

Omega-3, -6, and -9 Fatty Acids Influence Platelet Activity under Psychological Stress

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Summary

Background. This study investigated the effects of unsaturated fatty acid (FA) supplementation and psychological stress on the total phospholipid FA composition and activation markers of platelets in a rat model.

Material and methods. Thirty healthy female Wistar rats were divided into three groups for two weeks: one group received ω -3+ ω -6 FAs without stress, another received

ω -9 FAs with stress, and the third received ω -3+ ω -6 FAs with stress. Supplementation provided 1.05 mg C18:1 ω -9, 0.62 mg C18:3 ω -6, 5.74 mg C20:5 ω -3, and 1.79 mg C22:5 ω -3 per 100 g of body weight. All animals had unrestricted access to food and water.

Results. The results indicated that C22:6 ω -3 FA levels (%) were significantly higher in the stress-free ω -3+ ω -6 group than in the stressed ω -3+ ω -6 group (Mdn. 0.84 vs. Mdn. 0.32, $p = 0.049$). Malondialdehyde levels (μ g/L) were also elevated in the stressed ω -3+ ω -6 group relative to the stress-free group (Mdn. 563.69 vs. Mdn. 493.42, $p = 0.049$). Furthermore, platelet-granulocyte aggregate formation (%) was higher in the stressed ω -3+ ω -6 group than in the stressed ω -9 group (Mdn. 5.90 vs. Mdn. 2.95, $p = 0.037$).

Conclusions. These findings suggest that psychological stress modifies platelet membrane FA composition and may enhance platelet activation and oxidative stress, regardless of FA supplementation status.

Keywords: fatty acids, platelet activation, stress.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential components of a balanced diet that play a critical role in human and animal growth and development. Recently, there has been increasing scientific interest in the impact of dietary unsaturated fatty acids (FAs) on platelet membrane composition, platelet function, and the risk of thrombosis [1, 2].

Among these, omega (ω)-3 and ω -6 PUFAs are well known for their cardioprotective effects. Supplements rich in these FAs have long been used to slow the progression of cardiovascular diseases (CVDs) and reduce associated complications [3]. These FAs are readily incorporated into cell and platelet membranes [4]. A higher dietary intake of α -linolenic acid (C18:3 ω -3) promotes the synthesis of eicosapentaenoic acid (C20:5 ω -3) and docosahexaenoic acid (C22:6 ω -3), which are two key FAs that help maintain physiological haemostasis and possess antiplatelet and anticoagulant properties [5].

In addition to cardiovascular benefits, PUFAs influence immune function by forming platelet-leukocyte aggregates (PLAs). PLAs play a dual role: they activate inflammation by

facilitating receptor binding, promoting leukocyte chemotaxis, and contributing to thrombosis [1, 6]. Given their involvement in inflammation and thrombosis, PLAs may serve as valuable biomarkers for predicting and diagnosing diseases.

Importantly, ω -3 PUFAs have been linked to mental health and cognitive function, mainly because of C20:5 ω -3 and C22:6 ω -3 roles. These FAs are abundant in brain lipids, maintain membrane fluidity, and regulate neurotransmitter signalling pathways, including the dopaminergic and serotonergic systems. Their reduction in cell membranes has been implicated in the development of inflammation, autoimmune disorders, and neuropsychiatric conditions [7–9].

In the oxidative stress (OS) context, PUFAs, particularly long-chain variants, are susceptible to peroxidation. One of the key by-products of this process is malondialdehyde (MDA), a widely used and reliable marker of lipid peroxidation and OS [10, 11]. MDA, formed through non-enzymatic reactions, can interact with biomolecules, such as proteins and DNA, disrupting their structure and function. Elevated MDA levels have been associated with various pathological conditions, including acute myocardial infarction, atherosclerosis, and diabetes [12, 13].

Given the interconnected roles of PUFAs in platelet function, inflammation, OS, and mental health, it is crucial to investigate how these effects are influenced by psychological stress, which affects both the cardiovascular and immune systems. In this experiment, a model of chronic mild psychological stress was employed by repeatedly rotating cages. The exposure period was set at two weeks to simulate a brief yet continuous stressor, sufficient to induce measurable physiological changes without causing overt harm. This method is a standard model for inducing stress in animal studies. Therefore, the present study aimed to evaluate the impact of unsaturated FA supplementation on platelet membrane composition and biomarkers of platelet activation under psychological stress.

