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Long-term supplementation with EPA and DHA changes the fatty acid profile of rat tissues, upregulates NRF2 expression and downregulates TGFβ expression

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ABSTRACT

We studied the influence of long-term treatment with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on the liver, and kidney lipogenesis, inflammation and antioxidative gene expression. Male Wistar rats were divided into three groups on the basis of the different n6/n3 fatty acid ratios achieved by using different dietary oil blends: the control (CON, n6/n3 ratio was ~7), the N6 group (n6/n3 ratio was ~50) and the DHA group (n6/n3 ~1). Treatment significantly influenced the fatty acid profile of the liver and kidney tissues. The most characteristic changes were an increased content of EPA and DHA in the DHA group in both tissues, of the kidney and liver. The expression of transforming growth factor beta (TGF-ß) was downregulated in the liver tissue by long-term EPA/DHA supplementation. This could be attributed to a decrease in the production of arachidonic acid-derived proinflammatory mediators, and an increase in EPA and DHA derived eicosanoids. DHA and EPA supplementation also significantly increased expression of the NRF 2 gene. This finding suggests that n3 PUFA could influence the activation of the NRF 2 pathway, which is important in cell antioxidative defense. In conclusion, we have shown that long-term dietary supplementation with DHA and EPA could influence lipid metabolism, inflammation and antioxidative defense. Therefore, the long-term addition of dietary DHA and EPA could potentially influence the most important pathological processes in aging.

Key words: n6/n3 ratio; NRF2; DHA; lipogenesis; oxidative stress; aging

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Introduction

Polyunsaturated fatty acids are an important family of lipid compounds, which constitute and influence the physical properties of lipid bilayers, and consequently cell functions (LOPEZ et al., 1995; CALDER, 2006; DAS, 2006). In addition, they are precursors for inflammatory mediators, and act as signaling molecules in cells (SHAIKH and BROWN, 2013). Therefore, a change in the composition of fatty acids could result in different pathological processes in tissues (FRITSCHE, 2015; LEE et al., 2016). The fatty acid composition of different tissues could be influenced by diet, *de novo* lipogenesis and bioconversion. Fatty acids derived from diet or generated *de novo* are further metabolized by a series of highly regulated steps of desaturation, elongation and b oxidation, into different saturated and unsaturated fatty acids (JUMP, 2009; TU et al., 2010). The bioconversion rate of linolenic acid into the important n3 long chain PUFAs (polyunsaturated fatty acids, eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA) is very low, which implies that a certain percentage of these fatty acids must be obtained from the diet (BRENNA, 2002). There has been a great deal of research focused on the potentially protective role of n3 PUFA, EPA and DHA against cardiovascular diseases, different metabolic disorders (diabetes, non-alcoholic fatty liver disease), cancer, neurological disorders and inflammatory processes, and related diseases (BONAFINI et al., 2015; OPPEDISANO et al., 2020). The alteration of the PUFA n6/n3 ratio in favor of n3 PUFA could have an important role in the prevention and treatment of metabolic disorders, such as metabolic syndrome (MAŠEK et al., 2018; STARČEVIĆ et al.; 2018, SAKAMURI et al., 2020). Lipid metabolism also has an important role in the aging process. There are many scientific studies about the role of PUFA in the development of senile changes in the nervous and musculoskeletal systems, and the beneficial effects of their supplementation on degenerative changes during aging (LOPEZ et al., 1995; CUTULI et al., 2016; LALIA et al., 2017).

The purpose of this study was to investigate the influence of long-term supplementation with EPA and DHA on the liver and kidney lipid metabolism. Our focus was on characterization of the efficiency of a long-term diet with EPA/DHA for prevention of oxidative and inflammatory injury during aging.

