THE EFFECTS OF DIABETES ON RETINAL VERY-LONG-CHAIN POLYUNSATURATED FATTY ACIDS

by

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ABSTRACT

Diabetic retinopathy is the most common cause of vision loss among people with diabetes and the leading cause of vision impairment and blindness among adults. The human retina is known to have a unique lipid profile enriched in very-long-chain polyunsaturated fatty acids (VLC-PUFAs) which appear to promote normal retinal structure and functions. Significantly decreased levels of VLC-PUFAs and low n-3/n-6 ratios have been found in the retina of patients with age-related macular degeneration (AMD); however, the effects of diabetes on retinal VLC-PUFAs are not clearly understood. In this study, we studied the differences in retinal lipid compositions in diabetic eyes as compared to age-matched controls in human and animal models. The VLC-PUFA levels and n-3/n-6 ratios were unchanged in postmortem diabetic retinas in comparison to age-matched control retinas, in part due to the limited number of human donor eyes available for this project. To further study the effect of omega-3 supplementations on VLC-PUFA levels in diabetic models, we chose diabetic Nile rat (spontaneous diabetes) and Akita mouse (genetic diabetes) models. After a month of supplementation with omega-3 PUFAs, VLC-PUFA levels and n-3/n-6 VLC-PUFA ratios increased in diabetic Nile rats in comparison to age-matched control rats. Our results in the present study indicate that VLC-PUFA levels are lower in diabetes and diabetic retinopathy and that dietary supplementation with omega-3 PUFAs may help to improve dyslipidemia.

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ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Diabetes mellitus (DM), a growing public health concern, has reportedly affected nearly 422 million people around the world in 2014 (1). Diabetic retinopathy is a major complication of diabetes and the leading cause of blindness in 30-60-year-olds. Diabetic retinopathy results from chronic diabetic damage to tiny blood vessels in the retina caused by high blood sugar (2). Diabetic retinopathy has two phases: an early proliferative phase characterized by increased vascular permeability and hemorrhages and a late proliferative phase characterized by retinal neovascularization, which is accompanied by vision loss (3).

The role of lipids in the progression of diabetic retinopathy began to be documented in the 1950s. Later studies involving diabetes control and complication trials also confirmed that dyslipidemia (altered serum lipid levels) is associated with the progression of diabetes (4). Dyslipidemia is a complex disorder involving abnormal levels of lipids and altered fatty acid compositions in the plasma that arise due to metabolic disproportion partly caused by the imbalance of insulin levels. Although a few studies have shown a correlation between dyslipidemia and diabetes, the association is not well understood. Both systemic and retina-specific fatty acid profiles are affected by diabetes and have been suggested to contribute to the progression of diabetic retinopathy. Hence, there is a need to study the alterations of the fatty acid compositions

in both retina and serum to understand the disease process and to facilitate development of effective therapeutics.

Among the fatty acids, long-chain-polyunsaturated fatty acids (LC-PUFAs) have been gaining the interest of researchers and supplement companies with regard to inflammation and human health. Omega-3 (n-3) LC-PUFAs (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) are anti-inflammatory and omega-6 (n-6) LC-PUFAs such as arachidonic acid (AA) are proinflammatory in nature. The balance between these two fatty acids plays a pivotal role in disease progression and health. Mouse model studies, human clinical trials, and epidemiological studies have demonstrated that the intake of n-3 LC-PUFAs such as DHA, EPA, or fish (a major source of both DHA and EPA) has a protective effect against the progression of many lifestyle-related retinal diseases like age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma (2, 5, 6)

Very-long-chain polyunsaturated fatty acids (VLC-PUFAs) are a recently recognized new class of fatty acids that are considered very important for retinal membrane fluidity and maintenance of the highly curved membrane disks of the photoreceptor outer segments (Figure 1.1).

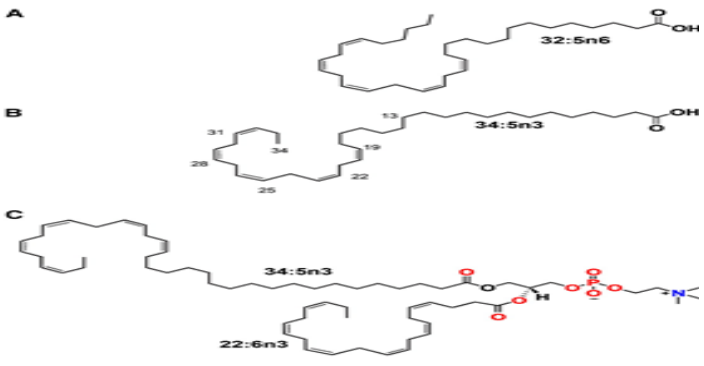

Figure 1.1 VLC-PUFA structures

VLC-PUFAs are nondietary fatty acids with chain length greater than 24 carbons which have been identified in the vertebrate retina and only a few other tissues such as testes. Until recently, very little attention had been paid to these rare C26-C38 retinal lipids due to their low abundance (<2% of all retinal fatty acids) and technical difficulties in measuring their retinal levels by standard gas chromatography (GC) methods. These lipids exhibit a unique hybrid structure, combining a proximal end with a typical saturated fatty acid character and a distal end more characteristic of common PUFAs. These rare fatty acids cannot be synthesized *de novo* in vertebrates and are rarely consumed in normal diets. They are synthesized *in vivo* from specific precursors such as α-linolenic acid (ALA), EPA (20:5 n-3), linoleic acid (18:2 n-6), and AA (20:4 n-6) through the action of an enzyme known as ELOVL4 (Figure 1.2).

In a recently published study from our laboratory, we observed that diet can profoundly influence retinal lipid (VLC-PUFA) composition. For example, an outlier subject who consumed 7g of fish oil/day showed a six-fold increase in EPA/AA ratio, n-3/n-6 LC-PUFA ratios in serum, and a subsequent increase in n-3/n-6 VLC-PUFA ratio in retina, indicating a strong impact of diet on n-3/n-6 VLC-PUFA ratios in retina. Significant correlations were observed between serum n-3/n-6 LC-PUFAs ($r=0.91$, $p<0.001$) and n-3/ n-6 VLC-PUFA ratios (7). Moreover, significant reductions in retinal VLC-PUFA levels and n-3/ n-6 ratios were noted in AMD donor eyes relative to age-matched controls. With this background, we next decided to explore if retinal VLC-PUFA levels and their n-3/n-6 ratios were also altered in diabetes and diabetic retinopathy. We hypothesize that diabetes and diabetic retinopathy affect the retinal lipid profile in the eyes and that these alterations can be ameliorated by dietary supplementation or an n-3 fatty acids rich diet. For this investigation, we chose human donor eyes to study the differences in retinal lipid profiles along with respective serum, as well as animal models (spontaneous diabetes and genetic diabetes models) for n-3 fatty acid supplementation. We studied the

