B).

Data Collection Procedures

Study materials were mailed to individuals who agreed to participate. The mailer included instructions, a demographic form, a consent form, a dietary questionnaire, and a blood mailer package inclusive of return shipping. Participants completed the study documents and had their blood sample drawn at a location of their convenience. All study participants were instructed to fast (i.e., abstain from eating) for a minimum of four hours. Blood sample collection was primarily scheduled for Monday through Thursday to allow for overnight shipping by express courier to the RFSW biochemistry laboratory for fatty acid analysis; all study documents were returned with the sample. Study participants living in the Dallas area completed their study documents and had their blood drawn at the RFSW. Upon receipt of the blood samples and documents, a unique, coded

identification number was assigned to each study participant. Data from fatty acid analysis, the questionnaire, and basic demographics (i.e., gender, age) were entered and maintained in a de-identified manner in Excel databases. Original, source documents were filed in a study-specific binder in the possession of an RFSW investigator and access was restricted to protect participant privacy.

Blood Lipid Analysis

Assessment of retinal DHA levels is not feasible in living, intact human tissue. Thus, blood lipid levels, plasma and/or red blood cells (RBC) are customarily used as a surrogate index. Connor, Lin, and Neuringer (1993) and Makrides, Neumann, Byard, and Gibson (1994) showed that fatty acid profiles obtained from neural tissue and RBCs were highly correlated in both monkeys and rats. Sarkadi-Nagy et al. (2004) similarly demonstrated that levels of DHA in plasma and RBCs are highly correlated with tissue levels in the brain, liver, and retina of neonatal baboons, such that RBC-DHA accretion is highly predictive of retina-DHA accretion ($r^2 = .92, p < .001$).

Fatty acid extraction. Blood samples were drawn from an antecubital vein of participants who had fasted for at least 4 hours. Six to ten mL of blood was collected in a Vacutainer® tube containing 1.8 mg/mL spray-dried potassium ethylenediamine tetra-acetic acid (EDTA) as anticoagulant. Plasma and red blood cells were separated immediately by centrifugation (3,000 X *g* X 10 min at 4° C) and measured aliquots prepared for analysis. Detailed lipid analysis procedures have been reported previously for this laboratory. (Hoffman, Uauy, & Birch, 1995). Briefly, blood fatty acid analysis

involved solvent extraction of lipids according to methods derived from Bligh and Dyer (1954), using methanol:chloroform (2:1) containing butylated hydroxytoluene (BHT; 0.02%) as antioxidant and the resulting lipid extract was derivatized with 14% Boron Trifluoride-Methanol.

Gas chromatography. The resulting fatty acid methyl acids were quantified using capillary-column gas chromatography and flame ionization detection on a Varian CP3800 gas chromatograph equipped with a 30-meter capillary column containing Omegawax stationary phase (Supelco, Bellefonte, PA). The peak identification was confirmed by comparison of retention times to an expanded standard mixture of fatty acids (i.e., saturates, monounsaturates, and omega-3, -6, -9 polyunsaturates) prepared from GLC68A (NuChek Prep, Elyson, MN) enriched with 11 individual fatty acid methyl ester standards allowing identification of 30 different fatty acids. Mass (i.e., μg fatty acid/ml packed RBC) and percent of total fatty acids were determined by comparing individual peak areas to an internal standard (i.e., 10 μg of 23:0 fatty acid) and downloaded to Excel spreadsheets. All chromatographic analyses were subjected to quality control and questionable analyses were re-run. For illustration, a figure is provided (Appendix C) comparing a representative Usher RBC fatty acid chromatogram to the GLC68+11 fatty acid standard inclusive of peak identification labeling.

Dietary Questionnaire

A questionnaire entitled "DHA and EPA Food Frequency Questionnaire©" [DHA/EPA FFQ] was used to assess dietary intake of omega-3 rich foods. This

questionnaire was developed by Martek Biosciences Corporation and used by RFSW with permission for research purposes (Appendix D).The instrument was previously validated in a group of 67 healthy adult volunteers wherein the FFQ scores were positively correlated ($p<0.0001$) with RBC fatty acid levels as well as plasma phospholipids (Benisek, Bailey-Hall, Oken, Masayesva, & Arterburn, 2002).

The DHA/EPA FFQ, comprised of seven, self-report items, asks the individual to estimate consumption of specific fish, shellfish, liver, poultry, and egg yolks on a weekly or monthly basis, as well as intake of DHA and/or EPA due to dietary supplements (e.g., fish oil capsules, DHA capsules). The questionnaire classified fish consumption into three categories based on the content of DHA/EPA; such that type 1 (e.g., salmon, mackerel, tuna), type 2 (e.g., bass, flounder, grouper), and type 3 fish (e.g., cod, haddock, shellfish) represented high, medium, and low content, respectively. Liver, poultry, egg yolk, and supplement consumption were reported as separate category items. Portion sizes were based on a 3-ounce serving and egg yolks were counted individually including those used in baking. Each of the seven items was scored for DHA and EPA content and a summed total for DHA and EPA intake was tabulated (i.e., mg/day).

All study participants were allowed to complete the DHA/EPA FFQ questionnaire on their own or with the assistance of a companion or study personnel. Assistance constituted reading the questionnaire aloud to the study participant and marking responses on the answer sheet. The completed questionnaires were checked for completeness and scored by study personnel. The calculated food items subtotals and the

total summed intake for EPA and DHA were entered into Excel spreadsheets along with basic demographic data (i.e., age, gender). All electronic data were checked against source documents. Aberrant questionnaire responses were highlighted in the Excel spreadsheet, double-verified against the source document, and if data entry confirmed the response was marked for participant re-contact. A total of 31 participant entries were flagged for re-contact for moderate-to-high liver consumption (i.e., $\geq$ 5 servings/month). A study coordinator contacted the highlighted participants by phone or email and the corrected/verified responses was initialed and dated on the source document, and the Excel spreadsheet updated accordingly.

Data Analysis

Statistical Software

The Statistical Package for Social Sciences (i.e., SPSS©) version 16.0 software was used to analyze the data. Significance was determined at $p \leq .05$ for all analyses.

Measures

In the present study, affectation status was the independent variable. The levels of independent variable included normal control, Usher syndrome, Usher clinical subtype (i.e., type I, type II, type III), and Usher genotype (i.e., IB, IC, ID, IF, IG, IIA, IIC, IID & IIIA). The dependent variables were blood levels of DHA (i.e., RBC-DHA; percent of total fatty acids) and calculated dietary DHA consumption (i.e., DHA intake; mg/day).

Descriptive Statistics

Descriptive statistics were calculated to describe the characteristics of the Usher and normal cohorts. Frequency statistics were calculated for gender and DHA and/or EPA supplementation. Measures of central tendency and variability were calculated for consumption of type 1 fish and the calculated EPA intake. Among the Usher syndrome cohort frequency statistics were used to assess distribution between clinical subtypes as well as independent genotypes.

