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Maternal fish-oil supplementation reduces presenilin 1 level and the amyloid-beta burden in adult 5xFAD offspring without major changes in brain fatty acids

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Abstract: Omega-3 fatty acid interventions show potential benefits in Alzheimer's disease (AD) when initiated during its early stages. This study investigated whether maternal diet supplemented with omega-3-rich fish oil (FO) could delay or reduce amyloid beta (Aβ) formation, a key feature of AD, in 5xFAD transgenic offspring. Dams received FO during mating, pregnancy, and lactation. Brain tissues from female offspring were collected at 2 and 6 months of age. The findings indicated a shift in amyloid precursor protein processing, evidenced by increased soluble amyloid precursor protein α (sAPPα) levels, suggesting a transition from amyloidogenic to non-amyloidogenic pathway. FO influenced the expression of presenilin 1 and 2 but did not impact Aβ levels in 2-month-old mice. However, FO reduced the Aβ burden in the brains of 6-month-old animals. Lipidomic analysis revealed that 5xFAD mice have unimpaired omega-3 acquisition during gestation and lactation in comparison to non-transgenic littermates. However, a response to FO supplementation was found in non-transgenic offspring only, indicating that alterations in brain lipids are not the primary mechanism of FO-induced Aβ decline in 5xFAD. In conclusion, FO did not prevent or delay amyloid pathology in genetically predisposed animals but did mitigate its progression, suggesting mechanisms that warrant further investigation.

Keywords: fish oil; maternal treatment; brain fatty acids; omega-3; Alzheimer's disease

Abbreviations: AD – Alzheimer's disease; FAD – familial AD; APP – amyloid precursor protein; PSEN1, 2 – presenilin 1, 2; CTFα – C-terminal fragment alpha; BACE1 – β-secretase; PUFA – polyunsaturated fatty acids; DHA – docosahexaenoic acid; CNS – central nervous system; FO – fish oil

INTRODUCTION

Alzheimer's disease (AD) is a prominent age-associated neurodegenerative condition marked by a persistent progression of memory loss and cognitive decline. At present, this type of dementia affects about 50 million individuals, and it is projected that the number of patients with dementia will reach 115.4 million in 2050, which is a huge burden for society [1,2]. Most AD cases are sporadic and lack a clear genetic etiology, primarily affecting individuals aged 60 and above. Only a modest 5-10% of AD cases are inherited, linked to familial AD (FAD), and stemming from autosomal dominant genetic mutations in three key genes: amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2).

Under normal conditions, APP is cleaved via the non-amyloidogenic pathway by α-secretase, releasing membrane C-terminal fragment alpha (CTFα) and the larger, soluble APPα ectodomain. APP and its metabolites are pivotal in maintaining neuronal homeostasis, contributing to intracellular transport, signaling, and neuroprotection. In this non-amyloidogenic pathway, only a minor fraction of APPs undergo degradation into Aβ peptides by β- and γ-secretases [3]. However, in pathological scenarios, mutations in APP generate new enzymatic sites, rendering APP more susceptible

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to cleavage by β-secretase (BACE1), which initiates the amyloidogenic pathway [4]. Furthermore, mutations in PSEN1 and 2, the key components of the γ-secretase complex, exacerbate amyloidogenic APP proteolysis, leading to an elevated presence of the longer Aβ42 amyloid peptides at the expense of the shorter ones. Aβ42 peptide, considered more neurotoxic due to its enhanced propensity to misfold and subsequently form oligomers that accumulate into insoluble plaques, is a central player in the amyloid hypothesis of AD [5-7]. Accumulation of senile plaques is one of the defining hallmarks of AD pathology in the brain. These aggregates in the neuropil of the brain lead to dystrophy of the neurites, inflammation, and neuron cell death.

Modern human lifestyles and diets have been recognized as significant contributors to chronic and inflammatory non-communicable diseases that plague contemporary society [7]. One characteristic of the Western diet is the excessive intake of omega-6 fatty acids (n-6), which surpasses omega-3 (n-3) intake by a factor of 20. This dietary pattern has sparked extensive research on the role of polyunsaturated fatty acids (PUFA), particularly omega-3s. Within the omega-3 family, docosahexaenoic acid (DHA) has a crucial role. It is the most abundant in the central nervous system (CNS) and essential in regulating neuronal differentiation, neurite outgrowth, synapse formation, and photoreceptor biogenesis [8]. DHA is indispensable for neurological growth and development during fetal and early postnatal stages of life [8-11]. However, with the aging process and in age-related conditions, there is a notable decline in n-3 content in the brain [7]. Thus, post-mortem examinations of AD patients reveal lower levels of omega-3 fatty acids.

Experimental results from various transgenic animal models and *in vitro* studies have demonstrated that omega-3-rich fish oil (FO), as well as omega-3 or DHA supplements, can alleviate AD pathology by reducing amyloid plaque burden, tau pathology (another hallmark of AD), neurodegeneration, inflammation, and by enhancing neurogenesis and synaptic plasticity [12-20]. Regarding the effects of n-3 in the human population, strong epidemiological, preclinical, and *in vivo* evidence points to an inverse relationship between omega-3 intake and AD incidence, slower rates of cognitive decline, and a reduced risk of developing dementia [1,21, 22]. However, there are conflicting findings, as some clinical studies have failed to consistently demonstrate the direct benefits of FO and other DHA supplements in the prevention or treatment of AD [7,22,23].

As the main causes of contradictory results in studies investigating the effect of omega fatty acids in AD, the latest literature highlights two factors: (i) the untimely start of nutritive intervention and (ii) the source of the fatty acids for supplementation [24]. The timing of intervention comes from the fact that when a diagnosis is made based on symptoms, the disease has progressed to a phase that is difficult to treat and too late to prevent [25]. Emerging evidence suggests that omega-3 supplementation in the early stages, during mild cognitive decline, shows promise [26]. In line with this, the most recent epidemiological research involving middle-aged individuals underscores the significance of timely action, as omega-3 supplementation during younger years appears to safeguard the brain against a range of indicators of brain aging in both structure and function seen in middle age [21].