Materials and methods

Animals, experimental design, and diets. The experiments were performed in accordance with the Croatian Animal Welfare Act and were approved by the Croatian National Ethics Committee and the Ministry of Agriculture, Republic of Croatia (authorization EP 107/2017). Animal experiments were performed on 18 male, Wistar HAN rats (initial live weight, 170±6 g, 7 weeks of age). The animals were kept in the animal laboratory facility of the Institute for Medical Research and Occupational Health, Zagreb, Croatia. After 2 weeks of acclimation, the rats were divided according to the dietary treatment that included different n6/n3 fatty acid ratios. Different n6/n3 ratios were obtained with the usage of sunflower/linseed/fish oil blends. The resulting groups were: control (CON, n6/n3 ratio was ~7), the N6 group (n6/n3 ratio was ~50) and the DHA group (n6/n3 ~1, supplemented with fish oil) (Table 1). The rats were kept in polycarbonate cages (61 x 43 x 20 cm, L x W x H) and housed under the conditions of a 12 h light/dark cycle at 25±2°C. Access to food and water (tap water) was unlimited to all. The rats were weighed in plastic containers at weekly intervals, always at the same time (8 h), using an electronic balance with 0.5g precision.

Sample collection. After 18 months the rats were sacrificed under Narketan/Xylapan anesthesia (Narketan, 80 mg kg^{-1} b.m. and Xylapan, 12 mg kg^{-1} b.m., i.p., Vetoquinol, Bern, Switzerland) followed by exsanguination. Blood was collected by cardiac puncture into ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was obtained by centrifugation at 1500×g for 5 min. Immediately after the animals were sacrificed, the liver tissue was divided into two samples: (1) stored in an RNA preserving agent (RNA later, Thermo Fisher Scientific, Waltham, MA, USA) for extraction of mRNA, and (2) frozen at -80°C for the fatty acid analyses.

Analysis of the lipid composition. Total lipids from the liver and kidney tissue were extracted using a chloroform/methanol mixture (2:1, v/v) (FOLCH et al., 1957). After extraction, the lipids were dried under N_2, dissolved in the same mixture (150 µL) with the addition of 0.3 mg/mL BHT, and stored at -80 °C. The analyses of fatty acids were performed by GC-MS (QP2010 Ultra, Shimadzu, Kyoto, Japan), equipped with a BPX70 capillary column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m long, SGE, Austin, TX, USA). Analytical conditions were set as described previously (MAŠEK et al., 2017). Nonadecanoic fatty acid (C19:0) was used as an internal standard. The results of fatty acid composition were expressed as the percentage of total fatty acids.

Table 1. Fatty acid composition (% of fatty acids) of diets supplemented to the rats

Fatty acids	CON	N6	DHA
Palmitic (C16:0)	7.15	7.36	15.25
Stearic (C18:0)	2.76	2.21	4.25
Oleic (C18:1n9)	28.97	29.10	31.20
Linoleic (C18:2n6)	52.15	58.92	26.38
Linolenic (C18:3n3)	7.39	1.21	0.86
Eicosapentaenoic (C20:5n3)	nd	nd	9.05
Docosahexaenoic (C22:6n3)	nd	nd	10.95
n6/n3 ratio	6.92	48.69	1.26

nd, below quantification level

Desaturation indices. The Δ9 desaturase activity was estimated as the product to precursor ratio: Δ9 (18) = 18:1n9/18:0

Analysis of liver gene expression by real-time quantitative PCR. Total RNA was isolated from the frozen liver tissue using the SV Total RNA Isolation System (Promega GMBH, Mannheim, Germany) according to the manufacturer's instruction. The purity and quantity of the RNA samples were checked by spectrophotometry (BioDrop µLITE, BioDrop, Cambridge, UK). One step RT qPCR reaction (One-Step SYBR PrimeScript RT-PCR Kit II) of total RNA was conducted according to the manufacturer's manual (Perfect Real Time, TaKaRa Bio Inc. Shiga, Japan). The qPCR was performed using a Stratagene MxPro3005 (Agilent Technologies, US and Canada) thermocycler. The ratio of the relative expression of target genes to the housekeeping genes was calculated using the ΔΔCt method ($2^{(-\Delta\Delta CT)}$) and values were normalized to housekeeping genes, β-actin and cyclophilin (LIVAK and SCHMITTGEN, 2001). All reactions were performed in triplicate. The primer sequences used in the qPCR reaction are listed in Table 2.