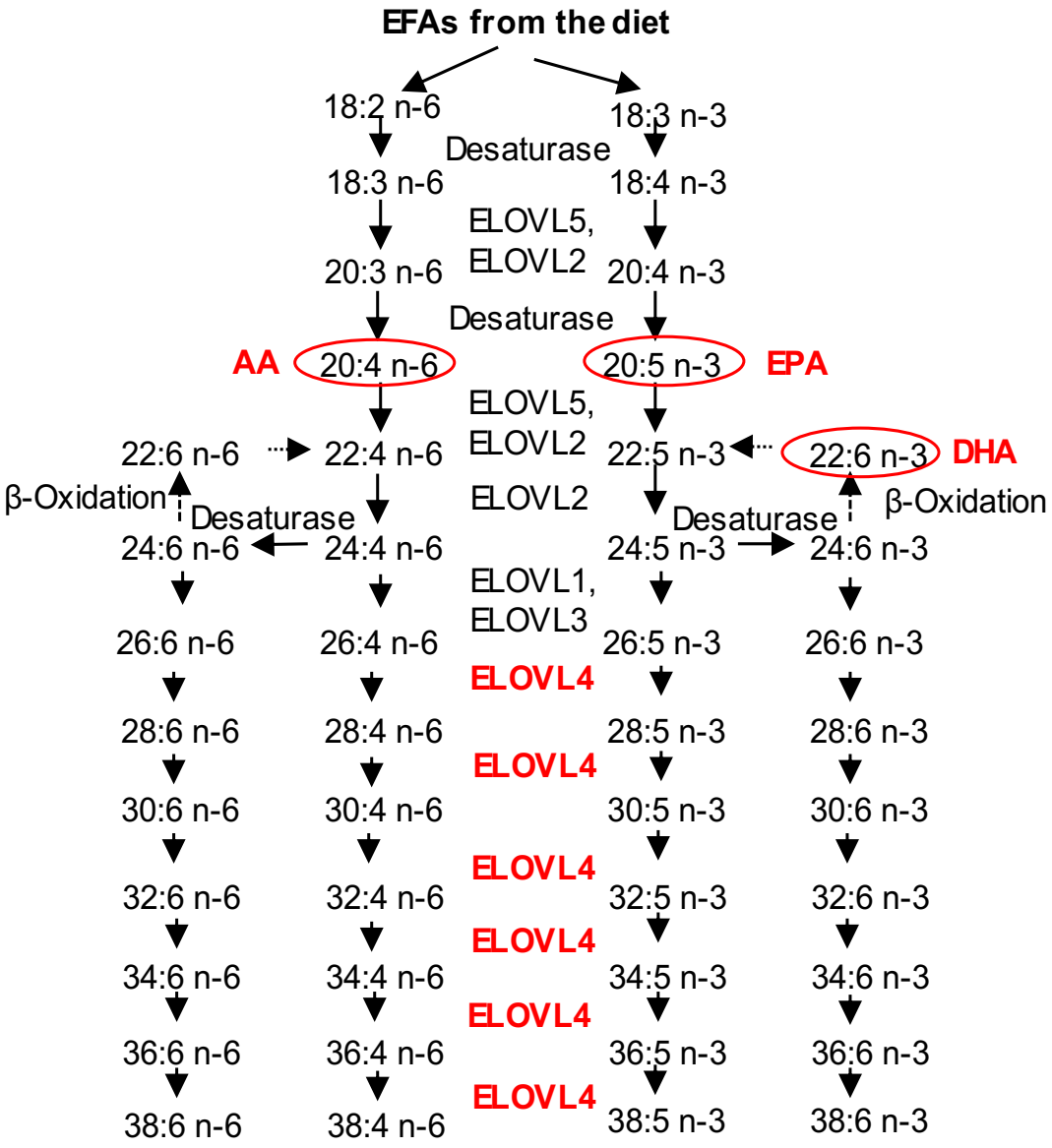

Figure 1.2. Pathways of VLC-PUFA synthesis via ELOVL4

LC-PUFA, VLC-PUFA levels and their n-3/n-6 ratios, glucose levels, serum adiponectin, and gene expression levels of AdipoR1, a regulatory switch for DHA (8) and ELOVL4, an elongase required for the biosynthesis of very long-chain polyunsaturated fatty acids (9).

CHAPTER 2

METHODS

2.1 Materials

All chemical reagents, such as methanol, hydrochloric acid, isopropanol, n-hexane, and diethyl ether, were of gas chromatography mass spectrometry (GC-MS) grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). All standards including the internal standards such as tridecanoic acid (13:0) and Supelco-37 (a commercial mixture of fatty acid methyl esters (FAMEs)) were purchased from Matreya (Pleasant Gap, PA, USA). The internal standard, tridecanoic acid, was dissolved in nonane at a concentration of 1.0 mg/ml. Fish oil for supplementation was purchased from Nordic Naturals, Inc. (Watsonville,CA, USA). EPA (98%) and DHA (98%) were purchased from Avanti Chemicals (Alabaster, AL, USA). A Mini BeadBeater-16 and the associated mixing beads were purchased from BioSpec Products (Bartlesville, OK, USA). Silica gel, glass-encased solid phase extraction (SPE) cartridges (500 mg/6 ml) were purchased from Sorbent Technology (Atlanta, GA, USA). A glucometer was purchased from Bayer HealthCare LLC (Pittsburgh, PA, USA). Serum adiponectin levels were measured by Mouse Adiponectin/Acrp30 Immunoassay (R&D Systems, Minneapolis, MN, USA). All assays were performed according to the manufacturers' protocols.

2.2 Sample Collection

2.2.1 Human Donor Eye Samples

All human donor eyes were collected from the Utah Lions Eye Bank. Donor eyes with large drusen, severe macular atrophy, macular hemorrhage, or any grossly visible chorio-retinal pathologic abnormalities were excluded. Six mm punches of peripheral retina and serum samples were collected. All punched human retinal tissues were stored in tubes and kept at -80°C. We collected diabetic human donor eyes (n=7) between the ages of 76-90. As controls, age-matched control-eyes (n=22) and serum were collected using the same procedures.

2.2.2 Nile Rat Model

The Nile rat (*Arvicanthis niloticus*) develops type 2 diabetes in a similar progression to humans. In general, Nile rats were fed on guinea pig diet in a special room set up with 37°C temperature and 70% humidity. Male Nile rats are a good model for spontaneous diabetes because male Nile rats fed Prolab RMH 2000 diet with low fiber and high fat for 2 months develop hyperinsulinemia and hyperglycemia leading to diet-induced type 2 diabetes. After confirmation of diabetes by blood glucose level estimation, all diabetic rats were divided into two groups and fed RMH 2000 to the control group (n=4) and RMH 2000 + 3 % fish oil (1560 mg of n-3 fatty acids/5ml) to the experimental group (n=4). After 4 weeks of supplementation, all rats were sacrificed, and serum, eyes, and other organs were collected. Eyes were dissected under a light microscope to separate retinal pigment epithelium (RPE) from retinas, and collected samples were frozen at -80 °C for future analysis of LC-PUFAs and VLC-PUFAs.