Inferential Statistics

Independent samples *t*-test were used to assess differences between cohorts in instances of two groups (i.e., HO1, HO4), and an Analysis of Variance (ANOVA) was used when assessing differences between more than two groups (i.e., HO2, HO3). Pearson Product Moment Correlation analysis was used to assess relationships between calculated dietary DHA and blood-DHA levels (i.e., HO5, HO6). Finally, a Z Stat was used to assess for differences in strength of correlation between dietary DHA to blood-DHA levels for the normal control group compared to the Usher syndrome group (i.e., HO7). As this statistic is not assessed by SPSS, the calculation was conducted using the following equation as described by Kshirsagar (1972) for testing the equality of two correlation coefficients Z Stata = $(Z_1 - Z_2) / \text{sqrt}((1/(N_1-3)+(1/(N_2-3))$ where $Z_i = \tan^{-1}(r_i)$ and r_i = sample correlation coefficient.

CHAPTER IV

RESULTS

The present study was a descriptive, retrospective, cross-sectional, quantitative design to investigate blood levels of DHA in patients with Usher syndrome. Dependent measures were percent RBC-DHA (i.e., percent of total fatty acids) and daily dietary DHA intake (i.e., mg/day) as assessed by blood fatty acid analysis and the DHA/EPA Food Frequency Questionnaire. Descriptive statistics were used to characterize the study participants and dependent variables. Statistical comparisons were made between the Usher syndrome cohort and normal control participants, between Usher syndrome clinical subtypes, and between Usher syndrome genotypes for RBC-DHA levels. Difference in dietary DHA intake was assessed between the Usher syndrome cohort and normal controls. A relationship between RBC-DHA and dietary DHA intake was assessed independently among the Usher syndrome and normal control cohorts. The strength of these relationships were subsequently compared. A detailed review of the data will proceed including descriptive statistics and results of statistical hypothesis testing.

Demographic Characteristics

One hundred and ninety-seven participants completed the study protocol (n = 63 normal controls, n = 134 Usher patients). Twenty-three individuals were excluded from the study due to the reported use of DHA and/or EPA oral supplements on the DHA/EPA FFQ leading to final study cohorts of 60 normal controls and 114 Usher patients. Thus,

the DHA/EPA supplementation rate was 4.8% among normal control participants and 14.9% among Usher patients. Both groups reported similar consumption of type 1 fish on the DHA/EPA FFQ, 1.48 servings (SD = 1.83) and 1.50 servings per month (SD = 2.04), normal and Usher, respectively. The calculated EPA dietary intake was also similar, namely, 64.70 (SD = 60.08) for the normal cohort and 60.69 (SD = 47.18) for Usher patients.

Descriptive characteristics were summarized for both cohorts (see Table 1). More than half of the study participants were female, 60.0% and 56.1% among the normal control and Usher cohorts. The racial/ethnicity distribution for normal participants was 86.7% White, 0% Black, 6.7% Asian, and 6.7% Hispanic/Latino. The Usher cohort was similarly distributed with 80.7% White, 0% Black, 1.8% Asian, and 3.5% Hispanic/Latino participants. The mean age of the normal cohort was 41.77 years (SD = 13.42) with a range of 13 to 67 years of age whereas the mean age of the Usher cohort was 47.28 (SD = 14.32) years with a range of 14 to 80 years of age. Several Usher syndrome participants failed to disclose a racial categorization or current age (n =16 race, n =14 age).

Table 1

Descriptive Personal Characteristics of Normal and Usher Syndrome Cohorts

		Normal Control Participants (n = 60)		Usher Syndrome Participants (n = 114)	
		Frequency	%	Frequency	%
Gender					
	Male	24	40.0	50	43.9
	Female	36	60.0	64	56.1
Race					
	White	52	86.7	92	80.7
	Black	0	0	0	0
	Asian	4	6.7	2	1.8
	Hispanic/Latino	4	6.7	4	3.5
Age					
	10-25 yrs	7	11.7	8	7
	26-40 yrs	21	35.0	19	16.7
	41-55 yrs	23	38.0	47	41.2
	56-70 yrs	9	15.0	20	17.5
	70+	0	0	6	5.3

Note: Frequencies not adding to 114 and percentages not adding to 100, reflect missing data in the Usher cohort; n = 16 did not give race and n = 14 did not give age.

Hypothesis Testing

Seven null hypotheses were tested in this study. The results will be illustrated with tables, plots, text descriptions or a combination thereof. Tables 2 through 5 describe null hypotheses 1 through 4. Figure 1 describes null hypotheses 5 and 6.

Null Hypothesis 1: There will be no significant difference between blood levels of DHA (RBC-DHA percent of total fatty acids) in patients with Usher syndrome compared to normal controls.

Independent samples *t*-test demonstrated that null hypothesis 1 was not supported as mean RBC-DHA was significantly reduced in the Usher cohort ($M = 3.70$) compared to the normal cohort ($M = 4.09$). As illustrated by Table 2, $t_{(172)} = 2.40, p < .05$.

Table 2

Independent Samples t-test of Mean Percentage RBC-DHA Between Usher Syndrome and Normal Control Participants

	n	Mean	SD	Minimum	Maximum
Normal Controls	60	4.09	1.03	2.51	7.36
Usher Syndrome	114	3.70	1.01	1.77	6.58

Note: $\alpha = .05, t = 2.40, p = .017$

Null Hypothesis 2: There will be no significant difference in DHA blood levels between the clinical subtypes of Usher syndrome.

One hundred eleven Usher patients were clinically ascribed to one of the three clinical subtypes (i.e., USHI, USHII, USHIII), three individuals were classified as atypical and were excluded from this data analysis. A one-way ANOVA between the three clinical subtypes demonstrated no significant differences of RBC-DHA between the groups supporting null hypothesis 2. As described by Table 3, $F_{(2,108)} = .160$ was not significant ($p = .852$).

Table 3

One-way ANOVA of Mean Percentage RBC-DHA Between Usher Clinical Subtypes

	n	Mean	SD	Minimum	Maximum
USHI	26	3.76	1.23	1.77	6.32
USHII	80	3.63	0.93	2.00	6.58
USHIII	5	3.67	0.33	3.29	4.10

Note: $\alpha = .05$, $F = .160$, $p = .852$

Null Hypothesis 3: There will be no significant difference in DHA blood levels between the major known genotypes of Usher syndrome.

Genotype characterization was available for 75 Usher syndrome patients (75.8%), ascribing their genetic pathology to one of seven genes. As several of the genotype groups were composed of small sample sizes, a non-parametric analysis of variance for independent groups (i.e., Kruskal-Wallis) was used to assess ranked differences in RBC-DHA levels. Hypothesis 3 was supported as $\chi^2 = 11.84$ was not significant ($p = .07$).

