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n-3 PUFA Supplementation Alters Retinal Very-Long-Chain-PUFA Levels and Ratios in Diabetic Animal Models

Aruna Gorusupudi[1,*], **Fu-Yen Chang**[1,*], **Kelly Nelson**[1], **Gregory S. Hageman**[1,2], **Paul S. Bernstein**[1,2]

[1]John A. Moran Eye Center

[2]Sharon Eccles Steele Center for Translational Medicine

Abstract

Scope: Long chain (LC)-PUFAs act as precursors for special class of retinal lipids known as Very-long-chain (VLC)-PUFAs and the effect of diabetes on retinal VLC-PUFA levels has been unexplored. In order to understand the supplemental effect of n-3 PUFAs, on decreasing levels of VLC-PUFAs due to diabetes, we chose Nile rat which develops diabetes spontaneously and Akita mouse, a genetic diabetes model.

Methods and results: Human retinal punches from donors were collected from eye bank, lipids were extracted and analyzed to study the alterations in VLC-PUFAs and their n-3/n-6 ratios. Nile rats were fed high fat diet to induce hyperglycemia, following which an n-3 PUFA rich diet was fed to the experimental group for two months. Diabetic male Akita mice and WT mice were fed with 5% fish oil mixed in with their chow for two months to observe the effect of n-3 PUFAs. Results indicate that VLC-PUFA levels were lower in human diabetic and retinopathic retinal punches compared to age-matched controls. With supplementation of n-3 PUFAs, there was a significant increase in n-3/n-6 VLC-PUFA ratios in both animal models compared to diabetic controls.

Conclusion: Dietary supplementation with n-3 LC-PUFAs help to prevent progression of diabetes and associated retinopathy.

Graphical Abstract

Corresponding Author: Paul S. Bernstein, MD, PhD, 65 Mario Capecchi DriveSalt Lake City, Utah 84132Phone: (801) 581-6078Fax: (801) 581-3357E- paul.bernstein@hsc.utah.edu.

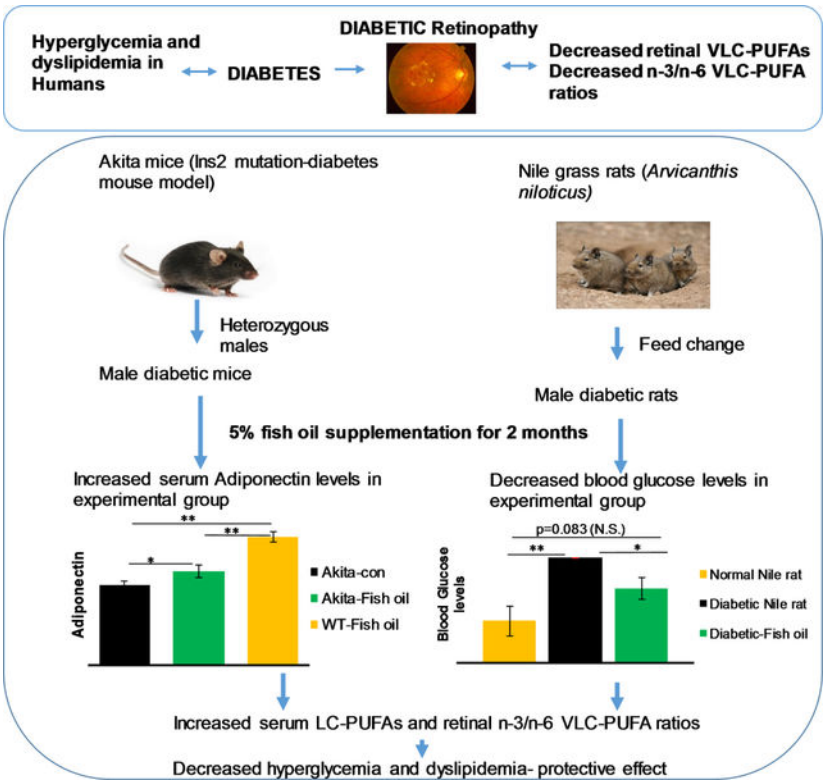
AUTHOR CONTRIBUTIONS

AG and PB conceived the hypotheses and designed the experiments. FC collected the data and performed the analysis. GH contributed the human retinal punches and serum samples and reviewed the manuscript. AG and PB wrote the manuscript. KN cared for the animals in the experiments and helped with manuscript preparation.

*Authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

CONFLICTS OF INTEREST

The authors do not have any conflicts of interest. GH is a founder and shareholder of Voyant Biotherapeutics LLC.



Layman Summary: VLC-PUFAs are a special class of non-dietary fatty acids present in the vertebrate retina and testes. We used diabetic animal models to observe the effect of n-3 LC-PUFAs on replenishing VLC-PUFAs and observed that n-3 PUFAs not only help to improve n-3 VLC-PUFAs but also decrease blood glucose levels and improve diabetic conditions in these animal models.

Keywords

VLC-PUFAs; n-3/n-6 VLC-PUFA ratio; diabetic retinopathy; Nile grass rats; n-3/n-6 LC-PUFA ratio

1. Introduction:

Diabetes mellitus (DM), a growing public health concern, reportedly affected nearly 422 million people worldwide in 2014[1]. Diabetic retinopathy (DR) is a major complication of diabetes and the leading cause of blindness in 30–60 year-olds. DR results from chronic diabetic damage to small blood vessels in the retina caused by above normal blood sugar levels[2]. DR has two phases: a non-proliferative phase characterized by increased vascular permeability and hemorrhages, and a proliferative phase characterized by retinal neovascularization, both of which are accompanied by vision loss[3].