2.2.3 Akita Mouse Model

Akita mice (C57BL/6J) have the Ins2 mutation which resulted in a single amino acid substitution in the insulin 2 gene which causes misfolding of insulin protein. Akita mice develop diabetic symptoms, such as hyperglycemia, polydipsia, and polyuria, soon after weaning. Four-week-old Akita mice start to display progressive loss of ß cell function, ß cell mass reduction, and overt hyperglycemia. For our supplementation experiments, we had a very small number of control mice (n=2) and experimental (n=4) mice. Three-month-old Akita mice were fed with EPA + DHA (3% of the diet) for 4 weeks. After supplementation, all mice were sacrificed, organs were harvested, and retinas were separated from RPE and used for LC-PUFA and VLC-PUFA analysis.

Mice were genotyped by PCR amplification of genomic DNA from tail biopsy by specific primer pairs for C57BL/6-Ins2^{Akita}/J (fwd: 5'-TGC TGA TGC CCT GGC CTG CT-3' ; rev: 5'- TGG TCC CAC ATA TGC ACA TG-3')

2.3 Extraction of Lipids and Analysis

Retina samples were homogenized with a Biospec Beadbeater. The samples (100 µl) and internal standards (50 µg of tridecanoic acid) were added in 12 ml glass tubes with 4 ml hexane-isopropanol (3:2 v:v) and sonicated for 5 min in an ice water bath. After centrifugation at 3,000 rpm for 5 min, the extracted solution supernatant was transferred to a clean vial and then dried under a stream of nitrogen. The dried film was dissolved in 200 µl hexane, and 2 ml of 4% HCl in methanol was added. The tubes were flushed with argon and incubated at 80°C for 4 hr to form fatty acid methyl esters (FAMEs) and then allowed to cool. The FAME mixture was extracted three times with 1 ml distilled water and 2 ml hexane. The hexane layers were combined and dried under nitrogen gas. Silica gel, glass-encased SPE cartridges were subsequently used to clean

the FAMEs extracts. The cartridge was activated with 6 ml of hexane before loading samples. The crude FAMEs extract was dissolved in 200 µl of hexane and loaded onto the activated cartridge. The cartridge was washed with 6 ml hexane, and the eluate was discarded. Then, the FAMEs were eluted with 5 ml hexane:ether (8:2), and the eluate was evaporated under nitrogen gas. The dry film was dissolved in 180 µl of hexane and centrifuged for 3 min at 14,000 rpm to remove particles prior to GC-MS analysis. For LC-PUFAs analysis, 1 µl of sample was injected into the GC-MS instrument. For VLC-PUFAs analysis, the sample was dried with nitrogen again and re-dissolved in 20 µl of nonane, and 5 µl samples were injected into the GC-MS instrument.

The Thermo Trace GC-DSQ II system (Thermo Fisher Scientific, Waltham, MA, USA) consists of an automatic sample injector (AS 3000), gas chromatograph (GC), single quadrupole mass detector, and an analytical workstation. The chromatographic separation was carried out with a Rxi-5MS coated 5% diphenyl/95% dimethyl polysiloxane capillary column (30 m × 0.25 mm i.d, 0.25 µm film thickness) (Restek, Bellefonte, PA, USA).

For LC-FA analyses, we used the following mass spectrometry conditions (Method A): 1 µl from a 200 µl sample was injected into the GC-MS using a splitless mode; the septum purge was on; the injector temperature was set at 200°C. The column temperature was programmed as follows: initial temperature 60°C; 5 degree/min to 170°C; 1 degree/min to 180°C; 2 degree/min to 240°C; 4 degree/min to 290°C; and a hold at 290°C for 5 min. Transfer line temperature was 290°C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. Mass spectrometry conditions were as follows: electron ionization (EI) mode with full scan (FS) and selected ion monitoring (SIM, *m/z* 79, 108, and 150) because *m/z* 79, 108, and 150 are typical ions of VLC-PUFAs, and n-3 and n-6 PUFAs can be identified by comparing the ratio of the *m/z* 108 and 150 ions; ion source temperature, 200°C; multiplier voltage, 1182 V; detector delay, 10 min. For

peak identification, the data were obtained by collecting the full-scan mass spectra within the scan range of 50-650 amu, and these peaks were identified by comparing their mass spectra with those in the standard solution and the National Institute of Standards and Technology (NIST) library. For the quantification of LC-FAs, the data were obtained by FS. Authentic reference compounds were used to calculate the mol percentage of every peak.

For VLC-PUFAs analyses (Method B), the identification method was described in our previous paper (10). We extracted mixed VLC-PUFAs from bovine retina as the VLC-PUFA standards to establish retention times because commercial standards are not available, and identification of each VLC-PUFA in retinal samples was achieved as described by Liu et al (10). For the quantification of VLC-PUFAs, we used similar mass spectrometry conditions to the LC-FAs method but with a larger injection volume of 5 µl from a 20 µl sample which had been concentrated from a 200 µl original volume. The column temperature program was similar to the LC-FAs method but held at 290°C for 35 min. Mass spectrometry conditions were similar to the LC-FAs method, but the detector delay time was 22 min.

The low amounts of VLC-PUFAs that are present in the mammalian retina elute very late from the GC-MS, and standards are not available commercially which means that their quantitation can be particularly challenging. We found that we had to perform two separate GC-MS runs linked by common C24 VLC-PUFAs. With Method A, the complete set of LC-FAs up to 22 carbons in length and two C24 FAs (24:1n-9 and 24:0) can be quantified under full scan mode, and when the chromatogram is re-analyzed under SIM mode (m/z 79, 108, and 150), we can identify and quantify all n-3 and n-6 LC-PUFAs, and even the C24 VLC-PUFAs become detectable and can be quantified by comparing their mol percentages relative to the C22 LC-PUFAs. The C24 VLC-PUFAs can then be used as the common link between Method A and Method B because they

are present in both GC-MS chromatograms. All of the VLC-PUFAs measured by Method B can be subsequently quantified relative to the total LC-FAs determined by Method A after correcting for the effects of carbon-chain length and the degree of unsaturation on the response of the mass spectrometer.

2.4 Genotyping

The TaqMan platform (Applied Biosystems, Grand Island, NY, USA) was used for genotyping. 7900HT and SDS 2.4 software were used to run the amplification and genotype data. The SNPs, rs381253, and rs10753929, used in this study have been selected based upon their association with ELOVL4 and AdipoR1 as reported in the earlier literature (11, 12).

2.5 Statistical Analysis

Variables were expressed using mean and the SEMs, and the differences were compared by independent t tests. Statistical analyses were performed using Microsoft Excel Software (2016, Microsoft, Redmond, WA). All data are presented as mean ± SEM. Significance was set at $P < 0.05$.

CHAPTER 3

RESULTS

3.1 Effect of Diabetes on Retinal Lipid Profiles in Human Eyes

Seven diabetic subject samples were collected (age between 76-90), and their serum and retinal punches were analyzed by GC-MS. The retinal VLC-PUFA levels and n-3/n-6 VLC-PUFA ratios in human age-matched control versus diabetic retinal punches are shown in Figure 3.1. VLC-PUFA levels and n-3/n-6 VLC-PUFA ratios were unchanged in diabetic retinas in comparison to age-matched control retinas. The lack of statistical significance may be due to the limited number of diabetic human samples.