Regarding the origin of n-3, the primary source for humans is in the consumption of preformed forms of DHA from marine fish, algae, grass-fed poultry, eggs, meat, and dairy products. The conversion rate of alpha-linolenic acid, abundant in flaxseed and other plants, into DHA, is notably low and insufficient for maintaining brain function and structure [1, 21]. Due to contemporary dietary habits, supplementation with omega-3 in the form of oils becomes a necessity. Yet, the brain may have limited uptake of triglyceride-DHA found in fish oil supplements, which leads to innovative omega-3 molecules designed to mimic the natural forms of lipids as a potential therapeutic nutrient [1]. In infants, breast milk feeding is the most effective means of enhancing DHA levels, as maternal milk provides DHA and EPA in more complex structures than fish oil, with enhanced bioactivity and bioavailability [23,27].

To overcome these barriers, this study was designed to investigate the effects of maternal supplementation with n-3-rich fish oil during preconception, pregnancy, and lactation on the adult offspring in a 5xFAD transgenic mouse model. This approach affords a unique opportunity for early intervention and exploration of the potential of maternal FO diets, where DHA and other n-3 fatty acids are delivered in milk as a natural matrix. The study aimed to assess if such dietary modifications can influence transgene expression in

5xFAD mice and have lasting effects on AD-related markers in the brain or even postpone the onset of AD pathology. APP processing was examined at the onset of amyloid deposition in young, 2-month-old animals, while the brain fatty acid content was assessed in adult 6-month-old animals to determine whether changes in the neural membrane, where APP processing takes place, were associated with changes in amyloid beta level. 5xFAD is a widely used model that recapitulates many AD phenotypes and has an early and aggressive presentation. Soluble Aβ42 is detectable by 1.5 months, and its levels sharply increase with age. Such accelerated pathology is the consequence of 5 mutations in human APP and PSEN1 knock-in genes, as rodents normally do not develop Aβ plaques. Since females express slightly more APP than males, probably due to an estrogen response element in the Thy1 promoter used to drive transgene expression, and generate higher levels of Aβ [28], we conducted the studies on females. This is also in accordance with the incidence of AD in the human population, considering that AD is twice as common in women as in men [29].

MATERIALS AND METHODS

Ethics statement

In this study, we used 5xFAD mice (obtained from Jackson Laboratory, Bar Harbor, ME, USA) bearing five familial AD mutations in 2 human transgenes (3 in the APP gene; Sweden, Florida, US, and London, UK), and 2 in the PSEN1 gene (M146L and L286V) under the transcriptional control of the neuron-specific murine Thy-1 promoter. Amyloid plaques, accompanied by gliosis, are seen in mice as young as two months, and neuron loss and behavioral deficits begin around 6 months [28]. 5XFAD transgenic male mice were crossed with C57BL/6xSJL female mice, and genotyping was performed by PCR of tail DNA according to the supplier's protocol. The animals (3-4 mice per cage) were housed under standard conditions (23±2°C, 60-70% relative humidity, 12 h light/dark cycles, free access to food and water). All animal procedures followed the EU Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes that were approved by the Ethical Committee for the Use of Laboratory Animals (Resolution No. 01-06/13) of the Institute for Biological Research, University of Belgrade. Minimal numbers of animals were used, and all efforts were made to minimize animal suffering.

Experimental design

All animals were raised on standard rodent chow [18]. Mature females were administered 100 μL of commercial fish oil for human consumption (12% DHA, 18% EPA; Dietpharm Atlantic Group, Croatia) with a defined composition [18] one week before placement with a male in the same cage for mating. Supplementation was performed by oral gavage and lasted until weaning, 28 days after giving birth. Only female pups were taken for the experiment. At the end of the treatment (age of 2 and 6 months), animals were anesthetized (100 mg/kg, Ketamidor, Richter Pharma, Austria; 16 mg/kg Xylased, Bioveta, a.s., Czech Republic), killed and perfused with saline solution. The cortex, the brain structure among the first to be affected by amyloid pathology, served as the source of proteins, mRNA, and for enzyme-linked immunosorbent assay (ELISA). Halves of brains were used for phospholipid analysis to avoid the pooling of samples as higher amounts of tissue are required.

Lipid extraction and phospholipid analysis

For extraction of total lipids, one half of the brain was homogenized in 3 mL of chloroform/methanol (1:2 v/v) [18]. After centrifugation, the supernatants were collected and evaporated to dryness. The residues were dissolved in 3 mL of chloroform/methanol (2:1 v/v) and 3 mL of chloroform/methanol/KCl (4:2:1 v/v) and centrifuged to eliminate low molecular weight contaminants. After centrifugation, the upper layer was discarded, and the lower lipid layers were used for further analysis. During extraction, 2, 6-di-tert-butyl-4-methylphenol (BHT) was added to the solvents (10 mg/100 mL) to protect lipids from oxidation. The phospholipid fraction was isolated by one-dimensional thin-layer chromatography (TLC) on silica gel GF plates (Merck, Darmstadt, Germany) using a neutral lipid solvent system of hexane/diethyl ether/acetic acid (87:12:1 v/v), and methyl esters of PL fatty acids prepared by transmethylation.