The role of lipids in the progression of DR 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. Dyslipidemia is also an established risk factor for cardiovascular diseases and is strikingly common in patients with DM, affecting almost 50% of this population[5]. In addition to hyperglycemia and hypertension, dyslipidemia is a modifiable cardiovascular risk factor that remains largely uncontrolled in patients with DM. Although a few studies have shown a correlation between dyslipidemia and diabetes, the association is not well understood. Most clinical guidelines recommend tight control of dyslipidemia, especially in high risk patients[6]. Both systemic and retina-specific fatty acid profiles are affected by diabetes and have been suggested to contribute to the progression of DR. Hence, we deemed it important 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, while 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 intake of n-3 LC-PUFAs such as DHA, EPA, or fish (a major source of both DHA and EPA) has protective effects against the progression of many lifestyle-related retinal diseases like age-related macular degeneration (AMD), DR and glaucoma[2, 7, 8].

Very-long-chain polyunsaturated fatty acids (VLC-PUFAs) are a recently recognized new class of fatty acids that are considered important for retinal membrane fluidity and maintenance of the highly curved membrane disks of the photoreceptor outer segments (Figure 1). VLC-PUFAs are non-dietary fatty acids with chain lengths greater than 24 carbons; they have been identified in the vertebrate retina and 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 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 (Figure 1). 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 (18:3 n-3), 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 elongase 4 (ELOVL4) (Figure 2).

In a recently published study from our laboratory, we observed that diet can profoundly influence retinal lipid (VLC-PUFA) composition[9]. For example, one subject who consumed 7g of fish oil/day showed a six-fold increase in EPA/AA and n-3/n-6 LC-PUFA ratios in serum, and a subsequent increase in n-3/n-6 VLC-PUFA ratios in retina, indicating a strong impact of diet on n-3/n-6 VLC-PUFA ratios in retina[9]. Moreover, significant reductions in retinal VLC-PUFA levels and n-3/n-6 ratios were noted in donated eyes from individuals with histories of AMD relative to age-matched controls. With this background, we sought to

explore whether retinal VLC-PUFA levels and their n-3/n-6 ratios were also altered in diabetes and DR.

We hypothesized that diabetes and DR affects the retinal lipid profile in the eyes and that these alterations can be ameliorated by dietary supplementation of n-3 PUFAs. For this investigation, we chose human donor eyes to study the differences in retinal lipid profiles and animal models (spontaneous diabetes and genetic diabetes models) for n-3 LC-PUFA rich fish oil supplementation. We studied the 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[10] and ELOVL4, an elongase required for the biosynthesis of VLC-PUFAs[11].

2. Materials and Methods:

2.1 Chemicals:

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). Silica gel, glass-encased solid phase extraction 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. Prolab RMH 2000 diet, Custom made-fish oil mouse diet and control diet were purchased from Lab Supply, TX, USA and Labdiet, MO, USA respectively. Fish oil (1,560 mg of PUFA/5mL) for Nile rat supplementation was purchased from Nordic Naturals, Inc. (Watsonville,CA, USA).

2.2 Human Donor Eye Samples:

All human donor eyes and respective blood were collected from the Utah Lions Eye Bank by the Steele Center for Translational Medicine under an IRB-approved protocol. Donor eyes with large drusen, severe macular atrophy, macular hemorrhage, or any grossly visible chorio-retinal pathologic abnormalities were excluded. 6 mm punches of extramacular 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=17) between the ages of 76–90. As controls, age-matched control-eyes (n=22) and serum were collected using the same procedures.

2.3 Nile Rat Model:

Nile rats (also known as Nile grass rats) (*Arvicanthis niloticus*) are diurnal animals with cone-rich retinas, and they are a validated model of spontaneous diabetes which, in many ways, mimics the human form of diabetic condition and better than streptozotocin-induced mouse or rat models of diabetes. In general, Nile rats were fed with guinea pig diet in a special room set up with 37°C temperature and 70% humidity. Male Nile rats with a blood

glucose level of 250 mg/dL on guinea pig chow were fed with Prolab RMH 2000 diet (low fiber and high fat) for 2 months to develop hyperinsulinemia and hyperglycemia leading to diet-induced DM. After confirmation of diabetes(blood glucose levels more than 500 mg/dL), all diabetic rats were divided into two groups one group fed RMH 2000 as control and experimental group was fed RMH 2000 plus fish oil (n=6/group). After two months of supplementation, all rats were sacrificed, serum, eyes, and other organs were collected. Eyes were dissected under a light microscope to separate retinal pigment epithelium from retinas, and collected samples were frozen at −80 °C for future analysis of LC-PUFAs and VLC-PUFAs.

2.4 Akita Mouse Model:

Akita mice (C57BL/6J) have an Ins2 mutation which results in a single amino acid substitution in the insulin 2 gene which causes misfolding of the insulin protein. Akita mice develop diabetic symptoms, such as hyperglycemia, polydipsia, and polyuria soon after weaning. 3-month-old Akita mice were fed with fish oil diet for two months. After supplementation, all mice were sacrificed, and organs were harvested. Retinas were separated from retinal pigment epithelium 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’). All the procedures used in this study were approved by appropriate Institutional Animal Care and Use Committees and were carried out according to National Institutes of Health guidelines.