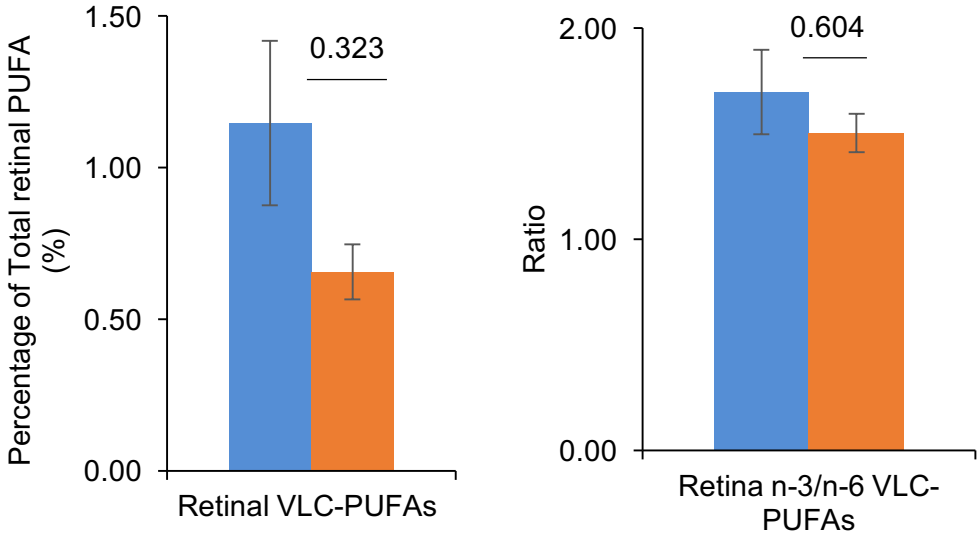

Figure 3.1. VLC-PUFA levels in human retinal punches (■ Control retina; ■ Diabetic retina)

We compared the serum lipid profiles of diabetics and age-matched controls (Figure 3.2). When we compared systemic biomarkers like EPA/AA, DHA/AA, and n-3/n-6 LC-PUFA ratios, no differences were observed in diabetic serum when compared to age-matched control serum. We next focused on the major VLC-PUFA precursors of retinal VLC-PUFAs as follows: 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, and 22:5n-3 for n-3 VLC-PUFAs 18:3n-6, 20:3n-6, 20:4n-6, and 22:4n-6 for n-6 VLC-PUFAs, and the n-3/n-6 VLC-PUFA precursors in diabetic serum samples were significantly lower in comparison to age-matched control serum ($p<0.001$).

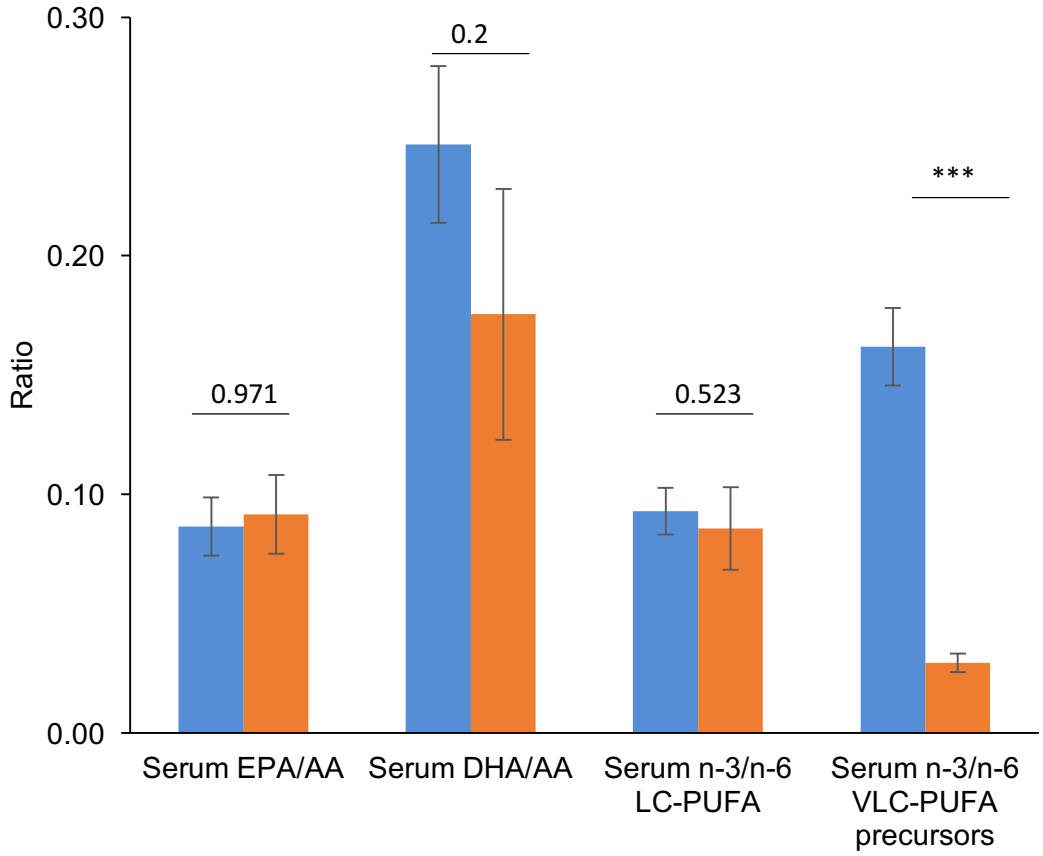

Figure 3.2. Comparison of serum lipid biomarkers in diabetes subjects with age-matched control subjects (■ Control retina; ■ Diabetic retina) (P values: ***P<0.001)

DNA was available for these subjects studied, so we examined whether variants in ELOVL4 and AdipoR1 have any influence on diabetic retinopathy or lipid profiles, but we found no significant relationships, nor did we find any statistically significant influences for diabetic retinopathy (all P values for comparisons were >0.05). Among the seven diabetes subjects, we had three patients with diabetic retinopathy. Therefore, we compared diabetic retinopathy subject data with non-retinopathy diabetes subjects as shown in Figure 3.3. We found there is no significance in retinal VLC-PUFA levels and n3/n6 VLC-PUFA ratios in retinopathy subjects in comparison to non-retinopathy diabetes subjects (Figure 3.3).