Table 4 summarizes the descriptive statistics for each genetic subtype. As a trend toward significance was observed, post-hoc analysis using Mann-Whitney U tests were initiated between the genotype groups with the greatest difference in mean RBC-DHA, namely type ID ($M = 2.41$) and IIC ($M = 3.06$) genotypes as compared to type IF ($M = 5.24$). Significance for each of these two comparisons was determined as $p \leq .05$. However, great caution should be exercised in interpreting these outcomes as anything more than a casual observation as each of these groups are composed of very small sample sizes and comparisons were merely of exploratory nature.

Table 4

Kruskal-Wallis Assessment of RBC-DHA Between Usher Syndrome Genetic Subtypes

	n	Mean	SD	Minimum	Maximum	Mean Rank
Type IB	14	3.84	1.22	2.05	6.32	41.00
Type IC	2	3.36	0.24	3.19	3.52	32.50
Type ID	3	2.41	0.92	1.77	3.47	12.83[a]
Type IF	3	5.24	0.63	4.53	5.72	68.50[b]
Type IIA	44	3.62	0.93	2.00	5.71	37.91
Type IIC	5	3.06	0.64	2.09	3.54	26.30[a]
Type IIIA	4	3.63	0.30	3.29	4.02	41.88
Total	75	3.64	1.01	1.77	6.32	

Note: Mean, *SD*, minimum, and maximum values given as percent of total fatty acids. Kruskal-Wallis analysis of variance $\alpha = .05$, $\chi^2 = 11.84$, $p = .07$. Mean ranks with different superscripts denote which groups significantly contributed toward the observed trend as assessed by Mann-Whitney U post-hoc analysis, $U = 0$, $p = .025$ (ID vs. IF) ; $U = 0$, $p = .05$ (IIC vs. IF).

Null Hypothesis 4: There will be no significant difference between the calculated milligrams of dietary DHA consumed/day in patients with Usher syndrome compared to normal controls.

Null hypothesis 4 was supported by an independent *t*-test comparing the daily dietary DHA intake between Usher syndrome and the normal control cohorts (see Table 5). The mean DHA intake of 112.11 mg/day for normal controls was not significantly different than 103.16 mg/day for Usher patients as illustrated by $t_{(172)} = .64$, ns. The DHA/EPA FFQ documented highly variable intakes between participants in each comparison group.

Table 5

Independent Samples t-test of Mean Calculated Daily Dietary Intake of DHA Between Usher Syndrome and Normal Control Participants

	n	Mean	SD	Minimum	Maximum
Normal Controls	60	112.11	112.00	15.00	836.00
Usher Syndrome	114	103.16	71.78	0.00	376.00

Note: $\alpha = .05$, $t = .64$, $p = .52$

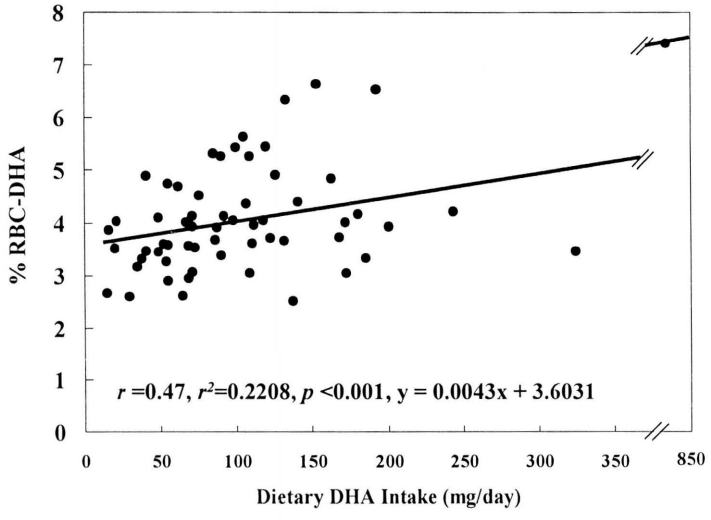
Null Hypothesis 5: There will be no significant relationship between the calculated milligrams of dietary DHA and the blood-DHA levels in normal controls.

A Pearson's Product Moment Correlation demonstrated that null hypothesis 5 was not supported as there was a significant moderate correlation between these dependent measures. The relationship between dietary DHA intake and RBC-DHA levels is described by $r_{(60)} = .470, p < .001$. The Figure 1A scatterplot illustrates the spread of the data and includes a trendline to highlight the positive relationship between the variables. Thus, as dietary DHA intake increases blood DHA levels also appear to increase.

Null Hypothesis 6: There will be no significant relationship between the calculated milligrams of dietary DHA and the blood-DHA levels in patients with Usher syndrome.

Similarly, null hypothesis 6 was not supported as a Pearson's Product Moment Correlation demonstrated a significant moderate correlation between the dependent variables. The relationship between dietary DHA intake and RBC-DHA levels among the Usher syndrome cohort is described by $r_{(114)} = .433, p < .001$. The Figure 1B scatterplot illustrates a positive relationship between the variables such that as dietary DHA intake increases the blood levels of DHA also increase.

A.



B.

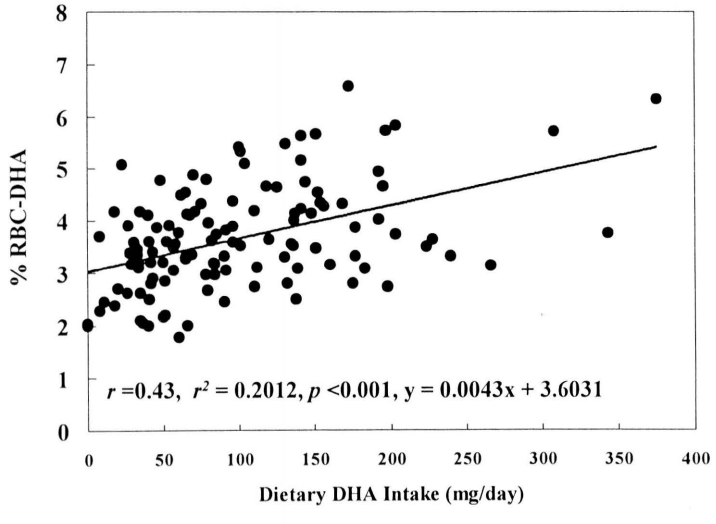


Figure 1. A. Correlation of dietary DHA intake and RBC-DHA level in normal cohort (n = 60). B. Correlation of dietary DHA intake and RBC-DHA level in Usher cohort (n = 114).

Note: Double hash lines on the Fig. 1A x-axis represent a break in the continuous scale.

Null Hypothesis 7: There will be no significant difference between the strength of relationship of the calculated milligrams of dietary DHA to blood-DHA levels for the normal control group compared to the Usher syndrome group.

Calculation of the Z Stata statistic demonstrated that there was no difference in the strength of relationship computed for the normal and Usher cohorts, thereby supporting null hypothesis 7. Comparison of $r = .470$ (normal) and $r = .433$ (Usher) was conducted to assess differences. The calculated $z = -0.285$ did not equal or exceed the critical z of 1.65 needed to substantiate significance at the $\alpha = .05$ level.