2.5 Dosage Administration:

For Nile rats, the above mentioned fish oil (5%) was mixed with pulverized RMH 2000 diet and fed to the experimental group for two months. For Akita mice, a 5% fish oil mouse chow (AIN-93G), custom-made by Test Diet was used for feeding to the experimental Akita and wild type (WT) mice. On average, the Nile rats and Akita mice were given 1.2 g/kg body weight/day and 3.13 g/kg body weight/day of n-3 PUFA, respectively.

2.6 Extraction of Lipids and Analysis

Retina samples were homogenized with a Biospec Beadbeater, and internal standards (50 μg of tridecanoic acid) were added along with 4 ml hexane-isopropanol (3:2 v:v). After centrifugation at 3,000 rpm for 5 min, the supernatant was transferred to a clean vial and then dried under a stream of nitrogen. To the dried film, 4% HCl in methanol was added and incubated at 80°C for 4 hr to form FAMEs. The FAME mixture was extracted with hexane and dried under nitrogen gas. Silica gel, glass-encased solid phase extraction cartridges were subsequently used to clean the FAMEs extracts. The cartridge was initially washed with hexane, and the eluate was discarded after which the FAMEs were eluted with hexane:ether (8:2) and dried. The dry film was dissolved in hexane, and 1 μl of sample was injected into the GC-MS instrument for LC-PUFA analysis. For VLC-PUFA 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. Analyses employed a Thermo Trace single quadrupole GC-MS DSQ II system (Thermo Fisher Scientific, Waltham, MA, USA). 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). The GC-MS analyses protocol is detailed in an earlier publication[12].

2.7 Statistical Analysis:

Differences between groups were evaluated by one-way ANOVA or Student's t-test with $p<0.05$ as significant. All statistical analyses were conducted using Graph Pad Prism version 7 software. Data are reported as mean ± SEM.

3. Results:

3.1 Effect of Diabetes on Retinal Lipid Profiles in Human Eyes:

Seventeen diabetic and twenty-two age-matched control human retinal punches (age between 76–90) and their respective serum were extracted for lipids using the procedure elsewhere described, and analyzed by GC-MS[9]. 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 3a and 3b. The VLC-PUFA levels were significantly lower in retinal punches from samples derived from donors with diabetic retinopathy (DM-DR) (n=8), as compared to those derived from diabetic donors without retinopathy (DM-No-DR) (n=9) and age-matched controls (n=22).

The n-3/n-6 VLC-PUFA ratios were significantly reduced in DM-DR retinas compared to DM-No-DR and age-matched control retinas. The present data also showed a decreasing trend in n-3/n-6 retinal LC-PUFA ratios in DM-DR compared with DM-No-DR and age-matched controls. As shown in Figure 3c, the retinal n-3/n-6 LC-PUFA and n-3/n-6 VLC-PUFA precursor ratios dropped in DM-DR. The major VLC-PUFA precursors (LC-PUFAs responsible for synthesis of VLC-PUFAs in the presence of ELOVL4) for retinal VLC-PUFAs were as follows: 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, and 22:5n-3 for n-3 VLC-PUFAs and 18:3n-6, 20:3n-6, 20:4n-6, and 22:4n-6 for n-6 VLC-PUFAs. We compared the n-3/n-6 VLC-PUFA precursor ratios in retina, and observed a decrease in the ratios, indicating the loss of n-3 LC-PUFAs during retinopathy. We compared the serum lipid profiles of diabetics and age-matched controls. The serum biomarkers like EPA/AA, DHA/AA and n-3/n-6 LC-PUFA also dropped in a similar trend to retinal LC-PUFA ratios. The n-3/n-6 LC-PUFA and n-3/n-6 VLC-PUFA precursor ratios in DM-DR serum samples were significantly lower than those in age-matched control serum ($p<0.05$) (Figure 3d).

We investigated whether the loss of VLC-precursors or lack of the enzyme ELOVL4 are responsible for decreased VLC-PUFA levels in the retinopathy retinas by genotyping the retinas for ELOVL4 variants (responsible for elongation of LC-PUFAs to form VLC-PUFAs). We did not observe any significant relationship between ELOVL4 variants and retinal lipid profiles. We also genotyped genetic variations in the AdipoR1 gene (responsible for transport of n-3 LC-PUFAs to the retina) and again did not observe any significant associations with abnormal lipid profiles.

3.2 Effects of Fish Oil Supplementation on Diabetic Nile Rats:

Due to the limited availability of diabetic human donor eyes, we chose to investigate the effect of fish oil supplementation in diabetic animal models. For this experiment, we chose Nile rat because they develop DM in a similar progression to humans and are a good model for spontaneous diabetes. As mentioned in the Methods section, we supplemented a group (n=6) of diabetic male Nile rats with 5% fish oil mixed in diet, with the other group (n=6) receiving no fish oil as control.

Even though the VLC-PUFA levels did not change significantly (Figure 4a) with fish oil feeding, the n-3/n-6 VLC-PUFA ratio improved in the retina (Figure 4b), indicating a slow recycling of VLC-PUFAs and an improved n-3 VLC-PUFAs and n-3 LC-PUFAs in the retina, which may be a key factor in decreasing dyslipidemia in the retina. The fish oil supplemented group had remarkably higher retinal ratios of EPA/AA, DHA/AA, n-3/n-6 LC-PUFA and retinal n-3/n-6 VLC-PUFA precursor ratios in comparison to the diabetic control group ($p<0.05$) (Figure 4c). Increased n-3/n-6 LC-PUFA levels in the retina shows that in spite of being diabetic, the transport process of EPA and DHA from fish oil diet to the retinas is functional in these rats.