3.2 Effects of Fish Oil Supplementation on Diabetic Nile Rats

Due to limited availability of donor diabetic eyes, we chose to investigate the role of fish oil supplementation in animal models of diabetes. For this experiment, we chose

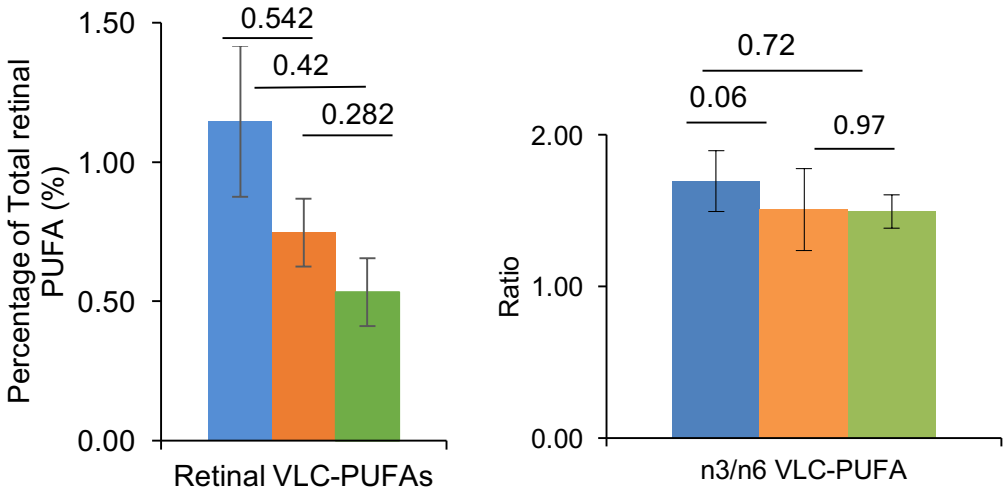

Figure 3.3. Comparison of retinal VLC-PUFAs in diabetes subjects with age-matched control subjects (■ Control retina; ■ Diabetic non-retinopathy retina; ■ Diabetic retinopathy retina) (P values: *P<0.05)

Nile rats (also known as Nile grass rats) because they are diurnal animals with cone-rich retinas, and they are a validated model of spontaneous diabetes which in many ways mimics humans better than Streptozotocin(STZ)-induced mouse or rat models of diabetes. As mentioned in the methods section, we supplemented the group of diabetic male Nile rats with 3% fish oil mixed in diet and the other group without fish oil. We observed that fish oil supplementation improves retinal n-3/n-6 VLC-PUFA ratios (Figure 3.4).

Figure 3.5 shows the serum lipid profiles in diabetes Nile rats after supplementation. After 4 weeks of feeding, the fish oil supplemented group (n=4) had significantly higher EPA/AA ratios and VLC-PUFA precursor ratios than the control diabetic rat group (n=4) ($p<0.001$).

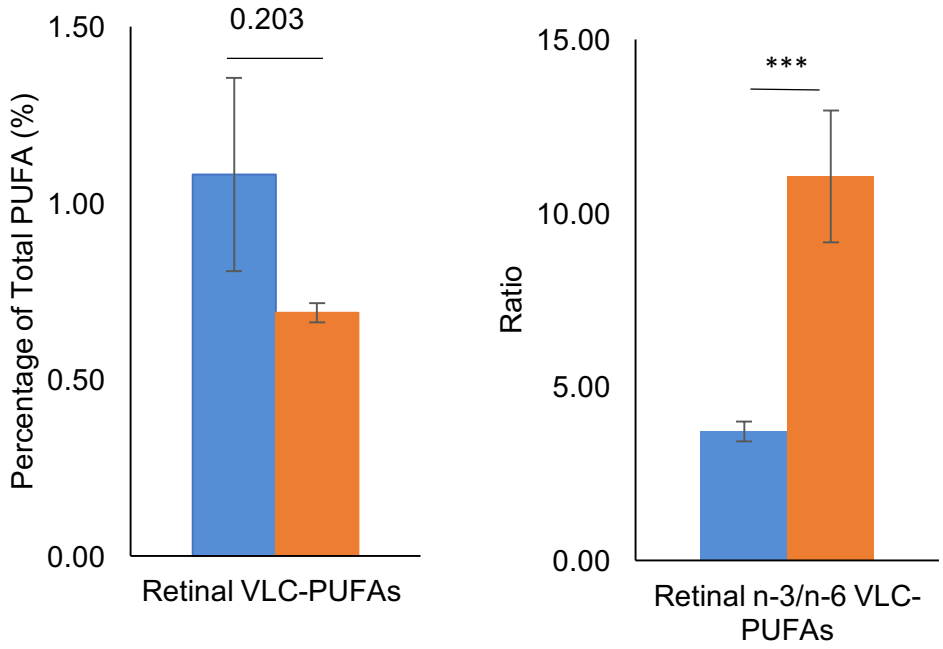

Figure 3.4. Comparison of retinal VLC-PUFA levels in fish oil supplemented diabetic Nile rats group with normal diet diabetic Nile rats group (■ Diabetic-ND; ■ Diabetic-Fish oil supplement) (P values: **P<0.01)

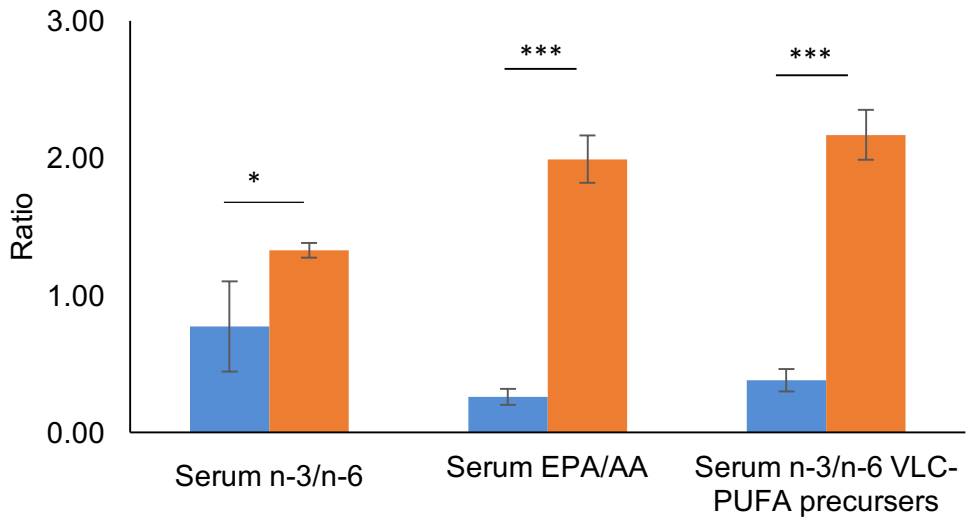

Figure 3.5 Comparison of serum lipid biomarkers in fish oil supplemented Nile rats with normal diet group (■ Diabetic-ND; ■ Diabetic-Fish oil supplement) (P values: *P<0.05; ***P<0.001)

In the retina, the fish oil supplemented group had remarkably higher EPA/AA and in comparison with the diabetic control group (p=0.005) (Figure 3.6). Thus, dietary fish oil supplementation improved serum VLC-PUFA precursor levels and retinal n-3/n-6 VLC-PUFA ratios, indicating diet could play a significant role in improving dyslipidemia. We also tested the glucose levels of Nile rats and found that serum glucose levels of the fish oil supplemented group were significantly reduced in comparison to the control group, consistent with beneficial systemic effects of fish oil on diabetes (Figure 3.7).