Fatty acid methyl esters were separated by gas-liquid chromatography on a Shimadzu chromatograph GC 2014 (Shimadzu Co., Japan), equipped with a flame ionization detector and GC column Trace TR-Fame (100 m x 0.25 mm ID, film thickness 0.2 μm) (Thermo

Fisher Scientific, USA). The injection port was set at 220°C, and the flame ionization detector was set at 260°C. The oven temperature was programmed at 180°C for 15 min and then from 180°C to 240 °C at a heating rate of 1.8°C/min. The total separation period was 78 min. Individual fatty acid methyl esters were identified by comparing sample peak retention times with a PUFA standard mixture (PUFA-2 mix, Supelco, Thermo Fisher Scientific, USA). Phospholipid and fatty acid profiles were expressed as the relative percentage areas of total fatty acids.

Western blotting

For Western blot analysis, a whole cell extract was obtained by homogenization of cortical tissue in 10 vol (w/v) of RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 10 mM EDTA, pH 8.0, 10 mM EGTA, pH 7.2, 0.5% Triton X-100) that contained protease-phosphatase inhibitors (Roche Diagnostic, USA). Protein concentrations were determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Twenty micrograms of proteins were loaded per lane, electrophoresed on 10% polyacrylamide gels, and transferred onto an Immobilon-P membrane (Merck Millipore, Germany). After blocking in 5% non-fat dry milk dissolved in Tris-buffered saline/0.1% Tween in (TBST) for 1 h, membranes were incubated overnight at +4°C with primary antibodies: mouse anti-sAPPα (Chemicon International, USA), mouse anti-APP (BioLegend, USA), mouse anti-Pen 2, mouse anti-nicastrin, mouse anti-PSEN1 and anti-PSEN2 (all from Santa Cruz Biotechnology, USA) in TBST. After washing in TBST, blots were incubated with the anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA) in TBST for 1 h at room temperature. For the loading control, each membrane was re-probed with mouse anti-β-actin antibody (Sigma, Germany). The signal was then detected by enhanced chemiluminescence (ECL; Amersham Bioscience, USA) and subsequent auto exposure on the iBright CL750 Imaging System. Final analysis was conducted utilizing iBright Analysis Software (Thermo Fisher Scientific, USA). The signal intensity of the target bands was normalized to the corresponding β-actin lane. This normalization process involved calculating the average normalized ratio of the control samples, which was then used to determine the relative ratio of all samples. As a reference point, the average expression of the control group was set to 1, and the experimental group was evaluated in terms of fold difference relative to the control group.

Real-time quantitative PCR

Total RNA from cortical tissues was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). One microgram of RNA was reverse-transcribed with a High-Capacity cDNA Archive Kit (Applied Biosystems, USA). PCR reactions in a total volume of 10 μL were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and the default thermal cycling mode in ABI 7500 (Applied Biosystems, USA). The primer sequences were as follows: Mfsd2a, F5'-agaagcagcaactgtccattt-3', R5'- ctcggcccacaaaaaggataat-3', and Actb, F5'-tggacatccgcaaagacctgtac-3', R5'-tcaggaggagcaatgatcttga-3' [18]. Dissociation curves were analyzed at the end of the assay to confirm the specificity of qRT-PCR reactions. Relative mRNA levels were calculated using the comparative Ct method and expressed as relative values.

Human Aβ42 specific enzyme-linked immunosorbent assay (ELISA)

Levels of soluble (the PBS fractions) and insoluble Aβ42 (5 M guanidine fractions) were measured using sandwich ELISA as per the manufacturer's instructions (Cat. No. KHB3442, Invitrogen, USA). Briefly, 50 μL of guanidine or PBS fractions of brain homogenates were added to a 96-well plate containing Aβ detection antibody and incubated for 3 h at room temperature. After the washing step, the plate was incubated with 100 μL of anti-rabbit IgG HRP. After adding 100 μL of chromogenic solution to each well, the absorbance was measured at 450 nm, and the level of Aβ1-42 in each sample was normalized to the protein content.

Statistical analysis

Data were analyzed using the GraphPad Prism ver. 8.00 (GraphPad Software, USA). For the data obtained by Western blotting and qRT-PCR, the statistical significance was analyzed by the Mann-Whitney U-test. For analysis of the lipid profile, the normality of the distributions of values obtained for each

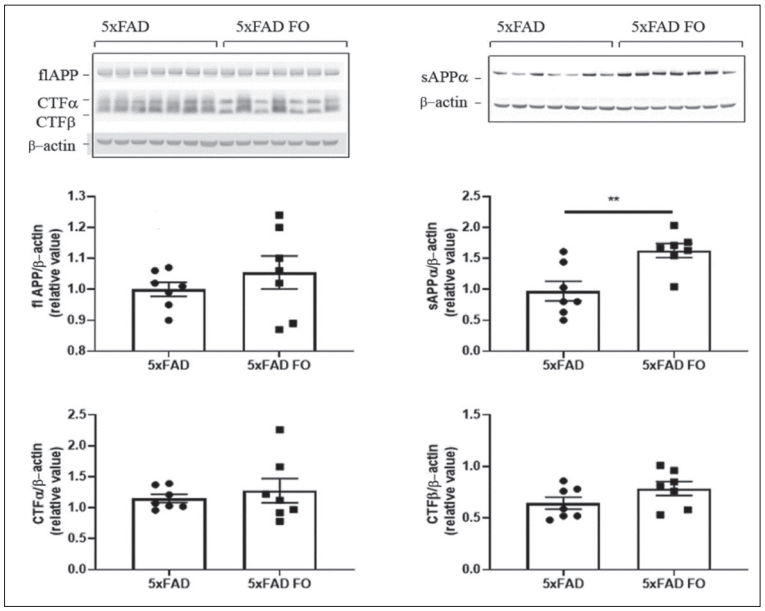

Fig. 1. The effects of FO maternal supplementation on APP expression and processing in the cortex of 5xFAD offspring. Representative Western blots and quantitative analysis of full-length APP (flAPP), C-terminal fragment α (CTFα), C-terminal fragment β (CTFβ), and soluble APPα (sAPPα). Values are presented as the mean±SEM. **P<0.01.