In our earlier study, we indicated that serum EPA/AA and n-3/n-6 LC-PUFA ratios serve as important biomarkers that correlate with the n-3/n-6 VLC-PUFA ratios in the retina, and this was likewise true in this experiment. Thus, dietary fish oil supplementation improved serum n-3 VLC-PUFA precursor levels (Figure 4d) and retinal n-3/n-6 VLC-PUFA ratios (Figure 4b), indicating that diet could play a significant role in improving dyslipidemia. We also tested the glucose levels of Nile rats and found that the 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 4e).

3.3 Effects of Fish Oil Supplementation on Diabetic Mouse Model:

The effects of fish oil supplementation on Akita mice (a genetic model for type 1 diabetes with a Ins2 gene mutation) were also assessed. Male Akita mice develop type 1 diabetes as early as 2 months old. In the present study, one group of Akita mice (n=6) was fed with fish oil chow (5%) for 2 months, and the other group (n=6) was fed a control chow (without added fish oil) to study the influence of fish oil supplementation on n-3/n-6 VLC-PUFA ratios, dyslipidemia, blood glucose and serum adiponectin. As a control, age-matched WT mice (n=6) were also fed with fish oil chow for 2 months. The results of the study indicate that the n-3/n-6 VLC-PUFA ratios improved significantly in fish oil fed Akita mice in comparison to the control diet fed Akita mice, although VLC-PUFA levels were not altered significantly in the former group compared to the latter (Figure 5a). As shown in Figure 5b, the n-3/n-6 VLC-PUFA ratio in fish oil fed WT mice was insignificantly lower than supplemented Akita mice, although the VLC-PUFA levels were higher in the former group compared to the latter group.

The retinal n-3/n-6 LC-PUFA ratios improved approximately threefold in the fish oil fed Akita mouse group in comparison to the control diet group. The n-3/n-6 VLC-PUFA precursor ratios in retina also improved significantly in the fish oil fed Akita mice compared

to the control diet group (Figure 5c). Since DHA levels are quite constant in the retina, we did not see any significant differences between the groups. In the serum, we observed significantly higher n-3/n-6 LC-PUFAs and VLC-PUFA precursor ratios in the fish oil supplemented group compared to the control diet group (Figure 6a)($P<0.05$). These results indicate that an increase in n-3 PUFAs in diet significantly decreases the levels of dyslipidemia in diabetes.

We also observed that serum adiponectin levels improved significantly in the fish oil supplemented groups in comparison to the control group ($p<0.05$). Higher serum adiponectin levels in WT mice are consistent with the previous results of serum adiponectin levels being lower in both mice and humans with diabetes (Figure 6b). We also tested serum glucose levels in all groups before sacrificing the animals, and observed that serum glucose levels improved in the fish oil supplemented group significantly in comparison with the control group (Figure 6c).

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 role 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[10, 13]. However, 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 have indicated that consuming fish[16] and possibly fish oil supplements[17] improved the disease phenotype, which suggests that fish oil supplementation with n-3 PUFA supplementation can actually reverse VLC-PUFA deficiency and improve vision. Epidemiological studies generally support the recommendation that consuming foods rich in n-3 LC-PUFAs may lower the risk of developing retinal degenerative diseases like AMD[7, 8], glaucoma[18] and DR[19], but prospective clinical intervention studies with n-3 PUFA supplements have been either negative or equivocal[20].

In the present study, the lipid profiles of seventeen diabetic donor retinal punches were compared with twenty-two age-matched control donor retinal punches. This is the first report to measure VLC-PUFA levels in human diabetic retinas, 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. 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-PUFA 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 a profound effect on VLC-PUFA levels even before retinopathy symptoms are detected. Since seven of the donors had DR, we compared diabetic donor eyes having no retinopathy (DM-No-DR) with those of the diabetic retinopathy donors and also with age-matched controls. Even though the numbers were small, we observed that retinal VLC-PUFA levels in diabetic retinopathy are lower than than

the DM-No-DR group (Figure 3a) indicating that DR affects retinal VLC-PUFAs as well as membrane permeability.

We also observed changes in serum LC-PUFAs and other lipid biomarkers. As shown in Figure 2, the fatty acids which elongate to give rise to n-3 VLC-PUFAs are considered to be n-3 VLC-PUFA precursors, and the same terms are used for the n-6 series. Compared to the age-matched control,subjects with diabetic retinopathy has reduced retinal and serum n-3/n-6 LC-PUFA and n-3/n-6 VLC-PUFA precursor ratio indicating dyslipidemia (Figure 6). AdipoR1, a receptor for adiponectin in the eye known to control the delivery of fatty acids to the retina, is also known to be associated with retinal degeneration[11, 23]. According to the present results, we did not observe any differences between AdipoR1 and ELOVL4 gene expressions in these diabetic patients and 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.