Statistical analyses. The experimental results were analyzed using the GraphPad Prism 8 program. Data were expressed as the means ± SD. Normality of distribution was tested with the Shapiro–Wilks test. ANOVA and the post-hoc Tukey test were used to determine statistical differences between the group means. Standard deviations of fold changes for the tested gene expression were determined according to the established procedure (LIVAK and SCHMITTGEN, 2001). Differences were considered significant at $P< 0.05$.

Table 2. Primer sequences and cycling conditions for polymerase chain reactions to evaluate the impact of the treatment in gene expression

Gene	Sequence 5' - 3'	Access. No.	Ann. Tm.	Cycles
TGFβ	F: AAT ACG TCA GAC ATT CGG GAA GCA	NM_021578.2	60°C	40
	R: AAT ACG TCA GAC ATT CGG GAA GCA			
Δ9D	F: ACA TTC AAT CTC GGG AGA ACA	NM_139192.2	60°C	40
	R: CCA TGC AGT CGA TGA AGA AC			
NRF2	F: CAC ATC CAG ACA GAC ACC AGT	NM_031789.2	60°C	40
	R: CTA CAA ATG GGA ATG TCT CTG C			
β-actin	F: CAT TGT CAC CAA CTG GGA CGA TA	XM_039089807.1	60°C	40
	R: GGA TGG CTA CGT ACA TGG CTG			
Cyclophilin	F: GGA TGG CAA GCA TGT GGT CTT TG	M19533	60°C	40
	R: CTT CTT GCT GGT CTT GCC ATT CCT			

TGFβ, Transforming growth factor beta; Δ9D, delta 9 desaturase; NRF 2, Nuclear factor erythroid 2-related factor 2

Results

After 18 months, the animals assigned to the different experimental diets showed significant differences (P<0.05) in body weight (Fig. 1). There was a significant weight reduction (P<0.05) in the experimental groups in comparison to the CON group.

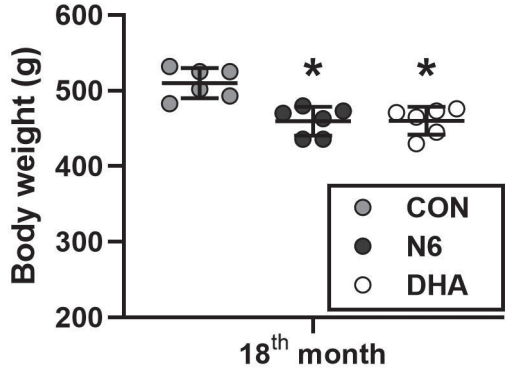

Fig. 1. The influence of different n6/n3 fatty acid ratios on the body weight of the experimental rats
Values are means ± SD. *P<0.05 for N6, and DHA versus the CON group

Quantification of individual fatty acids revealed significant differences between the experimental and control groups in the liver tissue (Fig. 2). The changes that were noticed in the liver tissue were: a significant increase in C18:1n9 (P<0.05) and a significant decrease in C18:2 n6 (P=0.05) in the N6 group compared to the CON group, and a decrease in the content of arachidonic acid and an increase in the content of DHA and EPA in the DHA group compared to the CON group.

It is noteworthy that a very low content (below quantification level) of EPA groups was observed in the CON and N6 groups after 18 months. In addition, differences between the N6 and DHA groups were also observed. The DHA group had significantly higher values for C18:2 n6 (P<0.05), C20:5 n3 and C22:6 n3 (P<0.001) and a lower content of C20:4 n6 (P<0.01).

Similar patterns were observed in the kidney tissue in the experimental groups. In the N6 group, a significant increase was visible in C 18:1n9 (P<0.001) and a significant decrease in C18:2 n6, C22:6 n3 and C20:5 n3 (P<0.05, P<0.01 and P<0.01) in comparison to the DHA group.

The desaturation index for C18 (C18:1n9/ C18:0) revealed a significant increase in the N6 group compared to the CON group (Fig. 2).