Additional Findings

Additional analyses were conducted to test for differences in percent RBC-DHA between Usher and normal control participants, to describe the distribution of genotypes among genetically characterized Usher participants, and to describe the characteristics of Usher and normal participants that were excluded from study participation due to nutritional supplementation. An independent samples t-test was used to assess for differences of EPA-DHA levels between the Usher and normal cohorts. Descriptive statistics were used to characterize genotype distribution and the attributes of excluded participants.

RBC-EPA

Comparison of mean percent RBC-EPA revealed no significant difference between the normal control ($M = .53$) and Usher syndrome cohorts ($M = .57$) as described by $t_{(172)} = -.79$, *ns*. Descriptive statistics for this comparison are listed in Table 6.

Table 6

Independent Samples t-test of Mean Percentage RBC-EPA Between Usher Syndrome and Normal Control Participants

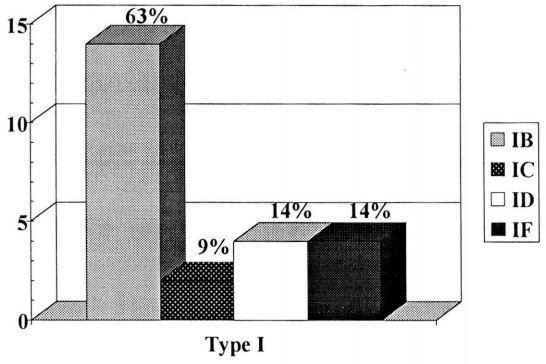
	n	Mean	SD	Minimum	Maximum
Normal Controls	60	0.53	0.24	0.22	1.51
Usher Syndrome	114	0.57	0.28	0.23	2.26

Note: $t = -.79$, $p = .43$

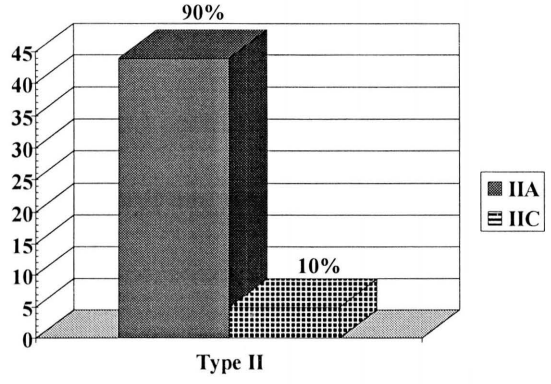
Genotypes

Overall, nearly 76% (i.e., 75/114) of the Usher cohort had genotyping data available for categorization. The percent genotyped among each clinical subtype were as follows: 85% type I, 61% type II, and 80% type III. The distribution of Usher participants according to clinical subtype and ascribed genotype is illustrated by Figure 2 bar graphs (i.e., A, B, C). For type I the genotype distribution was 63% IB, 9% IC, 14% ID, and 14% IF. For type II the genotype distribution was 90% IIA and 10% IIC. Of the successfully genotype type III participants, all were ascribed to the IIIA gene.

A.



B.



C.

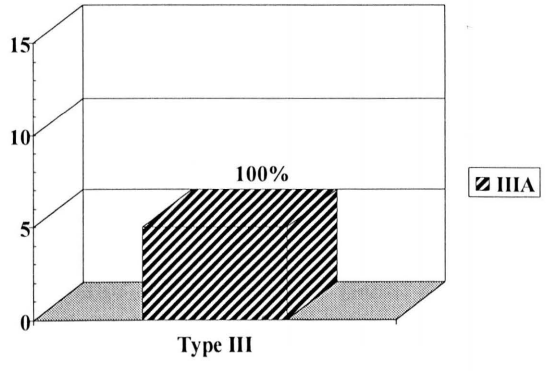


Figure 2. A. Distribution of genotyped Usher type I participants (n = 22). B. Distribution of genotyped Usher type II participants (n = 49). C. Distribution of genotyped Usher type III participants (n = 5).

Excluded Participants

Individuals were excluded from participation if at the time of study or at any time during the previous three months the individual reported consuming DHA and/or EPA nutritional supplements. Selected attributes, such as age, gender, DHA/EPA supplementation, dietary intake, and percent RBC-DHA are summarized for individuals excluded from the normal (n = 3) and Usher (n = 20) cohorts (see Table 7). The mean age of excluded individuals was 50.00 (SD = 9.90) and 53.53 (SD = 13.01) years for normal and Usher syndrome.

Among the three excluded normal control participants, one reported DHA supplementation (i.e., 385 mg/day) and two reported supplementing with EPA (M = 240 mg/day). Of the 20 excluded Usher syndrome participants, all were supplementing with DHA (M = 224.23 mg/day) and 14 individuals were also supplementing with EPA (M = 343.51 mg/day). The dosage of supplementation varied widely among the excluded Usher participants ranging from 5.25 - 600 mg/day DHA and 7.86 - 900 mg/day EPA.

The mean calculated daily DHA dietary intake was 33.33 mg/day (SD = 22.19) among the excluded normal controls and 102.05 mg/day (SD = 74.55) among the excluded individuals with Usher syndrome. The mean RBC-DHA level was 3.83% and 4.83% of total fatty acids for the normal and Usher exclusion groups.

Table 7

Descriptive Characteristics of Normal and Usher Cohort Exclusions by Age, DHA/EPA Supplementation, Dietary DHA Intake, and % RBC-DHA

	n	Mean	SD	Minimum	Maximum
Normal Control Participants (*n* = 3; 2 male, 1 female)					
Age (yrs)	3	50.00	9.90	43	57
DHA Supplement	1	NA	NA	385	385
EPA Supplement	2	240.00	0	240	240
Dietary DHA Intake	3	33.33	22.19	13	57
% RBC-DHA	3	3.83	0.61	3.16	4.35
Usher Syndrome Participants (*n* = 20; 5 male, 15 female)					
Age (yrs)	20	53.53	13.01	33	79
DHA Supplement	20	224.23	153.70	5.25†	600
EPA Supplement	14	343.51	273.81	7.86†	900
Dietary DHA Intake	20	102.05	74.55	14	304
% RBC-DHA	20	4.83	1.50	2.00	8.00

Note: Calculated mean, SD, minimum, and maximum values are based the number of individuals per line item. DHA/EPA supplementation and dietary DHA intake given as mg/day. †Individual ingested one fish oil capsule twice/month.

Summary

The present study found that blood DHA levels as indexed by percent RBC-DHA differed between a normal control cohort and a heterogeneous cohort of patients with Usher syndrome. However, the two groups did not differ in their daily dietary DHA intake. Subgroup comparisons of the Usher clinical subtypes and genotypes did not illustrate differences in RBC-DHA levels between these groups. The DHA intake calculated by the DHA/EPA FFQ demonstrated a significant positive relationship to the measured RBC-DHA levels for both study cohorts. The findings presented in this chapter will be further discussed in Chapter V.