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.[24] and Ebbesson et al.[25] demonstrated the influence of n-3 PUFAs in DM women and Alaskan Eskimos and suggested that n-3 PUFAs in serum can improve insulin sensitivity and glucose tolerance. Thorsdotter et al.[26] also correlated the amount of n-3 PUFAs 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 PUFAs as nutritional therapeutics against insulin resistance in humans. Given the above studies, we conclude that supplementation with fish oil to diabetic animal models will improve glucose tolerance, n-3/n-6 LC-PUFA ratios 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[27], 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 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[28], 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[29]. 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[29]. We hypothesized that the Nile grass rat would make 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 depicted in Figure 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. There is a significant increase in the retinal

EPA/AA and n-3/n-6 VLC-PUFA precursor ratios in the supplemented groups compared to the control diabetic group. To observe any differences in retinal VLC-PUFA levels, we may have to supplement fish oil for periods longer than two months. 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 presursor ratios ,, which shows the improvement in dyslipidemia in these animals. As discussed in the methods section, the glucose levels of normal guinea pig chow fed male Nile rats were approximately 250 mg/dL, which after feeding high fat RMH 2000 diet for 2 months increased to above 600 mg/dL ratsduring the course of the experiment, while supplementation with fish oil decreased serum glucose levels to 400 mg/dL, consistent with the positive effects of 5% fish oil (containing 1360 mg/5 mL oil) supplementation on glucose tolerance (Figure 4e).

Furthermore, we studied the effect of n-3 PUFAs in Akita mice which is an Ins2 genetic diabetes (Ins2) mouse model. Male heterozygous KO (*Ins2*$^{-/+}$) mice develop hyperglycemia in less than 2 months, while homozygous KO mice die before birth. Akita mice show various retinal pathologies characteristic of early non-proliferative diabetic retinopathy[30] and also exhibit increased frequency of apoptic retinal neurons, increased vascular permeability[31] and decreased retinal blood flow[32]. These properties could answer the question of whether or not availability of substrate or lack of enzyme leads to loss of VLC-PUFAs in diabetes. After supplementation of 3-month-old Akita mice with fish oil (5%), we observed that the serum and retinal lipid profiles improved in comparision to control Akita mice. The retinal VLC-PUFAs improved in fish oil supplemented Akita mice compared to the control Akita mice (Figure 5). The n-3/n-6 VLC-PUFA ratios improved significantly in fish oil fed groups (WT and Akita mice) in comparision to the control groups ..We also observed that retinal n-3/n-6 LC-PUFAs increased significantly in supplemented Akita mice compared to control Akita mice . 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[33, 34]. Our results are consistent with a clinical study in India that lower n-3/n-6 ratios in dietary lipids increase the prevalence of type 2 diabetes and that higher n-3/n-6 ratios may restore normal insulin action[35]. The serum n-3/n-6 LC-PUFA and n-3/n-6 VLC-PUFA precursor ratios improved significantly in the PUFA supplemented group compared to control Akita mice (Figure 6a).

Even though the differences in VLC-PUFA ratios in supplemented groups were not significantly different than control diabetic groups of Akita mice and Nile rats, we observed that n-3/n-6 VLC-PUFA ratios improved significantly in supplemented groups. Moreover, the serum lipid profile of diabetic supplemented groups had significantly higher n-3/n-6 LC-PUFA ratios compared to control groups. Previous experiments have shown that adding n-3 LC-PUFAs to the diet reduces the progression of retinopathy compared with controls[36, 37]. 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 twofold increase in retinal LC-PUFAs, which is in correlation with our studies. Also, we observed that serum adiponectin significantly improved in the fish oil supplemented group in comparison to the control group (Figure 6b), indicating the beneficial effects of n-3 PUFA supplementation in diabetic mice. Because of type 1 diabetes and hyperglycemia, the blood glucose levels for Akita mice were above 600 mg/dL before the

experiment, but the supplemented group experienced a significant reduction in serum glucose compared with control diabetic animals (Figure 6c). This indicates that improvement in the lipid profile decreases dyslipidemia which in turn reduces hyperglycemia. Further studies with histopathological observations and adiponectin levels, AdipoR1 and ELOVL4 mRNA expression, and assessment of genetic variations in these genes will give us better knowledge of the benefits of fish oil supplementaion in diabetic models.

In conclusion, these data confirm our prior findings that retinal VLC-PUFA levels and n-3/n-6 VLC-PUFA ratios are lower in degenerated retinas in comparison with age-matched controls[38], which indicates the need to understand the physiology of these special fatty acids. Humans have relatively less VLC-PUFAs compared to mice[12], and further loss of these active molecules may indeed induce physiological and structural disruption in the photoreceptors as seen in DR. In the present study, we observed that a decrease in serum and retinal n-3 PUFAs is a plausible reason for the loss of retinal VLC-PUFAs and decreased n-3/n-6 ratios. Therefore, we studied the importance of fish oil supplementation in diabetic animal models with respect to retinal VLC-PUFAs. The retinal and serum n-3/n-6 LC-PUFA ratios increased in both the Nile rat and Akita mouse groups supplemented with fish oil, indicating reduced dyslipidemia. We also observed that fish oil supplementation reduces hyperglycemia and improves blood glucose and serum adiponectin levels in diabetic animal models. Diet plays an important role in altering the serum n-3/n-6 LC-PUFA ratios which in turn influence the n-3/n-6 LC-PUFA and VLC-PUFA ratios and levels in retina with possible beneficial effects on retinal physiology and protection against degeneration. Our results clarify the biological mechanisms underlying the epidemiological studies which have shown that diets rich in n-3 fatty acids are protective against retinal degenerative diseases. Future studies involving diabetic retinopathy should seek to discover the advantages of VLC-PUFAs and their role in degeneration, as well as the effect of hyperglycemia on these special fatty acids.