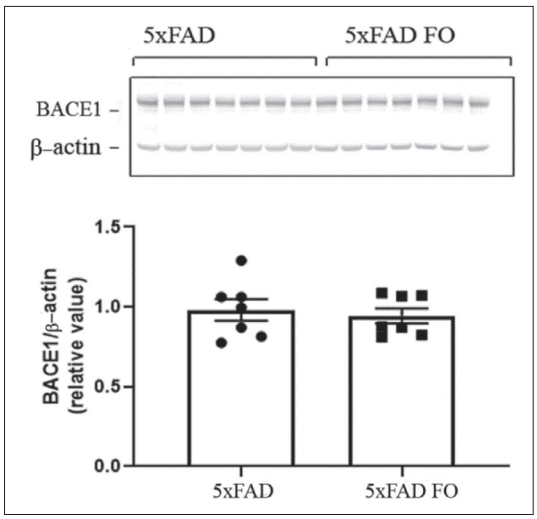

Fig. 2. Effects of FO maternal supplementation on BACE1 expression in the cortex of 5xFAD offspring. Representative Western blots and quantitative analysis of β-secretase expression (BACE1). Values are presented as the mean±SEM.

group/experimental treatment was determined using the D'Agostino-Pearson normality test. For multiple comparisons, two-way ANOVA (with the FO diet and genotype as factors), Tukey's post hoc test was used when the distribution was normal, while Kruskal-Wallis with Dunn's test was applied when the distributions were not Gaussian. All data are shown as the mean±standard error of mean (SEM). A P value of ≤0.05 was significant.

RESULTS

Maternal fish oil supplementation enhances the expression and processing of the APP in offspring

One of the defining characteristics of AD pathology is the proteolysis of APP protein, leading to the accumulation of Aβ peptides in the form of fibrillary plaques. To investigate whether perinatal maternal FO supplementation can delay or mitigate the emergence of AD pathology, we examined both the expression of full-length (fl) APP protein and its processing into Aβ peptides. Since the formation of the amyloid in the 5xFAD model begins at month 1.5 of age, we selected 2-month-old animals for our investigation to ensure that all animals had active transgenes and the initiation of plaque deposition. Western blot analysis revealed that maternal FO supplementation induced a significant increase (by 65%) in the soluble APPα fragment (P<0.01), which is a product of α-secretase activity in the non-amyloidogenic pathway (Fig. 1). However, the levels of the remaining membrane-bound C-terminal fragment α (CTFα), as well as the membrane fragment CTFβ, which originates from β-secretase cleavage in the amyloidogenic pathway, remained unchanged. These fragments travel at similar speeds, as illustrated in Fig. 1, and their mutual ratio and relationship with APP remained unaffected after treatment. We noted a slight but statistically non-significant increase in flAPP protein in the cortex of FO-supplemented 5xFAD mice.

Impact of maternal fish oil supplementation on β- and γ-secretase expression in offspring

In the context of amyloidogenic pathways in APP processing, examining the influence of maternal FO supplementation on β-secretase (BACE1) and the γ-secretase complex in young, 2-month-old offspring is crucial. Regarding BACE1 protein expression, there is no significant change in the offspring's brain following maternal FO supplementation (Fig. 2). There

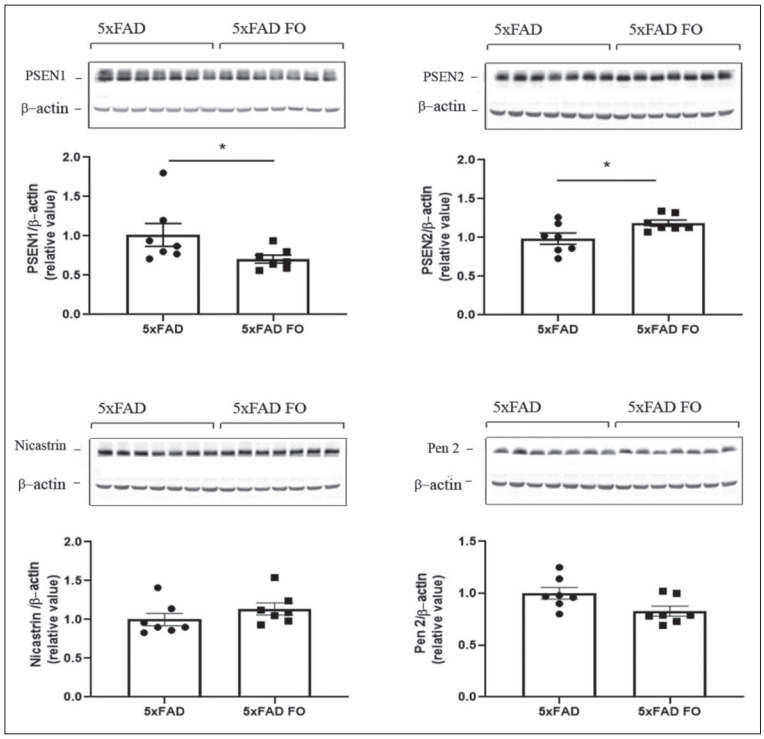

Fig. 3. Effects of the FO maternal supplementation on the expression of γ-secretase complex in the cortex of 5xFAD offspring. Representative Western blots and quantitative analysis of presenilin 1 and 2 (PSEN1, PSEN2), nicastrin, and presenilin enhancer 2 (Pen 2). Values are presented as the mean±SEM. *P<0.05.