CHAPTER V

DISCUSSION

The overriding finding of the present study is that the patient cohort with Usher syndrome had lower mean blood levels of DHA compared to normal. This finding is consistent with the general outcome of several previous studies (Bazan et al., 1986; Maude et al., 1998; Williams et al., 1989). However, significant differences were not observed in RBC-DHA levels between clinical or genotypic subgroups of Usher syndrome. A noteworthy finding was that daily dietary DHA intake between the normal and Usher cohorts was not significantly different. A significant positive relationship was observed between calculated daily dietary DHA intake and RBC-DHA levels among both the normal and Usher cohorts. The strength of this relationship was not different between the two groups.

The results of the present study contribute to our understanding of the omega-3 fatty acid status among patients with Usher syndrome and the association of dietary intake of omega-3 rich food sources. This study represents the first comprehensive comparison of blood DHA levels between each of the clinical subtypes. Furthermore, this is the first study to present blood DHA data from patients that have been genotypically-characterized. This chapter will recapitulate the purpose and overall design of the research study and discuss the findings presented in the previous chapter. The following

areas of discussion will follow: summary, limitations, conclusion, discussion and implications, and recommendations.

Summary

The purpose of this study was to assess blood fatty acid levels among patients with Usher syndrome and determine if RBC-DHA levels were significantly different from normal. The secondary purpose was to determine if differences in RBC-DHA were evident between clinical and genotypic subgroups of Usher syndrome. The relationship between dietary intake and blood fatty acid status was explored and compared between the normal and Usher study cohorts.

Study participants were comprised of a convenience sample of normal controls and patients with Usher syndrome recruited from the Southwest Eye Registry and Boys Town National Research Hospital databases, which drew from multi-state and nationwide patient populations, respectively. One hundred ninety-seven (i.e., 197) individuals were enrolled into the normal (n = 63) and Usher (n = 134) cohorts; data were excluded for 23 individuals due to DHA and/or EPA supplementation. This study used a retrospective, cross-sectional design.

Data collection for this collaborative project took place between January 2004 and May 2008 at the Retina Foundation of the Southwest, Dallas, Texas. The study protocol proceeded in the following manner:

1. Study materials were mailed to eligible individuals who agreed to participate.
2. The mailer included instructions, a demographic form, a consent form, a dietary questionnaire, and a blood mailer package inclusive of return shipping.
3. The study documents and "fasting" blood sample was drawn at a location of convenience to the participant.
4. The documents and blood sample was assigned a coded ID number unique to each participant.
5. Data were maintained in a de-identified manner in all databases.

Limitations

Several limitations exist within the current study and warrant further discussion. First, the study design relied on a convenience sample drawn from two genetic registries. Although both registries draw from multi-state or nationwide patient populations, no methods were employed to geographically randomize or balance enrollment, thus registry participants may not fully represent the breadth of Usher patients in the United States. All registry participants had previously volunteered for inclusion in these databases and subsequently consented to participate in this study. Therefore, these participants may also reflect an eager, motivated sub-population.

Secondly, this study included a survey instrument and self-report data thereby introducing several sources of potential error including recall bias and social desirability.

These issues can result in either under- or over- reporting of elements of interest and are a threat to the internal validity of the study. Furthermore, the cross-sectional study design provided dietary data and blood DHA levels from participants obtained at a single time point that may reflect current rather than long-term behavior.

This study was exploratory in nature involving a rare disease with numerous clinical and genotypic subtypes. Participants with Usher syndrome were enrolled based on their clinical diagnosis, genotypic data was divulged prior to data analysis. Small sample sizes, although anticipated for the most rare clinical and genetic subtypes, affected the power of some statistical comparisons.

Several weaknesses of the DHA/EPA FFQ became evident during the course of the study. First, the wording of one question was confusing and led to aberrant responses by a portion of the participants. "How many 3-oz servings of liver (chicken, turkey, or beef) do you eat monthly?" was alternatively understood as "how many servings of liver, chicken, turkey and beef are eaten monthly?" leading to abnormally high responses for this question. An additional weakness of the questionnaire is the absence of questions to query consumption of DHA-fortified foods. The DHA/EPA FFQ was developed prior to the infusion of omega-3-enriched commercial products, thus it does not fully index the daily dietary intake of DHA-rich foods.

A final limitation of the study is the effect of Diffusion of Innovation. The potential benefit of DHA in slowing retinal degeneration in retinitis pigmentosa might have influenced the dietary behavior of Usher patients leading to alteration of dietary

intake via increased DHA-rich food sources or nutritional supplements. Such dietary modifications could have weakened the ability to detect differences in blood DHA levels among patients with Usher syndrome. The current study noted a greater DHA/EPA supplementation rate among individuals recruited with Usher syndrome compared to normal controls.

Conclusion

As shown in Table 8, null hypotheses 1, 5, and 6 were rejected and the remaining hypotheses (i.e., 2, 3, 4, and 7) were accepted. This study concluded that (a) blood levels of DHA are significantly different between patients with Usher syndrome and normal controls, (b) blood levels of DHA are not different between the Usher clinical and genetic subtypes, (c) dietary intake of DHA was not different between the normal and Usher cohorts, (d) there was a significant relationship between daily dietary DHA intake and blood levels of DHA, and (e) the strength of relationship between these variables did not differ by affectation status.

Table 8

Summary of Null Hypotheses Results

	Not Rejected	Rejected
Hypothesis 1		X
Hypothesis 2	X	
Hypothesis 3	X	
Hypothesis 4	X	
Hypothesis 5		X
Hypothesis 6		X
Hypothesis 7	X	

Discussion and Implications

Demographic Findings

Individuals were enrolled to both groups of the study prospectively with no overt recruitment methodology to help balance age, gender and race/ethnicity between the two groups. The resulting demographic characteristics of the normal control and Usher cohorts were nonetheless quite similar. Several interesting things were noted, namely, study participants tended to be female, there were no black participants, and there were a greater number of older (>70 years) Usher participants than anticipated. The Usher cohort also had a greater tendency to omit disclosure of age and racial categorization. Whether

this reflects a reluctance to disclose the information or oversight is not known. The researcher's personal impression is the latter, as the amount of documentation required of each participant was particularly taxing on those individuals with visual impairment.

Null Hypotheses Findings

RBC-DHA. Null hypotheses one through three were concerned with comparison of RBC-DHA levels between the Usher and normal cohort (HO1), between the Usher clinical subtypes (HO2), and between the Usher genotypes (HO3). A statistically significant difference in RBC-DHA levels was found between the Usher and normal cohorts, such that patients with Usher syndrome had blood levels on average 10% lower than normal controls. This finding is consistent with the previous, albeit limited, reports of low blood levels of DHA, although the overall percentage reduction is not as striking as the Bazan et al. (1986) assessment of plasma phospholipids (64% of normal) in a heterogenous cohort of patients with Usher syndrome.