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Abbreviations:

AA	Arachidonic acid
AMD	Age-related macular degeneration
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
DR	Diabetic retinopathy
ELOVL4	Elongase 4

EPA	Eicosapentaenoic acid
LC	Long-chain
n-3	Omega-3
n-6	Omega-6
STGD3	Stargardt-3 disease
VLC	Very-long-chain
WT	Wild type

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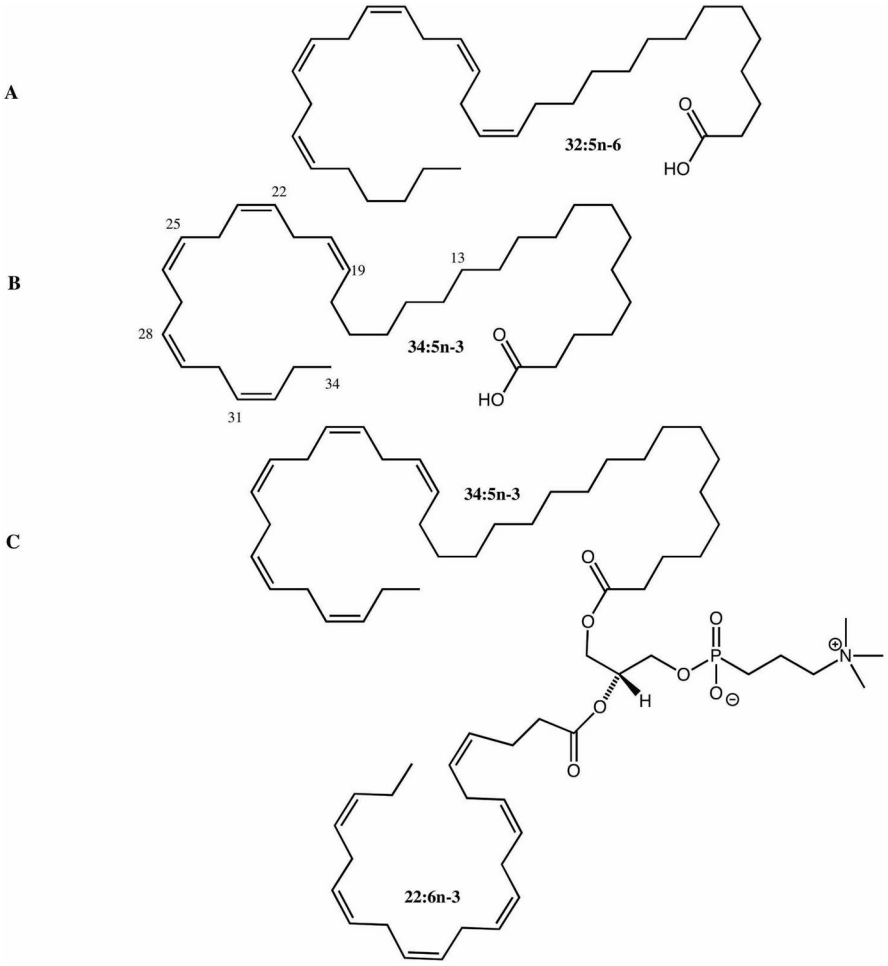

Figure 1:
VLC-PUFA structures (A. 32:5 n-6; B. 34:5 n-3; C. Phospholipid with 34:5 n-3 at sn-2 position and DHA at sn-1 position).