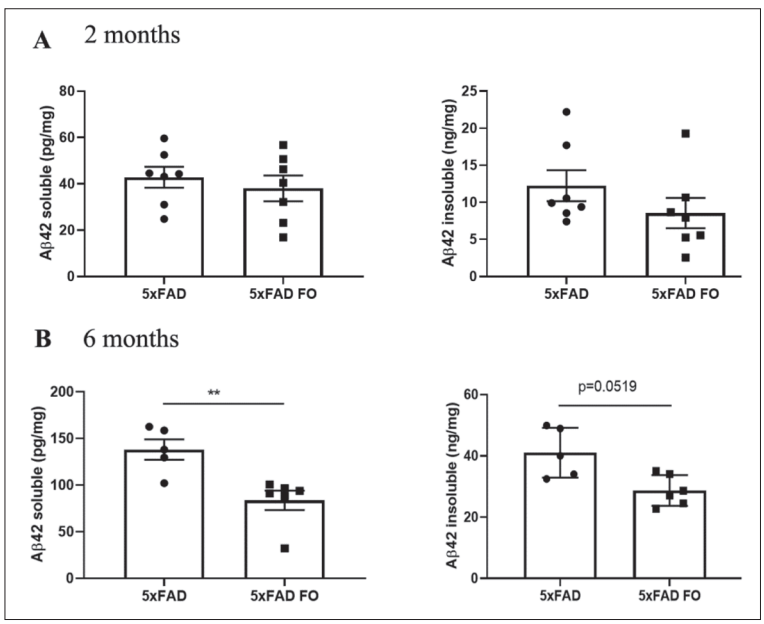

Fig. 4. Effects of the FO maternal supplementation on soluble and insoluble Aβ in the cortex of 2- and 6-month-old 5xFAD offspring. Values are presented as the mean±SEM. **P<0.01

were opposite changes in the levels of PSEN1 and PSEN2, with PSEN1 protein expression exhibiting a 20% decrease (P<0.05), while PSEN2 increased by 19% (P<0.01) after FO supplementation, as can be seen in Fig. 3. The expression levels of nicastrin and Pen2 proteins remained consistent after maternal FO treatment when compared to non-supplemented transgenic animals.

Maternal fish oil supplementation decreased the soluble and insoluble Aβ burden in 5xFAD offspring

In an effort to discern whether maternal FO supplementation could influence Aβ production and deposition in 5xFAD offspring, a sandwich ELISA assay was used to measure soluble Aβ as well as the insoluble one, found in the form of deposits in plaques.

Our findings indicate that long-term maternal FO supplementation did not alter the levels of either soluble or insoluble Aβ in 2-month-old offspring (Fig.4A). It's worth noting that the level of insoluble Aβ is approximately 100 orders of magnitude lower than that of the soluble Aβ, what can be attributed to the early stage of AD pathology in the 5xFAD animals at this age.

Based upon previous observations that a decrease in PS1 levels is strongly linked to Aβ reduction, particularly preceding changes in soluble Aβ, we conducted an assessment at a later time point-at the age of 6 months, nearly 5 months after the cessation of FO supplementation (Fig.4B). ELISA revealed that the 6-month-old offspring whose mothers received FO supplementation exhibited a 40% reduction in soluble Aβ levels (P<0.01) when compared to the non-supplemented group. As for insoluble Aβ, there was a 32% reduction, (P=0.0519). There was higher abundance of both species of Ab42 in 6-month-old mice when compared to their younger, 2-month-old transgenic counterparts as a consequence of disease progression.

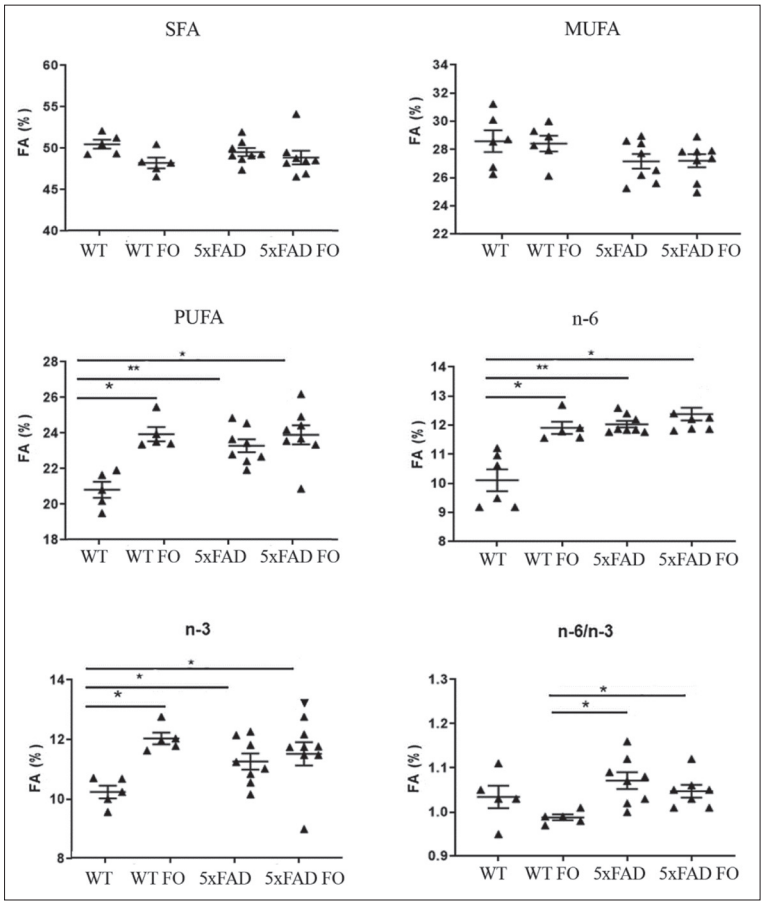

Fig.5. Effects of FO maternal supplementation on the fatty acid ratio in the brains of WT and 5xFAD adult offspring. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; n-6 – omega-6 polyunsaturated fatty acids – PUFA; n-3 – omega-3 polyunsaturated fatty acids. Values are presented as the mean±SEM. *P<0.05, **P<0.01.