No significant differences in blood DHA levels were observed between the Usher clinical subgroups. The mean RBC-DHA levels were strikingly similar between type I, type II, and type III ($M = 3.76, 3.63, 3.67$, respectively) patients. Reductions from mean normal were 8% for type I, 11% for type II, and 9% for type III, of these only the reduction for type II was significant ($p < .01$). Published reports regarding DHA levels among the Usher subgroups are conflicting and have focused solely on Usher type I and type II. The current findings are in contrast to those published by Maude et al. (1998) in which the Usher I patients were found to have striking reductions of DHA in both plasma

and RBC phospholipids and DHA levels for Usher II patients were found to be no different from normal controls. However, Connor et al. (1997), in a study of more limited size, demonstrated that the Usher II group exhibited the greatest reduction in sperm DHA levels (24% of normal) in a retinitis pigmentosa cohort that included patients with Usher I and Usher II.

Comparison of the Usher syndrome genotypes found no statistical difference between the seven available groups. However, a trend ($p = .07$) was suggestive that differences might be observed if group sizes were larger and parametric statistics could be utilized. An exploratory post hoc analysis did show that the two genotypes with the lowest mean DHA levels (ID, $M = 2.41$ and IIC, $M = 3.06$) were responsible for the largest contribution to the trend. The current study is the first to assess blood DHA levels according to genotype. As Usher syndrome is genetically heterogenous, caused by numerous genes from a variety of gene families, it is reasonable that blood DHA levels may vary according to genotype. Available data fall short of satisfactorily exploring this question, due in large part to limited sample sizes of the rarer genotypes. This area should be a topic of continued study, and indeed steps have been taken to prospectively increase sample size for each genotype with fewer than ten participants.

Dietary DHA intake. Null hypotheses four through seven were concerned with comparison of the DHA dietary intake data. Calculated DHA intake (mg/day) was assessed for differences between the normal and Usher cohort (HO4), correlations between the calculated DHA intake and RBC-DHA levels were assessed among normal

(HO5) and Usher (HO6) participants, and subsequently, the strength of relationship was assessed for differences (HO7).

No significant difference was found in daily dietary intake of DHA between the normal and Usher cohorts. Demonstrating equivalence of DHA intake was a vital element of establishing that dietary differences were not responsible for any observed differences in blood DHA levels. The calculated daily dietary intake for EPA was also not different between the two groups and the number of reported type 1 fish servings per month was nearly identical (1.48 normal, 1.50 Usher).

Significant moderate correlations were found between the calculated daily DHA intake and the measured RBC-DHA levels for both the normal (r = .470) and Usher (r = .433) cohorts. The current results indicated that increased dietary DHA intake was associated with higher blood DHA levels. These findings are consistent with survey validation data detailed by Benisek et al. (2002), although the strength of the correlations is attenuated in the current study by comparison (r = .716). However, this may merely reflect a less dynamic range in the variables among the current participants.

No significant difference was found between the normal and Usher cohorts in the strength of relationship between RBC-DHA and calculated daily DHA intake. This finding implies that the questionnaire worked equally well among the normal and the sight-impaired population. If the observed correlations had be significantly different it could have highlighted a problem with the questionnaire administration perhaps due to

the use in the special population or could have identified discontinuity between dietary intake and the measured blood DHA levels.

Additional Findings

RBC-EPA. As defined previously, EPA is an omega-3 biosynthesis precursor for DHA. The current study found that the mean RBC-EPA levels did not differ significantly between the Usher and normal cohorts. This finding is important as it establishes that despite normal RBC-EPA levels and dietary DHA intake equivalent to normal controls, the Usher cohort exhibited lower RBC-DHA levels suggestive of a block or downregulation of the biosynthetic pathway between EPA and DHA. This observation is consistent with the stable isotope findings of Hoffman et al. (2001) in xlRP patients in which a potential defect was pinpointed involving Δ^5-desaturase.

Clinical subtypes and genotypes. The distribution of Usher according to clinical subtypes in this study was 23.4% type I, 72.1% type II, and 4.5% type III. Reported distributions vary, but the current result is highly consistent with a study by Spandau and Rohrschneider (2002) in which the Usher type I to Usher type II ratio was found to be 1:3. There is also variability in the reported distribution of genotypes for Usher I and Usher II as would be expected if sampling different outbred populations. The current study experienced low enrollment numbers for several of the rare genotypes, but when viewed in an epidemiological perspective the distribution of the genotypes was very similar to that described by Roux et al. (2006) for Usher I and Kimberling (personal communication, July 15, 2008) for Usher II.

DHA supplementation. Interestingly, a higher rate of DHA/EPA supplementation was observed among the Usher cohort (14.9%) compared to the normal control group (4.8%). In reviewing this data, it was further noted that the three supplementers among the normal recruits were unaffected relatives of patients with Usher syndrome. These anecdotal findings suggest that individuals with Usher syndrome and their family members may be responding to the DHA supplementation research in retinitis pigmentosa. According to the adopter schema of Diffusion of Innovation (Rogers, 1995), these individuals likely represent innovators and a few early adopters. However, the current study was not designed to detect the DHA supplementation rate nor other dietary alterations among patients and families with Usher syndrome. These observations will merit further exploration and pose an interesting focus for follow-up study.

Currently, there is no cure for Usher syndrome and treatment options are very limited for this debilitating disease (e.g., cochlear implants, Vitamin A supplementation). The current study evaluated blood fatty acid levels (i.e., % RBC-DHA) and dietary DHA intake in a retrospective analysis of a large patient cohort. The data obtained from the study both clarifies and extends the empirical knowledge of the omega-3 fatty acid status in patients with Usher syndrome. Study findings are in some instances compatible and in others contradictory to the limited published data.

Key outcomes of this study are: (a) the observation of lower blood DHA levels in patients with Usher syndrome, (b) the first reported DHA levels of Usher type III, (c) the first reported DHA levels according to Usher genotype, (d) the observation of statistically

equivalent daily dietary DHA intake between patients with Usher syndrome and normal controls, and (e) the observation of statistically equivalent blood EPA levels between Usher and normal cohorts.

Recommendations

Results of this study indicate that several recommendations are in order. First, further research is needed on the blood DHA status of patients with Usher syndrome. Although this study demonstrated lower RBC-DHA among a heterogenous cohort of patients with Usher, small sub-group sample sizes hampered between group assessments. Therefore to maximize the utility of the current data, it is recommended that a focused effort be initiated to assess dietary intake and blood DHA levels in additional genotyped Usher patients, particularly those genetically ascribed to the rare subgroups. Concurrent to this recruitment effort, additional normal participants should be solicited to optimize the age-, gender, ethic/racial- matching of the comparison cohort. To this end, study investigators should increase the number of normal participants greater than 50 years of age. This recruitment should include a specific effort to solicit a subgroup of individuals greater than 70 years as this age category was absent from the current normative cohort.

Participants for this study were recruited from a multistate and a nationwide database in an effort to obtain a wide geographic representation of individuals, thereby increasing generalizability of the data at least with respect to individuals in the continental United States. The conduct of similar studies in geographically different populations should be encouraged in an effort to replicate these results and extend our

knowledge. Furthermore, additional research is suggested regarding the DHA/EPA food frequency questionnaire including modification to encompass DHA-fortified foods as noted by aforementioned limitations and the subsequent re-validation of the revised FFQ.