3.3 Effect of Diabetes on Retinal Lipid Profiles in Akita Mice

To study the effects of omega-3 fatty acids on diabetic retinopathy in another animal model of diabetic retinopathy, we used Akita mice. We used pure EPA (98%) and DHA (98%) to explore the effects of pure omega-3 fatty acids on diabetes and retinopathy. Three-month-old Akita mice were very sickly, and unlike wild-type mice

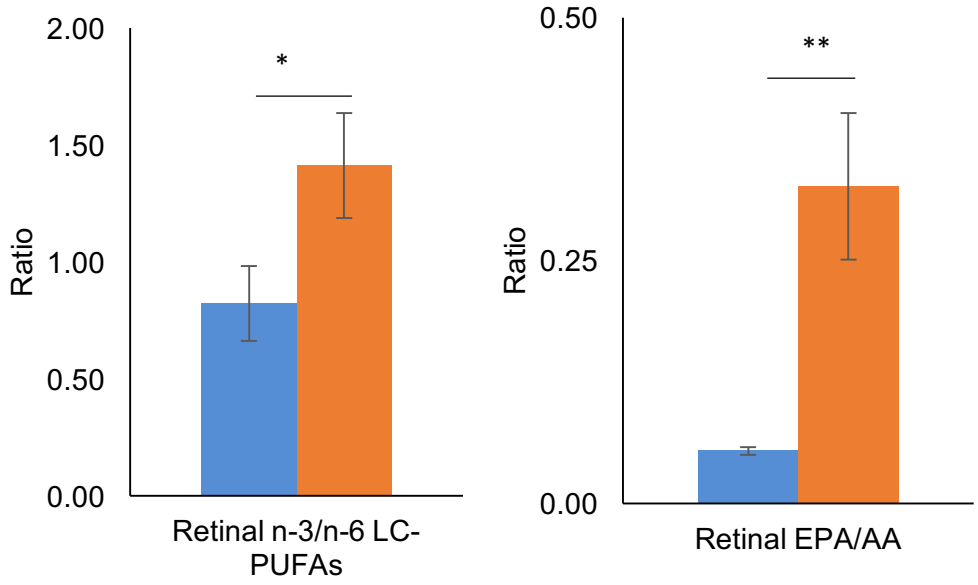

Figure 3.6. Comparison of retinal lipid profiles in fish oil supplemented diabetic Nile rats group with normal diet diabetic Nile rats group (■ Diabetic-ND; ■ Diabetic-Fish oil supplement) (P values: *P<0.05; **P<0.01)

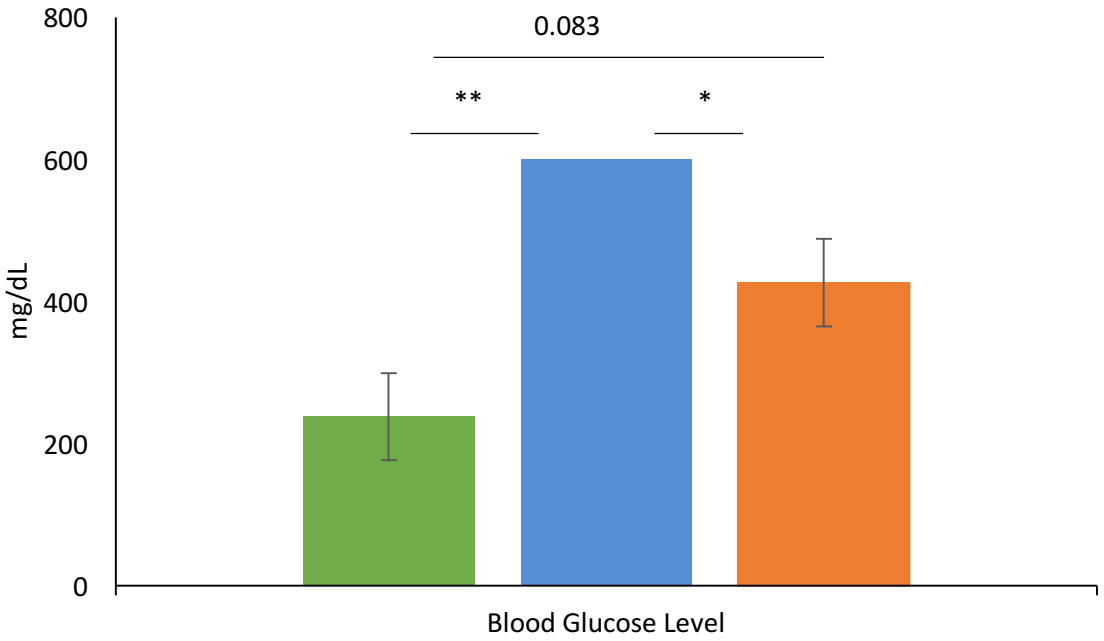

Figure 3.7. Comparison of glucose levels in fish oil supplemented Nile rats with normal diet group (■ Start of the experiment; ■ Diabetic-ND; ■ Diabetic-Fish oil supplement) (P values: *P<0.05; **P<0.01)

(WT), we could not detect any VLC-PUFAs in the retinas of Akita mice.

Akita mice do not produce insulin which leads to higher glucose levels and decreased adiponectin levels which in turn depletes the retinal VLC-PUFA levels and n-3/n-6 LC-PUFA ratios. Even though the Akita mice had undetectable retinal VLC-PUFA levels, we could confirm that retinal LC-PUFA levels were decreased in the Akita mice in comparison to the WT mice. These results indicate that these mice had very depleted levels of lipids in the retina (Figure 3.8).

In the serum, we also observed significantly higher serum EPA/AA, n-3/n-6 LC-PUFAs, and VLC-PUFA precursors ratios in EPA+DHA supplemented group in comparison to the control diet Akita group (Figure 3.9) ($P<0.05$). These results also indicate the important role that diet can play in diabetic dyslipidemia.

Serum adiponectin levels were significantly higher in the wild-type group when compared to the supplemented Akita mouse group ($p<0.05$). Higher serum adiponectin levels were lower in type-2 diabetes both in mice and humans (Figure 3.10).

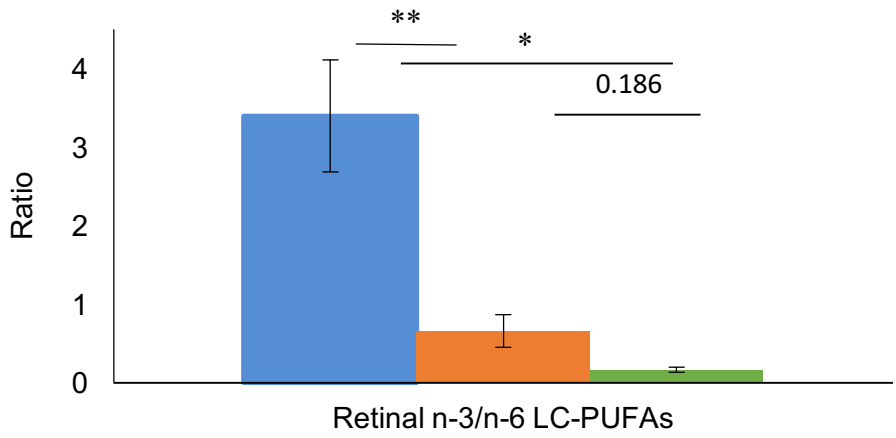

Figure 3.8. Comparison of retinal n-3/n-6 LC-PUFA levels in EPA+DHA supplement Akita mice group with control diet Akita group (■ WT- EPA+DHA; ■ Akita- EPA+DHA; ■ Akita-ND) (P values: **P<0.01)