The impact of maternal fish oil supplementation on phospholipid composition in offspring brains

PUFAs play a pivotal role in the composition of cell membranes in the CNS. The effects of omega-3 fatty acids, especially DHA, have been linked to alterations in neural membrane structure and fluidity, which lower the proximity of APP and β- and γ-secretase and lessen the cleavage of APP in lipid rafts [30]. We comprehensively analyzed brain phospholipids to investigate whether maternal n-3-rich FO treatment induces lasting changes in offspring. While no substantial alterations were detected in the individual or total sums of saturated (SFAs) and monounsaturated fatty acids (MUFAs), the concentration of PUFAs significantly increased following FO supplementation by 15% (P<0.05). This increase was observed exclusively in non-transgenic (wild-type, WT) animals, due to an elevation in both total n-3 (by 18%, P<0.05) and n-6 (by 18%, P<0.01) fatty acids (Fig. 5). Among the n-3 category, the levels of DPA and DHA exhibited the most notable increases (by 25%, P<0.05, and by 17%, P<0.05, respectively), while EPA remained unchanged (Fig. 6). The surge in n-6 levels was primarily attributed to adrenic acid, which exhibited a 29% increase (P<0.001) in WT offspring treated with fish oil, and arachidonic acid (AA), which showed a 15% elevation (albeit not statistically significant). In contrast, in 5xFAD animals, maternal FO treatment did not cause any changes in total SFAs and MUFAs or the levels of individual fatty acids when compared to 5xFAD control mice (Fig. 6). Of all PUFAs, the only change induced by FO in 5xFAD mice was the increase in DGLA level (28%, P<0.05) that did not significantly affect total n-6 and total PUFAs. Remarkably, the baseline total PUFA levels in non-treated 5xFAD mice were significantly higher (by 12%, P<0.05) than in non-treated WT animals (Fig. 5). This was due to elevated levels of linoleic (12%, P<0.05), n-6 adrenic (18%, P<0.01), and arachidonic (15%, P<0.001) n-6 fatty acids in the transgenic mice, as well as higher levels of n-3 DPA (by 25%, P<0.05) (Fig. 6).

Maternal FO supplementation has no impact on the omega-3 transporter Mfsd2a mRNA expression in offspring brain

Finally, we examined whether maternal FO supplementation influenced the expression of Mfsd2a, which was recently recognized as a pivotal transporter for omega-3 uptake in the brain [31]. At the transcriptional level, we observed no discernible alterations in Mfsd2a mRNA in either 6-month-old WT or 5xFAD animals following maternal FO supplementation (Fig.7). There was a slight increase in Mfsd2a expression in WT animals after FO supplementation, although it was not statistically significant. Notably, there appears to be a genotype difference, with 5xFAD non-treated animals

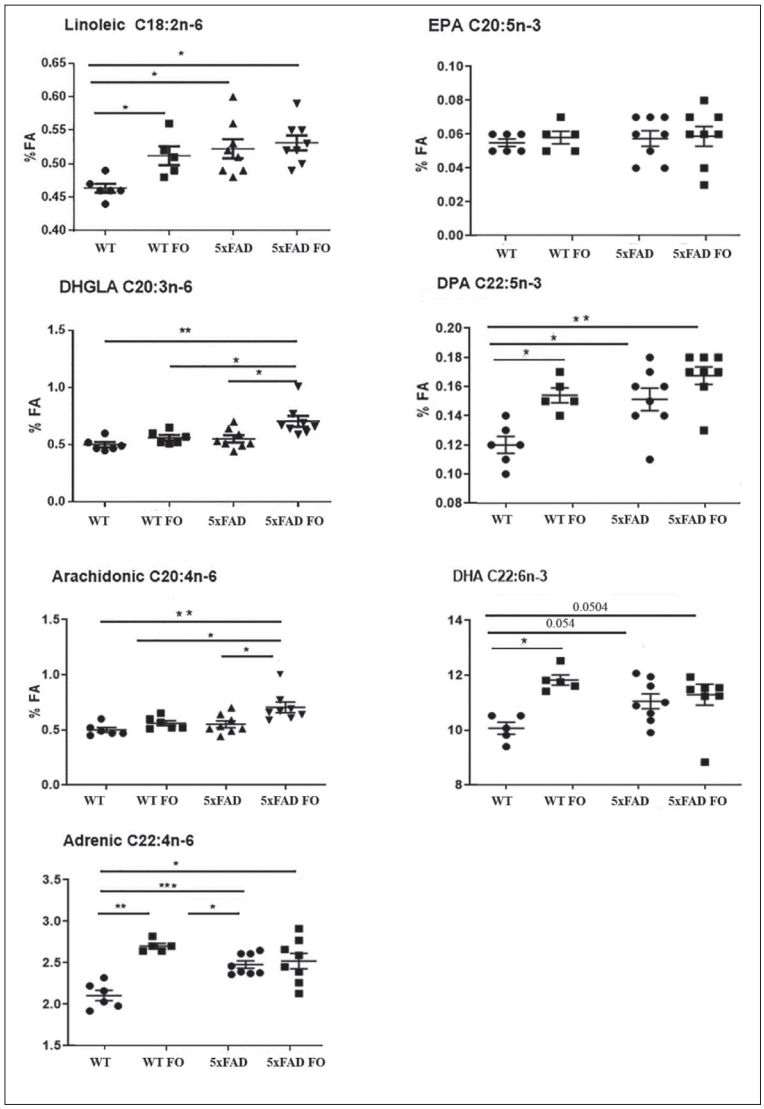

Fig.6. Effects of maternal FO supplementation on n-3 and n-6 PUFA levels in the brains of WT and 5xFAD mice. DHGLA – dihomo-γ linolenic acid; EPA – eicosapentanoic acid; DPA – docosapentanoic acid; DHA – docosahexanoic acid. Values are presented as the mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