There was some expectation that each of the Usher subtypes might exhibit uniquely different blood DHA levels and respond to DHA supplementation differently. It was thought that an understanding of these possible differences in DHA metabolism would provide assistance in determining first, the applicability of a clinical trial of DHA supplementation in Usher syndrome, but also help conceptualize the design of such a trial to retard retinal degeneration. The current data is suggestive that a clinical trial to elevate blood DHA levels, similar to that conducted in xlRP, would be appropriate. Thus, it is further suggested that the initial clinical trial design steps begin forthwith and concurrent with the recruitment of the additional normal and genotyped Usher participants. This latter data could subsequently assist in the refinement of the final study design.

Recommendations for health educators can be classified into three main functions: raising awareness, advocacy, and dissemination of potential preventative health messages. First, Usher syndrome is a rare disease that has garnered little public attention, yet it is a major etiologic contributor to deaf-blindness. Health educators can play a role in raising awareness among medical professionals (e.g., general practitioners, pediatricians, nurses), school health officials, and the public. With the initiation of universal infant hearing screening programs within the United States, congenital hearing loss is increasingly diagnosed during early infancy. Raising awareness of potential visual

problems among these "at risk" infants and children is needed to improve medical surveillance.

The onset of hearing loss is congenital in two of the three clinical Usher subtypes and vision loss is of juvenile onset. Any child with moderate to profound congenital hearing loss should have a thorough vision evaluation. Furthermore, in absence of other congenital malformations, and when history is unremarkable for maternal prenatal infection this vision evaluation should include an electroretinogram to assess the function of the retina. Health educators working with schools or associated with other pediatric populations should advocate for comprehensive vision evaluations for every deaf child.

Lastly, health educators can play a pivotal role in the dissemination of preventative and or therapeutic information to this special population of patients. Currently interventions for Usher syndrome are very few, but research in the areas of nutritional supplements and gene therapy are actively under evaluation. As these interventions illustrate efficacy, there will be a need to disseminate this information to clinicians and their patients with Usher syndrome. The deaf-blind population represents a special challenge and will need tailored messages and suitable channels for distribution. The theory of Diffusion of Innovation coupled with the Health Communication Process Model (Bensley & Brookins-Fisher, 2003) can be brought to bear to properly tailor the messages for dissemination.

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APPENDIX A

University of Texas Southwestern Medical Center IRB Letters of Continuing Renewal

SOUTHWESTERN
THE UNIVERSITY OF TEXAS
SOUTHWESTERN MEDICAL CENTER
AT DALLAS

Institutional Review Board

TO: David G. Birch, PhD
c/o Kirsten G. Locke, CRA, RN - Ophthalmology
Retina Foundation of the Southwest
9900 N. Central Exprwy., Ste. 400
Dallas, TX 75231

FROM: Albert Quan, MD
Institutional Review Board 4 – Chairperson
IRB - 8843

DATE: 13 August 2003

SUBJECT: **Continuing IRB Review – Expedited Approval**
IRB File Number: 0683-270
Project Title: Retinal Pathophysiology in Infants and Adults

The Institutional Review Board reviewed this research activity on an expedited basis. Your protocol and consent form(s) were approved for continuation for the period beginning 17 August 2003 and expiring on 16 August 2004. Your modification for personnel changes, dated 7/18/03, was also approved.

Please report to the IRB any unexpected or serious adverse events that occur during the study. Any proposed changes in this research must be submitted to the IRB for review and approval prior to implementation, except for immediate changes necessary to assure research subject safety, which must be reported to the IRB within two days.

This study will require continuing review from the IRB and a reminder will be mailed to you 60 days prior to the **expiration date of 16 August 2004.**

Should you have any questions, please telephone Reda Hall in the IRB office at 214.648.3378.

AQ/iw

5323 Harry Hines Boulevard, C1.206 / Dallas, Texas 75390-8843 / (214)648-3060 Fax (214)648-2171

UT SOUTHWESTERN
MEDICAL CENTER

Institutional Review Board

TO: David Birch, PhD
c/o Kirsten Locke, RN
Orthopaedic Surgery - 9057

FROM: George Buchanan, MD
Institutional Review Board 2 – Chairperson
IRB - 8843

DATE: July 27, 2007

SUBJECT: **Continuing Review Expedited Approval of Protocol/Project Summary, CR Form, Modification Dated June 11, 2007, Consent Forms, and Progress Report**
IRB File Number: 0683-27000
Project Title: Retinal Pathophysiology in Infants and Adults

The Institutional Review Board reviewed this research activity on an expedited basis. Having met the conditions as set forth by the IRB Chairman on July 16, 2007, your research protocol is now approved for continuation for a period of 12 months. This approval period will begin July 25, 2007 and last until July 23, 2008. If the research continues beyond approval period, the study will require continuing review from the IRB and a reminder will be mailed to you 60 days prior to the expiration date stated above.

Please Carefully Read Important Compliance Information Below:

All subjects must sign a copy of the attached IRB-approved and stamped consent form(s) and HIPAA Authorization, if applicable, before undergoing any study procedures, including screening procedures that would not otherwise be performed for a patient/subject's medical condition in a non-research context.

Important Note: Unless a verbal consent process or waiver of consent was approved, you must use a photocopy of the attached IRB-approved and stamped consent form(s) to document each subject's willingness to participate. Use of a copy of any other version of the consent form is prohibited.

A photocopy of the signed consent form(s) and HIPAA Authorization should be given to each participant. The copy of the consent form(s) bearing original signatures should be kept with other records of this research for at least five years past the completion of the study. For research involving treatment or invasive procedures, a photocopy of the signed consent form(s) should be on file in a subject's medical record.

Federal regulatory law requires that you report to the Institutional Review Board any unexpected and/or serious adverse events/unanticipated problems, as defined on the IRB website at http://www.utsouthwestern.edu/irb , that occur to research subjects or others during the course of

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www.utsouthwestern.edu

your study.

In the future, should you require a change or need to modify the research, including the informed consent document(s) and HIPAA Authorization, per federal regulation you must obtain prospective review and approval of the Institutional Review Board. For any change to the research, prior review and approval before implementing such changes is mandatory except when prompt implementation is necessary to eliminate apparent immediate hazard to a subject.

Approval by the appropriate authority at a collaborating facility is required before subjects may be enrolled on this study.

If you have any questions related to this approval or IRB policies and procedures, please telephone the IRB office at 214-648-3060.