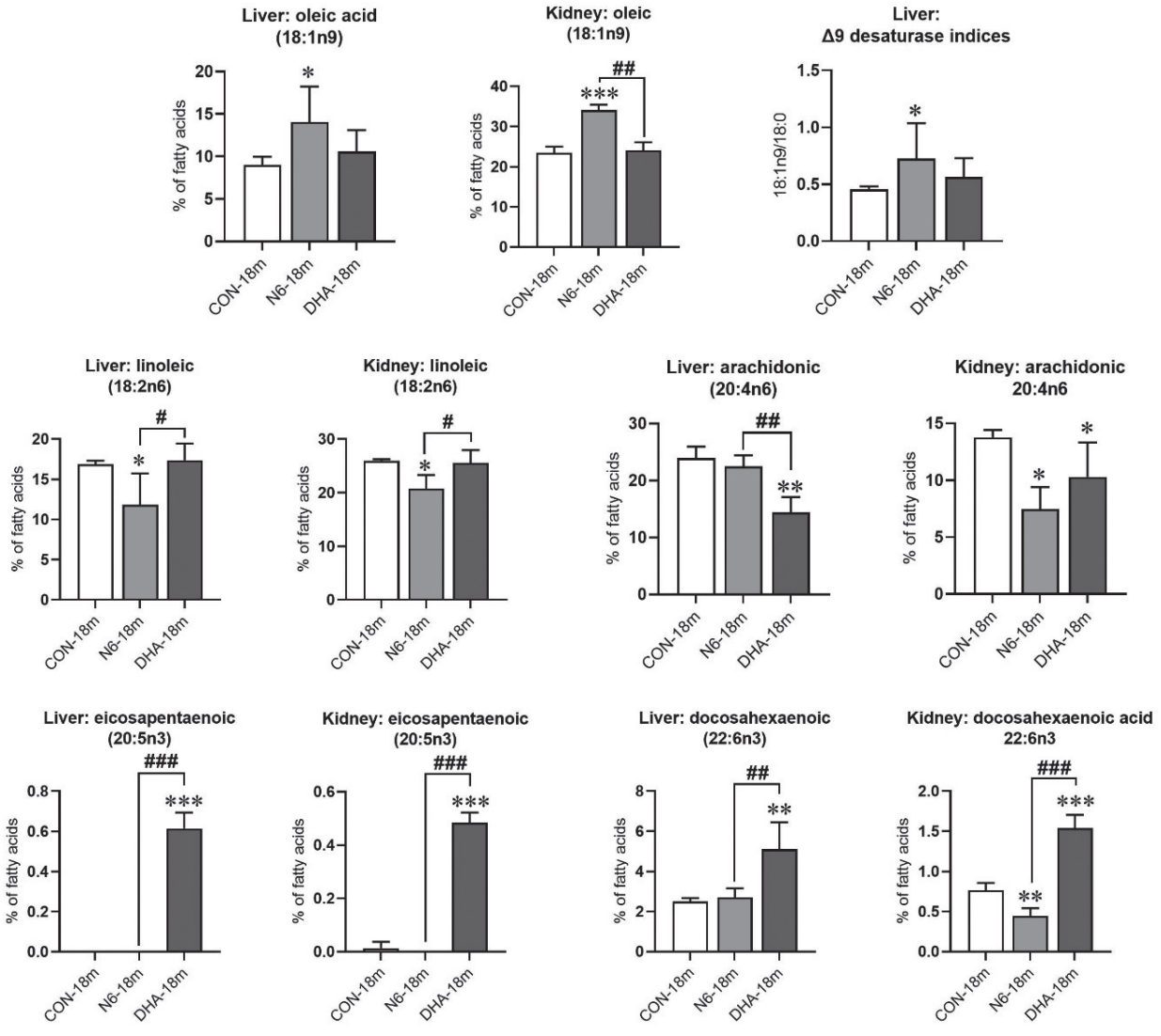

Fig. 2. The influence of long-term supplementation with different n3/n6 ratios on the fatty acid profile of the liver and kidney tissue

Values are means ± SD. *P<0.05, **P<0.01, ***P<0.001, for N6, and DHA versus the CON group and #P<0.05, ##P<0.01, ###P<0.001, for DHA versus the N6 group

In the liver tissue, we investigated the mRNA expression of Δ9 desaturase, transforming growth factor β (TGF β) and NRF2 (Fig. 3). Quantitative PCR revealed a significant increase in the Δ9 desaturase (steroyl-CoA desaturase-1) gene expression in the liver tissue in the N6 group compared to the CON and DHA groups (P<0.05) (Fig. 3). DHA/EPA supplementation in the diet showed a lowering effect on the TGF β expression in the liver tissue. The mRNA expression of NRF 2 decreased significantly in the N6 group in comparison with the CON group (P<0.05) in the liver tissue (Fig. 3). In contrast, the DHA group showed a significant increase in NRF 2 expression in comparison to the CON group.