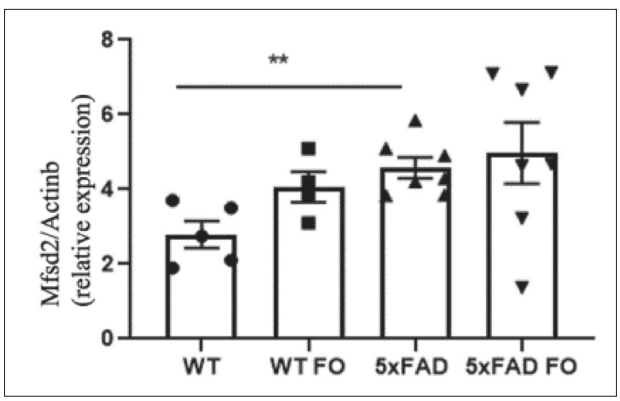

Fig.7. Effects of FO maternal supplementation on Mfsd2a mRNA in the cortex of adult 5xFAD offspring. Values are presented as the mean±SEM. **P<0.01.

exhibiting higher levels of Mfsd2a mRNA than WT non-treated animals (P<0.01).

DISCUSSION

In this research, we aimed to investigate the impact of long-term maternal FO consumption on amyloid beta pathology in adult female offspring using the 5xFAD mice model of AD. We sought to determine whether FO could delay the formation of Aβ and influence the expression of APP and its processing enzymes at the onset of AD. Our findings demonstrate that supplementation with FO during mating, pregnancy, and lactation enhanced non-amyloidogenic APP processing and reduced Aβ accumulation at later stages of AD. Analysis of brain fatty acids revealed that alterations in neuronal membrane composition might not be the primary mechanism by which FO supplementation exerts its beneficial effects since only minimal changes were observed in transgenic 5xFAD mice.

For our investigation, we utilized 5xFAD transgenic mice, a widely used animal model for AD due to its suitability for studying early diagnosis and treatment. However, this model presents a challenge for dietary interventions due to the early appearance of amyloid plaques at just 1.5 months of age [28,32]. It is widely acknowledged that early intervention is crucial for omega-3 treatment to display improvements in AD pathological markers in both animals and humans [2]. Also, recent studies have highlighted the importance of the source or the form of DHA or omega-3, suggesting that the therapeutic potential might be compromised by certain inappropriate forms that can affect brain uptake [33]. Thus, in our study, we deliberately administered omega-3-rich FO to dams during mating, pregnancy, and lactation to ensure that the mothers' reserves were adequately supplied with essential fatty acids, thereby facilitating the most effective and natural supplementation for the offspring.

Our research revealed that FO supplementation increased the production of soluble APPα generated by alpha-secretase in the

non-amyloidogenic APP cleavage pathway. Soluble APPα plays a neuroprotective role by competitively interacting with β-secretase, thereby preventing β-secretase-mediated APP degradation in the amyloidogenic pathway [2]. The shift from the amyloidogenic to the non-amyloidogenic pathway in 5xFAD mice induced by FO treatment aligns with similar effects observed with other natural products such as ginsenoside Rg1, hispidin, berberine, ligustilide, and polymethoxyflavones [34]. While our study showed an increase in α-secretase activity, the levels of CTF α and β residues in the plasma membrane after the shedding of soluble APP ectodomain by α- or β-secretase remained unchanged. Similar outcomes have been reported previously [35].

Inhibiting BACE is a widely investigated strategy for the prevention of APP cleavage; however, numerous BACE inhibitors tested in animal models have failed due to different adverse effects [2,4]. While some natural products like berberine or myricetin have shown promise by affecting BACE1[34] and both inhibiting its enzymatic activity and suppressing its expression, we did not observe any change in BACE1 expression after maternal FO treatment. Presenilins, components of the γ-secretase complex, are found downstream of BACE1 in the amyloidogenic APP pathway [4]. Our research notes that FO supplementation induced a distinct pattern of PSEN1 and PSEN2 protein expression in 2-month-old offspring. While activated PSEN1 typically cleaves CTFβ sequentially into shorter and shorter products, PSEN1 mutations can lead to an overall decrease in Aβ fragments or the generation of longer, more toxic fragments (Aβ42 *vs* Aβ40) that are prone to aggregation. The decline in PSEN1 observed in 5xFAD offspring agrees with previous findings suggesting that DHA and DPA ameliorate Aβ and Tau pathology by mechanisms involving reduced PSEN1 levels [12]. Interestingly, nicastrin and PEN-2 were not changed, implying that the effects of FO are specific to presenilin rather than affecting the whole γ-secretase complex.

PSEN2 is homologous in structure to PSEN1 and can also function as an APP protease but differs in substrate specificity and location [36]. PSEN2 modulates the Ca^{2+} shuttling between ER and mitochondria, regulating their physical interaction [37]. Therefore, elevated expression of PSEN2 may lead to an increase in ER/mitochondria contacts, impairing γ-secretase activity and reducing Aβ levels. This is in line with accumulating evidence suggesting that FAD is linked to an imbalance in cellular Ca^{2+} homeostasis [38]. Notably, while changes in presenilin levels did not affect the amount of soluble and insoluble Aβ measured by ELISA in the brains of 2-month-old offspring, they did have an impact on 6-month-old transgenic animals. This suggests that maternal FO supplementation may not affect the onset of the disease or the occurrence of amyloid pathology but could potentially affect the progression of the disease by reducing the occurrence of Aβ in later stages and thus possibly reducing the severity of symptoms.