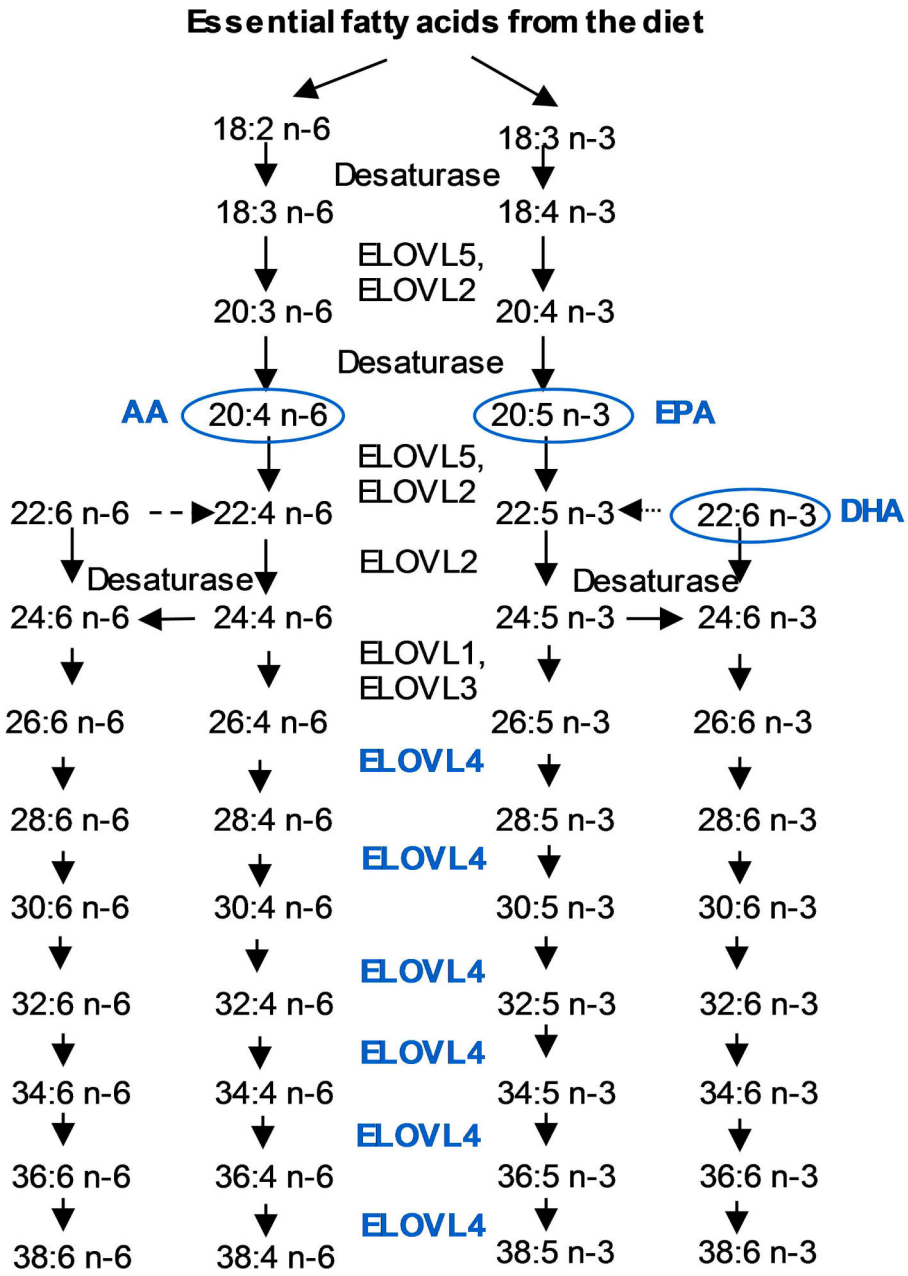

Figure 2:
Biosynthetic pathway of VLC-PUFAs in the mammalian retina.

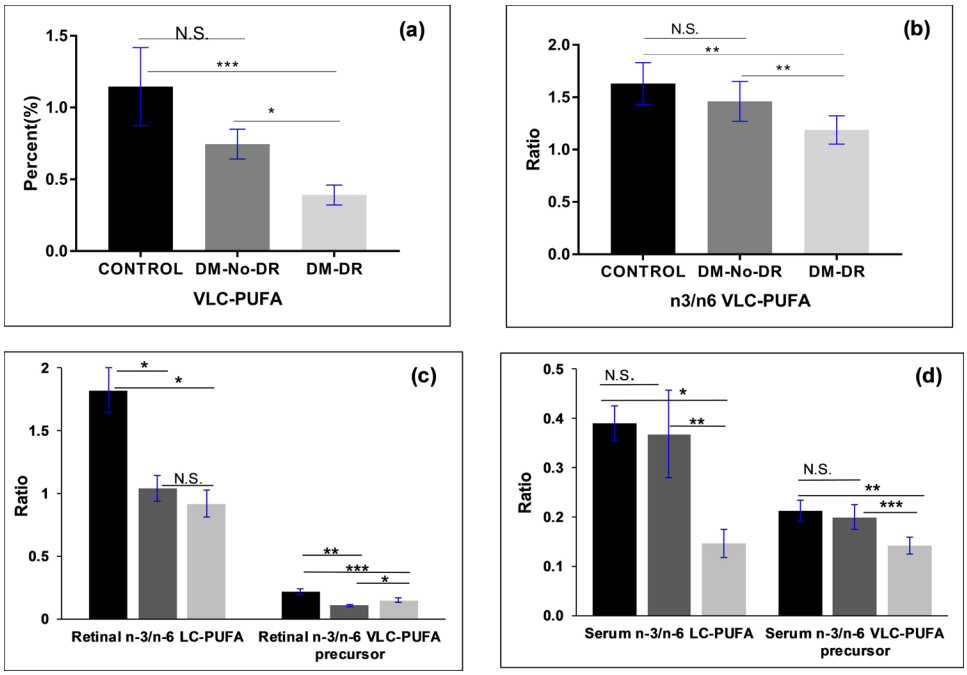

Figure 3:

Comparison of (a) retinal VLC-PUFA levels; (b) n-3/n-6 VLC-PUFA ratios (c) retinal LC-PUFAs (d) serums lipid biomarkers in age-matched controls (n=22), diabetic-No-Diabetic retinopathy (DM-No-DR) (n=9) and diabetic retinopathy (DM-DR) (n=8) donor retinal punches. (■-Age-Matched controls; ■-DM-No-DR; ■-DM-DR) (P values: ***P<0.005; **P<0.01; *P<0.05; N.S., not significant).

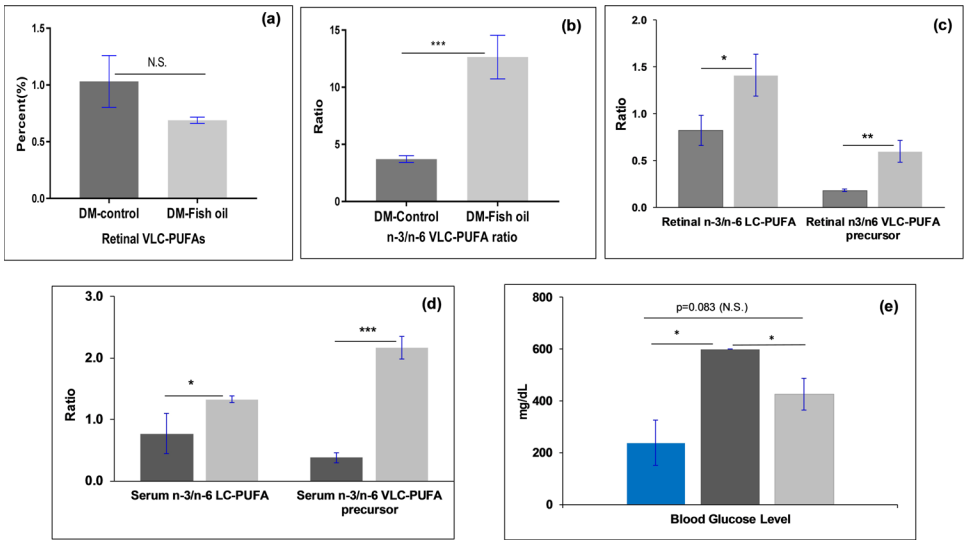

Figure 4:

Comparison of (a) retinal VLC-PUFA levels; (b) n-3/n-6 VLC-PUFA ratios; (c) retinal LC-PUFA ratiosratios; (d)serum lipid biomarkers; (e) blood glucose levels in n-3 PUFA rich fish oil supplemented diabetic Nile rats group (n=6) in comparison to control diabetic Nile rat group (n=6) (■-DM-control nile rats; ■-DM-Fish oil supplemented Nile rats; ■- non diabetic control Nile rats) (***P<0.005; **P<0.01; *P<0.05; N.S., not significant).

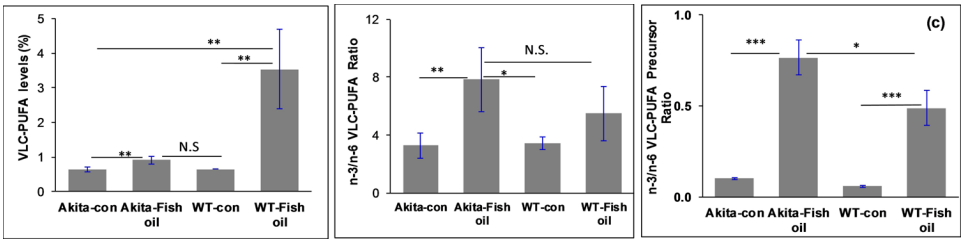

Figure 5:

Effect of fish oil supplementation on (a) retinal VLC-PUFA levels; (b) n-3/n-6 VLC-PUFA ratios (c) n-3/n-6 VLC-PUFA precursor ratios of Akita mice (n=6) and WT mice (n=6) in comparison to control Akita mice (n=6) and WT control mice (n=6) respectively (***P<0.005; **P<0.01; *P<0.05; N.S., not significant).

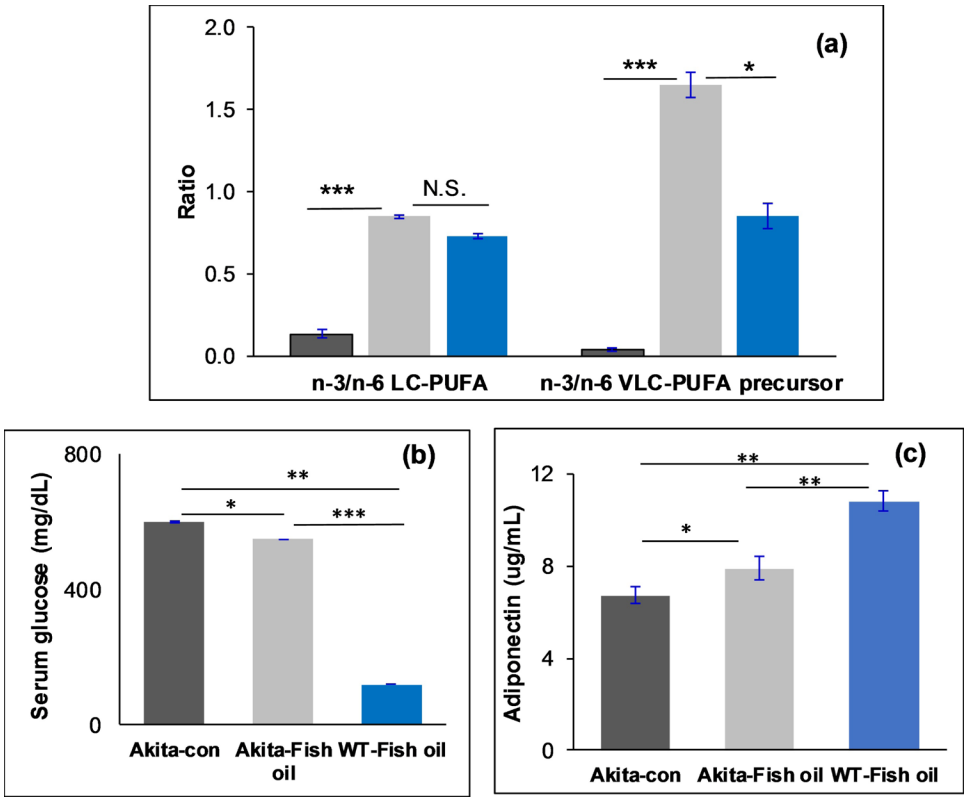

Figure 6:

Comparison of serum (a) lipid biomarkers; (b) Adiponectin levels (c) glucose levels in fish oil supplemented Akita mice group (n=6), fish oil supplemented WT mice (n=6) compared to Akita control group (n=6) (■- Akita-control; □- Akita-fish oil supplemented; ■- WT mice-fish oil supplemented) (***P<0.005; **P<0.01; *P<0.05; N.S., not significant).