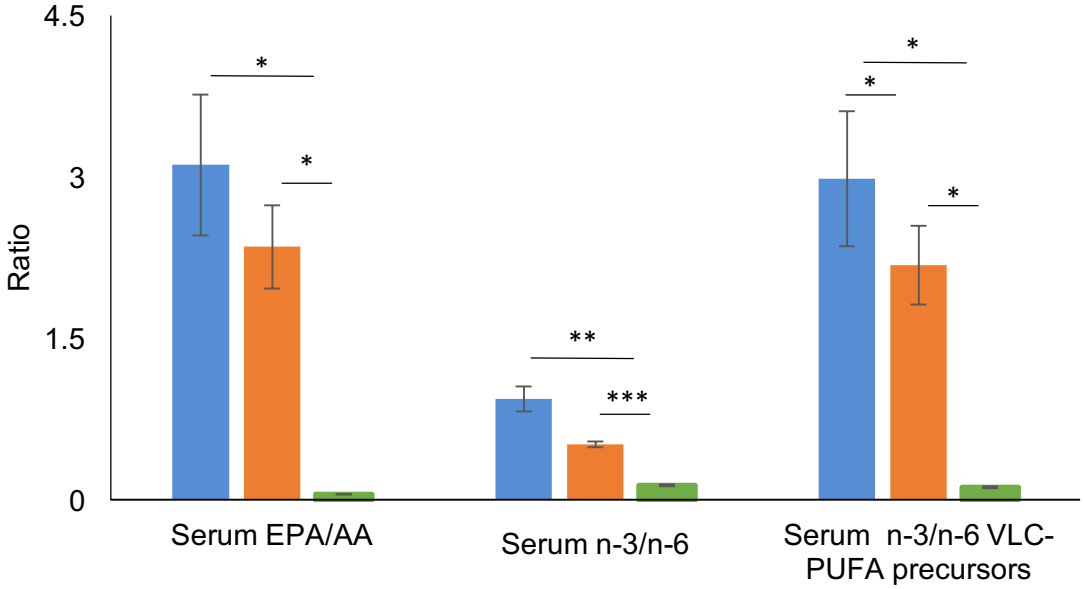

Figure 3.9. Comparison of serum lipid biomarkers in EPA+DHA supplement Akita mice group with control diet Akita group (■ WT-EPA+DHA; ■ Akita- EPA+DHA; ■ Akita-ND) (P values: *P<0.05; ***P<0.001)

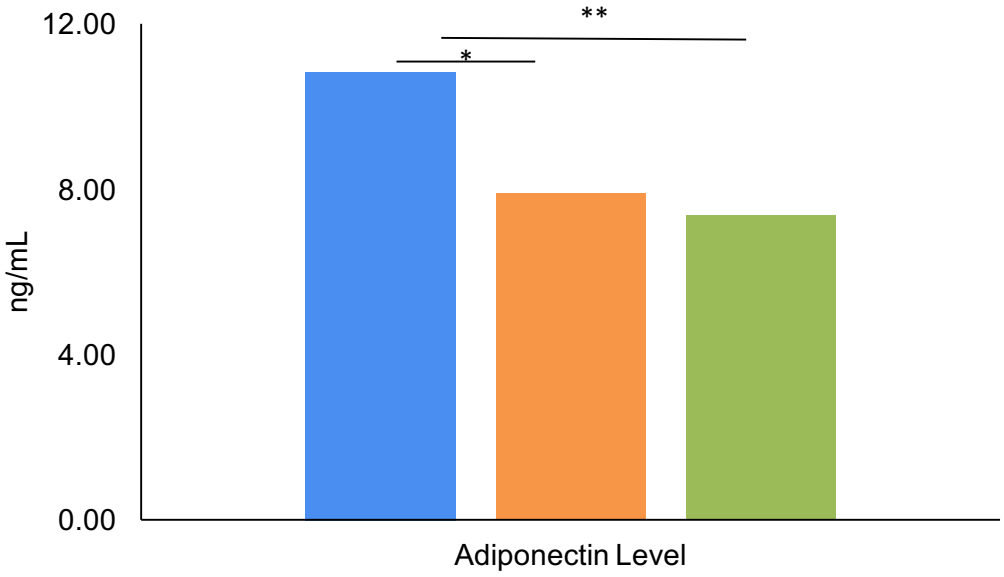

Figure 3.10. Comparison of Adiponectin levels in EPA+DHA supplemented Akita mice with normal diet group (■ WT-EPA+DHA; ■ Diabetic-EPA+DHA; ■ Diabetic-ND) (P values: *P<0.05; **P<0.01)

CHAPTER 4

DISCUSSION

Even with the unique presence and specificity of VLC-PUFAs in the human retina, there are controversies regarding their beneficial effects and the clinical significance of their roles in health and disease. There is considerable evidence that mutations in the ELOVL4 gene, required for the biosynthesis of VLC-PUFAs in retina, result in Stargardt-3 (STGD3) disease, a dominant juvenile macular dystrophy (8, 13), but the mechanistic process by which STGD3 disease occurs, either by protein aggregation or by VLC-PUFA deficiency, is not well characterized (14, 15). Our clinical trials with STGD3 patients indicated that fish consumption (16) and possibly fish oil supplementation (17) improved the disease phenotype, which indicates that fish oil supplementation/ n-3 PUFAs supplementation can actually reverse VLC-PUFA deficiency and improve vision. Epidemiological studies generally support the recommendation that consumption of foods rich in n-3 LCPUFAs is associated with a lower risk of retinal degenerative diseases like AMD (5, 6), glaucoma (18), and diabetic retinopathy (19), but clinical intervention studies with n-3 PUFA supplements have been either negative or equivocal (20).

In the present study, lipid profiles of seven diabetic donor retinal punches were compared with 22 age-matched control donor retinal punches. This is the first report to measure VLC-PUFA levels in diabetic donor punches, and we observed a decreasing trend in VLC-PUFA levels and n-3/n-6 VLC-PUFA ratios in diabetic retinas in comparison to age-matched control retinas. However, since the number of eye tissues is

limited, we could not obtain any statistically significant difference. In correlation with our study, Kady et al. (21) reported that intravitreal delivery of human ELOVL4 reduced the diabetes-induced increase in vascular permeability, thereby increasing VLC-ceramides in diabetes. Tikhonenko et al. (22) also observed a decrease in retinal VLC-PUFAs levels and a decrease in ELOVL4 expression levels as well as ELOVL4 protein levels in early diabetic animals in comparison to control animals. These studies indicate that diabetes has profound effects on VLC-PUFA levels even before detection of retinopathy symptom.