Membrane-mediated changes are the most well-established and understood consequences of omega-3 administration. DHA replaces cholesterol in the membrane, thereby preventing the close proximity of APP and BACE1 in lipid rafts, which reduces amyloid-related processing [7,30]. Lipidomic analysis of brain tissue revealed that maternal FO supplementation induced changes in PUFA content in the neural membranes of offspring that persisted for months after the supplementation was discontinued; however, these changes were genotype-dependent. In non-transgenic littermates (WT animals), we observed significant elevations in n-3 (DPA and DHA) and n-6 linoleic, arachidonic, and adrenic fatty acids. In contrast, in transgenic 5xFAD brains, only DGLA, an n-6 fatty acid, exhibited a slight but notable increase. The substantial increase in PUFAs, particularly DHA, in WT animals is to be expected and is in agreement with our earlier findings following short-term supplementation, and consistent with other animal models or human studies [39-41].

There could be several reasons for the absence of maternal treatment effects on fatty acids in the 5xFAD transgenic brains. Firstly, genotype-specific differences in basal fatty acid content between 6-month-old 5xFAD and WT mice might have a role. Transgenic animals already exhibit higher basal levels of long-chain PUFAs, possibly reaching a threshold where further elevation could be detrimental to brains undergoing pathological changes and experiencing blood-brain barrier (BBB) breakdown [23]. Recent studies have emphasized mitochondrial dysfunction in AD, which generates reactive oxygen species (ROS), leading to lipid peroxidation and ongoing membrane damage [10,16,23,38]. PUFAs are highly susceptible to peroxidation, with

even a minor percentage of DHA oxidation potentially counteracting its protective effects [42]. Oxidized lipids have been linked to the exacerbation of AD pathology by the elevation of expression of components of the γ-secretase complex, BACE1 expression, and activity [42]. Therefore, maintaining a stable lipid profile in 5xFAD mice that already exhibit BBB leakage [43] might be a reasonable explanation, as excessive DHA incorporated into neuronal membranes could heighten vulnerability to oxidative stress.

Altered lipid metabolism and fatty acid uptake in the brain, observed in both animal models and human studies on AD brains, could also contribute to the lack of an effect of maternal supplementation in 5xFAD animals [30]. Thus, our prior study revealed increased omega-3 levels in the livers of both genotypes following FO supplementation, but the anticipated rise in the brains of 5xFAD mice was absent [18]. Transcriptomic analyses in 7-week-old 5xFAD brains compared to non-transgenic ones revealed differential gene expression that was related to inflammation and synaptic transmission [32,44]. Fatty acids incorporated in the plasma membranes of the cells are susceptible to cleavage by phospholipase and serve as precursor molecules involved in synaptic function and inflammatory pathways [30,45]. The lack of elevation of omega-3 in 5xFAD transgenic brains after FO supplementation might be due in part to the higher demand and turnover of omega-3 into anti-inflammatory and neuroprotective metabolites, which are essential for coping with ongoing neurodegeneration [45,46].

Regarding fatty acid uptake, aging and age-related pathologies like AD are associated with reduced absorption of n-3 PUFA, diminished transport across the BBB, and decreased conversion of PUFA precursors to long-chain PUFAs in the brain [1]. Mfsd2a, a major transporter of long-chain fatty acids to the brain and an essential component of the BBB, displays altered expression in AD and other pathological conditions [47,48]. In our study, we observed significantly higher levels of Mfsd2a transcripts in 5xFAD compared to WT mice, which is consistent with the elevated baseline DHA and DPA levels. This mRNA elevation may be a compensatory mechanism due to impaired passive transport of fatty acids, highlighting the potential importance of Mfsd2a-dependent transport in compromised BBB conditions [47].

In 5xFAD mice, after FO supplementation, a notable increase was observed only in DGLA, which is known for its anti-inflammatory and anti-proliferation roles in diseases [49-51]. DGLA is present in some plant oils and is consumed in extremely small amounts [50]. However, it is found in human milk, where it may serve as a reservoir for upstream AA synthesis, which, opposite to its well-known role in inflammation, has a trophic role in times of increased nutritional demand [51-54]. Deficiency of DGLA has been associated with aging, diabetes, cancer, and other pathologies [49,50]. Thus, the rise in DGLA might be linked to its increased availability in maternal milk following FO supplementation and could be beneficial in aiding 5xFAD mice in handling developing AD-like pathology.

Different routes of omega-3 fatty acid transport to the brain and their different chemical binding forms (triglycerides, phospholipids, esters) raise questions about their bioavailability and metabolic efficacy in AD [23,39,53]. Different forms of omega-3 PUFAs impact their metabolic fate and distribution into brain tissue [23]. However, this is less pronounced in more extended studies or under low dietary intake [23,53], suggesting that adipose tissue deposits of DHA can affect its homeostasis and act as a constant source for the brain [23]. The DHA incorporated in phospholipids is the superior form for brain and eye uptake, and this is precisely the form in which dietary DHA is transported to the placenta and breast milk [23,39,53]. Administering FO to dams during mating, pregnancy, and lactation thus enabled us to overcome the challenges concerning the omega-3 fatty acid form and the short time frame available for preventive interventions in the 5xFAD model.

This study has some limitations. Although maternal treatment is advantageous, we did not analyze the dams' lipid status, assuming that administering FO to dams would meet the maternal reserves of DHA and neonatal requirements [1,18,27]. Additionally, we focused solely on female mice, as they have pronounced AD pathology compared to males due to estrogen receptor binding sites in the Thy promoter, which drives the expression of knock-in genes [28]. However, future experiments should also include males to simulate the human population better. Overall, our study showed that FO supplementation can improve some markers of AD pathology. The development of cost-effective

and safe interventions, including the long-term use of omega-3 nutraceuticals, alone or combined with other nutrients, may represent a relevant strategy to postpone or alleviate the appearance of more severe symptoms in chronic disease.

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Data availability: Data underlying the reported findings have been provided as a raw dataset, which is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Milanovic%20et%20al_9386-Raw%20Dataset.xlsx

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