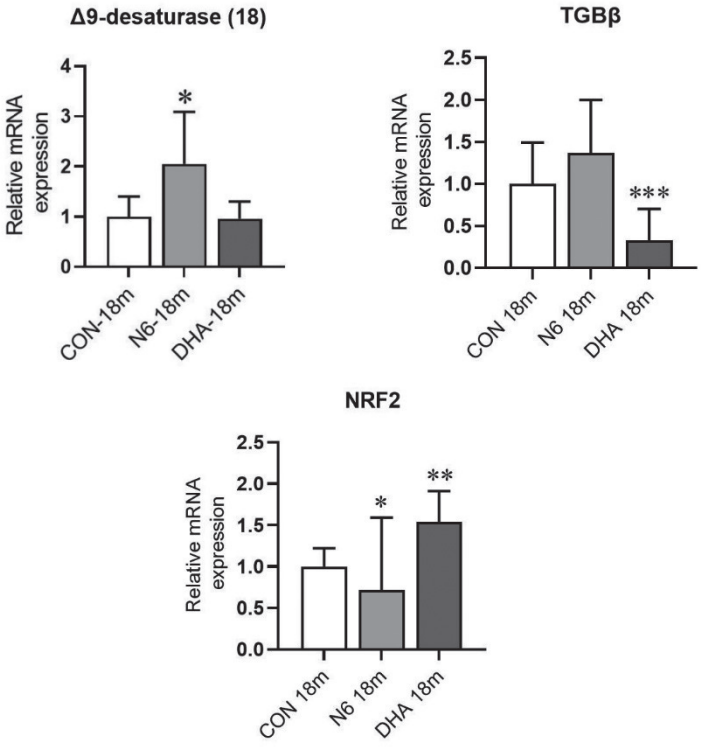

Fig. 3. The effects of treatment on mRNA expression of Δ9 desaturase, inflammation marker (TGFβ) and antioxidative response (NRF2) in the liver tissue

Values are means ± SD. *P<0.05, **P<0.01, ***P<0.001, for N6, and DHA versus the CON group and #P<0.05, ##P<0.01, ###P<0.001, for the DHA versus the N6 group

Discussion

The purpose of the present study was to assess the effect of long-term EPA/DHA supplementation on the fatty acid composition, inflammation, and oxidative stress markers in liver and renal tissue. The increase in lifespan leads to the emergence of chronic diseases, including those of the cardiac, immune, and nervous systems. In recent decades, the beneficial impact of EPA and DHA supplementation on the prevention of aging-related changes has been observed (VALENCAK and RUF, 2011). Our study included 18 month long EPA/DHA supplementation in order to study the effects of a long-term low dietary n6/n3 ratio.

Experimental rats fed a low n6/n3 ratio showed a significant reduction in body weight. Similar results were already documented from chronic feeding with a DHA-rich diet which reduced body weight (FELIX-SORIANO et al.; 2021, YANG et al., 2021). In our trial, we observed that long-term supplementation with a low n6/n3 fatty acid ratio (high quantities of DHA and EPA) changed the fatty

acid composition of liver and renal tissue. EPA/DHA supplementation decreased the content of oleic acid and Δ9 desaturase expression, indicating decreased *de novo* lipogenesis. This result supports the well-established role of PUFAs in the expression of Δ9 desaturase in rodents (HOFACER et al., 2012; MAŠEK et al., 2017; ROŠKARIĆ et al., 2021).