Three of seven donors had diabetic retinopathy. Hence, we compared diabetes with no retinopathy (No DR) with diabetic retinopathy donors and also with age-matched controls. Even though the numbers were small, we observed in Figure 3.3 that VLC-PUFA levels in diabetic retinopathy are lower than in the No DR group, indicating that diabetic retinopathy affects retinal VLC-PUFAs as well as membrane permeability.

In the present study, we also observed changes in serum LC-PUFAs and other lipid biomarkers. As shown in Figure 3.2, the fatty acids which elongate to give rise to n-3 VLC-PUFAs are considered as n-3 VLC-PUFA precursors, and the same term is used for n-6 VLC-PUFA precursors. Compared to the age-matched control serum, diabetic serum has reduced ratios of DHA/AA and n-3/n-6 VLC-PUFA precursors. The ratio of n-3/n-6 VLC-PUFA precursors indicates the presence of substrates for VLC-PUFAs in serum, and a significant reduction of this ratio indicates that there is not only a reduction in ELOVL4 expression levels in diabetes but also a decrease in n-3/n-6 VLC-PUFA precursors. AdipoR1, a receptor for adiponectin in the eye, known to control the infiltration of fatty acids to the retina is also known to be associated with retinal degeneration (9, 12) According to the present results, we did not observe any differences in AdipoR1 gene expression in these diabetic patients in relation to age-matched controls. These results from the human retinal punches indicate that impaired

insulin metabolism causes dyslipidemia, which affects n-3/n-6 VLC-PUFA ratios and VLC-PUFA levels as well as other serum biomarkers in humans.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) have long been studied for their therapeutic potential in the context of type 2 diabetes, insulin resistance, and glucose homeostasis. Crochemore et al. (23) and Ebbesson et al. (24) also demonstrated the influence of n-3 PUFA in type 2 diabetes women and Alaskan Eskimos and suggested that high dose of n-3 PUFAs in serum can reduce insulin sensitivity and glucose tolerance. Thorsdotter et al. (25) also correlated the amount of n-3 PUFA from cow's milk to the prevalence of diabetes in Nordic populations. In spite of all these population studies and mouse studies, there is a lack of concrete knowledge about the practical application of n-3 PUFA as nutritional therapeutics against insulin resistance in humans. Given the above studies, we hypothesize that supplementation with n-3 PUFAs/ fish oil to diabetic animal models improves glucose tolerance, n-3/n-6 LC-PUFA ratio in serum, and most importantly, counteracts the decreasing levels of VLC-PUFAs in the retina. Adiponectin is known to be the major regulator of lipid and glucose homeostasis through its insulin-sensitizing properties (26), and lower adiponectin contributes to vascular complications and diabetic retinopathy. Hence in this study, we also considered adiponectin as a biomarker for diabetes.

The Nile grass rat *(Arvicanthis niloticus*) is a diurnal rodent which is a becoming popular for studying circadian rhythms. It has a cone-rich retina with 30% cones in comparison to 3% cones in mice or rats (27), making it is a very useful model to study eye diseases, and it is also a good model to study metabolic syndromes and spontaneous diabetes (28). Diabetic Nile grass rats in the early phase of the disease develop hyperinsulinemia, and show a strong inverse correlation between plasma adiponectin and HbA1c levels (28). We hypothesized that Nile grass rats would be a good model to study diabetes and its effects on VLC-PUFAs. As discussed in the

methods section, we changed their regular guinea pig chow to an RMH 2000 diet, which altered their blood glucose levels and made all the male rats diabetic.

As discussed in Figure 3.4, the VLC-PUFA levels did not show any significant difference between the fish oil supplemented groups, and control diet groups, but the n-3/n-6 VLC-PUFA ratio altered significantly. The retinal n-3/n-6 LC-PUFA ratio improved from 4.5 to 11 in the fish oil supplemented group, indicating decreased levels of dyslipidemia and improved n-3 VLC-PUFA precursor levels (Figure 3.6). To observe differences in retinal VLC-PUFA levels, we might have to supplement fish oil for longer periods than 4 weeks. We also observed that fish oil supplementation improved serum lipid biomarkers like EPA/AA, n-3/n-6 LC-PUFA ratios, and n-3/n-6 VLC-PUFA presursors (Figure 3.5), which shows the improvement in dyslipidemia in these animals. As shown in Figure 3.7, the glucose levels at the start of the experiment were approximately 250 mg/dL, which increased to above 600 mg/dL in the control animals during the course of the experiment, while supplementation with fish oil increased glucose levels to just 400 mg/dL, consistent with positive effects of 3% fish oil (containing 1360 mg/5 mL oil) supplementation on glucose tolerance.

Further, we have studied the effect of n-3 PUFAs in Akita mice which is an Ins2 genetic diabetes (Ins2) mice model. Male heterozygous KO (*Ins2*$^{-/+}$) develop hyperglycemia in less than 2 months, while homozygous KO die before birth. Akita mice show various retinal pathologies characteristic of early non-proliferative diabetic retinopathy (29) and also exhibit increased frequency of apoptic retinal neurons, increased vascular permeability (30), and decreased retinal blood flow (31). These properties could answer the question whether availability of substrate or lack of enzyme leads to loss of VLC-PUFAs in diabetes. After supplementation of 3-month-old Akita mice with EPA + DHA (2:1), we observed the serum LC-PUFAs and retinal LC-PUFA improved in comparision to control Akita mice. Unfortunately, no VLC-PUFAs were

detected in the eyes from both the supplemented and control Akita mice, as opposed to the wild-type supplemented mice. In spite of the lack of detectable VLC-PUFA levels, we observed that retinal n-3/n-6 LC-PUFAs improved significantly in supplemented Akita mice compared to control Akita mice (Figure 3.8). In correlation with our study, earlier studies suggested that n-3 PUFA supplementation effectively reduced pathological retinal neovascularization and protected retinal neurons and retinal ganglion cells in various animal models. Our results are consistent with a clinical study in India that lower n-3/n-6 ratio in dietary lipids increases the prevalence of type 2 diabetes and that higher n-3/n-6 ratios may restore normal insulin action (32).

Even though the VLC-PUFAs were undetectable, interestingly, we observed that n-3 PUFAs supplementation in Akita mice improved the serum lipid profile. The n-3/n-6 LC-PUFA, EPA/AA, and n-3/n-6 VLC-PUFA precursor ratios improved significantly in PUFA supplemented group compared to control Akita mice (Figure 3.9). Previous experiments have shown that adding n-3 LC-PUFAs to the diet reduces the progression of retinopathy compared with controls (33, 34). These animal studies found that a 2% change in dietary intake of n-3 LC-PUFA (n-3 vs n-6 FA) resulted in a 2-fold increase in retinal LC-PUFA, which is in correlation with our studies. Also, we observed that serum adiponectin improved in the n-3 PUFA supplemented group in comparison to the control group, indicating the beneficial effects of PUFA supplementation in diabetic mice. The serum adiponectin levels were in the order of WT<EPA+DHA<Control.

We need to further study the effects of supplementation using larger numbers of animals, and the period of supplementation should be more than 2 months to study the effects of retinal VLC-PUFA levels. Further studies with hispathologic observations and adiponectin levels will give us better knowledge of the benefits of fish oil supplementation in diabetes models.

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