Attachments: Project Summary, CR Form, Modification Dated June 11, 2007, Consent Forms, and Progress Report

GB/ca

APPENDIX B

Texas Woman's University IRB Letter of Exempt Status

TWU
TEXAS WOMAN'S UNIVERSITY
DENTON DALLAS HOUSTON

Institutional Review Board
Office of Research and Sponsored Programs
P.O. Box 425619, Denton, TX 76204-5619
940-898-3378 Fax 940-898-3416
e-mail: IRB@twu.edu

April 25, 2008

Ms. Dianna K. H. Wheaton
6000 Gateridge Drive
Flower Mound, TX 75028

Dear Ms. Wheaton:

Re: *Omega-3 Fatty Acid Status in Patients Diagnosed with Usher Syndrome: A Descriptive Study of Red Blood Cell (RBC) Docosahexaenoic Acid (DHA) Levels in Usher Subtypes*

The above referenced study has been received and reviewed by the Texas Woman's University Institutional Review Board (IRB) and has been determined to be exempt from further review because it has been reviewed and approved by an IRB at the University of Texas Southwestern Medical Center in Dallas. All participants in the study will be recruited and the study will take place at the Retina Foundation of the Southwest in Dallas, Texas.

Another review by the TWU IRB is required if your project changes in any way, and the TWU IRB must be notified immediately regarding any adverse events. If you have any questions, feel free to call the TWU Institutional Review Board at the phone number listed above.

Sincerely,

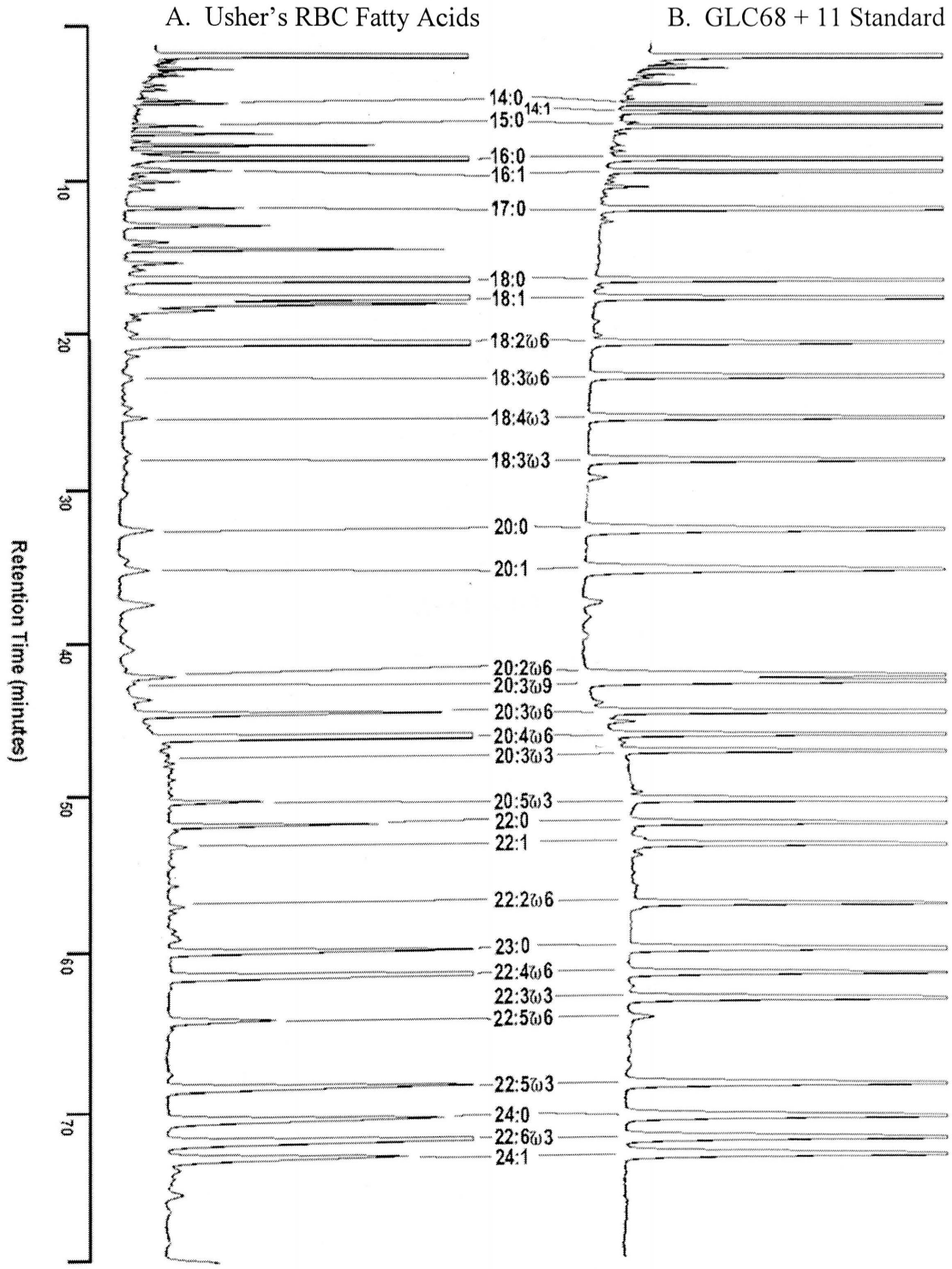
Dr. David Nichols, Chair
Institutional Review Board – Denton

cc: Dr. Kristin Wiginton, Health Studies
Graduate School

Think SUCCESS Think TWU

APPENDIX C

Fatty Acid Analysis Sample Chromatogram


A. Usher's RBC Fatty Acids
B. GLC68 + 11 Standard
Retention Time (minutes)
10
20
30
40
50
60
70
14:0
14:1
15:0
16:0
16:1
17:0
18:0
18:1
18:2ω6
18:3ω6
18:4ω3
18:3ω3
20:0
20:1
20:2ω6
20:3ω9
20:3ω6
20:4ω6
20:3ω3
20:5ω3
22:0
22:1
22:2ω6
23:0
22:4ω6
22:3ω3
22:5ω6
22:5ω3
24:0
22:6ω3
24:1

APPENDIX D

Letter of Permission for DHA/EPA Food Frequency Questionnaire

Martek®

Martek Biosciences Corporation

VIA FEDERAL EXPRESS

June 15, 2007

Dennis R. Hoffman, Ph.D.
Dianna K.H. Wheaton, M.S.
Retina Foundation of the Southwest
Suite 400
9900 North Central Expressway
Dallas, TX 75251

Re: Use of Dietary Questionnaire

Dear Dr. Hoffman and Ms. Wheaton:

Martek Biosciences Corporation hereby provides the attached Dietary Questionnaire developed and copyrighted by Martek for use in your research studies involving Retinitis Pigmentosa and allied retinal degenerative diseases. We would be appreciative if you would send us the Questionnaire results and associated blood fatty acid data when they are available.

Please indicate your acknowledgment of the above by signing an enclosed copy of this letter and returning it to the undersigned. Thank you very much.

Sincerely yours,

George P. Barker
Senior Counsel

Acknowledged as of the date first above written.

Dennis R. Hoffman, Ph.D.
Retina Foundation of the Southwest

Dianna K.H. Wheaton, M.S.
Retina Foundation of the Southwest

cc: Ed Nelson, M.D.

555 Rolling Hills Lane
Winchester, Kentucky 40391

(859) 744-0920
Fax (859) 744-8364
www.martekbio.com