Oxidative stress is recognized as a trigger for the development of many different metabolic disorders which are related to a prolonged lifespan, because adaptive response to oxidative stress declines with aging (LIGUORI et al., 2018). Therefore, we studied the effect of prolonged feeding with EPA/DHA on nuclear factor erythroid 2–like 2 (NRF2). NRF2 is a transcriptional factor that has emerged as the most important cellular defense pathway against oxidative stress (CIGLIANO et al., 2019). The potential effect of long-term EPA/DHA supplementation on NRF 2 expression and overall antioxidative defense was visible in our trial, because supplementation increased NRF2 mRNA expression in the liver tissue. Therefore, slowing the aging process by regulating transcription factor NRF2 could be an interesting strategy to attenuate age-related diseases (BRUNS et al., 2015; ROŠKARIĆ et al., 2021).

The content of arachidonic acid (ARA) was significantly decreased in the liver and kidney tissue in the group supplemented with EPA/DHA. Arachidonic acid is a precursor for a variety of bioactive lipid molecules that have an important role in the development of inflammation. In contrast, EPA-derived eicosanoids are less inflammatory, therefore, the EPA/ARA ratio could be used as an indicator of inflammatory processes. EPA content was extremely low in 18-month-old rats (CON group), as well as in the group fed a high n6/n3 ratio (N6 group). The supplementation with EPA/DHA significantly improved the EPA/ARA ratio by increasing the level of EPA in the liver and kidneys.

From the obtained results it is evident that prolonged feeding with different PUFAs could also influence the expression of TGFβ, which is a pleiotropic cytokine that plays an important role in physiological tissue homeostasis, and various pathological conditions (TOMINAGA and SUZUKI, 2019). The decrease in TGFβ expression observed in the DHA/EPA supplemented group could be an indicator of the capability of DHA and EPA to attenuate low-grade inflammation, which is nowadays considered as a hallmark of many metabolic diseases and tumors (MICCADEI et al., 2016). The key mechanism of DHA and EPA's anti-inflammatory action is a decrease in the production of ARA-derived proinflammatory mediators, and an increase in the production of eicosanoids originating from EPA and DHA, as well as EPA and DHA-derived resolvins, protectins and maresins (DUVALL and LEVY, 2016). Additionally, DHA and EPA exert anti-inflammatory effects that are not directly related to eicosanoid production, such as: modulation of inflammatory gene expression (CHANDRASEKAR and FERNANDES, 1994; ROBINSON et al., 1996), their influence on the cell surface expression of adhesion molecules (DE CATERINA et al., 1994), and inhibition of the synthesis of inflammatory cytokines (LO et al., 1999).

Conclusions

The positive effects of n3 PUFA on health could be attributed to many different pathways. Our trial showed that among these different pathways, long-term n3 PUFA supplementation could substantially influence lipid metabolism, oxidation, and inflammation. Therefore, the long-term addition of dietary DHA and EPA could potentially influence the most important pathological processes in aging.

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SAŽETAK

Istraživali smo utjecaj dugotrajnog dodatka EPA i DHA u hrani na ekspresiju gena upalnog odgovora, oksidacijski status i lipogenezu. Mužjaci Wistar štakora podijeljeni su u tri skupine: kontrolnu skupinu (CON, omjer n6/n3 bio je ~7), skupinu N6 (omjer n6/n3 ~50) i DHA skupinu (n6/n3 ~1). Dodatak je znakovito utjecao na sastav masnih kiselina tkiva jetre i bubrega. Najznakovitija promjena vezana je uz povećanje koncentracije EPA i DHA u tkivu bubrega i jetre kod DHA skupine. Ekspresija TGFß smanjena je u tkivu jetre nakon dugotrajnog dodatka EPA/DHA. Takav učinak EPA i DHA dodatka u hrani povezuje se s njihovim pozitivnim utjecajem na sintezu protuupalnih eikozanoida. Dodatak DHA i EPA također je znakovito povećao ekspresiju NRF2 gena. Ovo otkriće sugerira da n3 PUFA može utjecati na aktivaciju NRF2 puta koji je važan u antioksidacijskoj obrani stanice. Rezultati su pokazali da dugotrajni dodatak DHA i EPA može utjecati na metabolizam lipida, upalu i antioksidacijsku obranu. Stoga bi dugotrajno dodavanje DHA i EPA u hrani moglo pozitivno utjecati na sprječavanje razvoja patoloških procesa tijekom starenja.

Ključne riječi: n6/n3 omjer; NRF2; DHA; lipogeneza; oksidacijski stres; starenje