MATERIAL AND METHODS

Ethics statements

The protocol for rodent use was obtained from the State Food and Veterinary Service, the organisation responsible for ethics approvals for animal studies in Lithuania (Approval No. G2–160).

Subjects of the study

The experimental study randomly selected 30 mature (6-month-old) healthy female Wistar rats (weighing $244 \text{ g} \pm 16.9 \text{ g}$). Animals were housed in cages (cage dimensions: 800 cm^2 floor area, $\geq 250 \text{ cm}^2$ per rat, 18 cm height) of three rats each under standard conditions (i.e., room temperature $24^\circ\text{C} \pm 2$, light/dark cycle: 12/12 h, relative humidity: 40–60%). The experimental animals had free access to food and water. The cages and bedding were replaced weekly. The Department of Biological Models of the Institute of Biochemistry at the Life Sciences Center of Vilnius University provided the studied animals.

Study design

For the first 2 weeks, the study animals ($n = 30$) were subjected to an adaptation period with standard laboratory rodent chow (LLC Joniškio grūdai, product code: JE-83003920). The chow consisted of approximately 23% protein, 6.0% fat, 3.73% fibre, and 7.36% ash, and met all rodent nutrient requirements. After adaptation, the animals were randomly divided into three groups of 10 rodents each, which were socially housed with three animals per cage and treated under different experimental conditions for 2 weeks.

In the first group of rats ($n = 10$), a daily oral dose of a combined fish and evening primrose oil supplement (Equazen, Vifor Pharma Ltd., Wigan, WN05 0JZ, United Kingdom) was administered alongside standard chow. The dose was $30.8 \text{ } \mu\text{L}$ per 100 g of body weight, delivering 5.74 mg C20:5 ω -3, 1.79 mg C22:5 ω -3, and 0.62 mg γ -linolenic acid (C18:3 ω -6) per 100 g of body weight. These rodents were not subjected to any psychological stress.

In the second group ($n = 10$), rats received a daily oral dose of extra virgin olive oil (Carletti, Antico Frantoio Carletti, Italy) and standard chow. The administered volume

was $10 \text{ } \mu\text{L}$ per 100 g body weight, providing approximately 1.05 mg oleic acid (C18:1 ω -9) per 100 g. Additionally, this group was exposed to psychological stress by varying the number of rodents in the cages daily. On the first day, five rats were housed together; on the second day, they were separated, with one rat per cage. On the third day, five rats were housed together again, and on the fourth day, they were separated. Standardising psychological stress induction in laboratory animals ensures consistency and reliability in the results of research studies. Therefore, a well-established stress induction method was validated in previous studies by altering the number of laboratory animals in each cage daily [14]. This procedure lasted for 2 weeks, and this group served as the control group for comparison.

In the third group ($n = 10$), rats received, in addition to standard laboratory chow, a daily oral dose of $30.8 \text{ } \mu\text{L}$ per 100 g of body weight of a combined fish and evening primrose oil supplement (Equazen, Vifor Pharma Ltd., Wigan, WN05 0JZ, United Kingdom). This dose provided approximately 5.74 mg C20:5 ω -3, 1.79 mg C22:5 ω -3, and 0.62 mg C18:3 ω -6 per 100 g. These animals were also subjected to psychological stress for 2 weeks by varying the number of rats housed in a cage, as described for the second group of animals.

At the end of the two-week experimental period, blood samples were collected from all animals to determine the composition of platelet membrane FAs (%), MDA concentration ($\mu\text{g/L}$), and PLA formation (%).

Sample collection

Blood samples of all rats ($n = 30$) were collected in microvettes (BD Microtainer® Blood Collection Tubes, BD Biosciences, USA) by a terminal cardiac puncture with a 20 G needle. To determine the MDA concentration in the serum, rat samples were collected in a microvette using a coagulation activator without gel (total single-sample volume: $200 \text{ } \mu\text{L}$). Whole blood samples for platelet and leukocyte aggregate (PLA) measurements were collected in a microvette with ethylenediaminetetraacetic acid dipotassium salt (total single-sample volume: $200 \text{ } \mu\text{L}$). To determine the composition of platelet membrane FAs, blood samples were collected in a microvette with lithium heparin (total single-sample volume: $400 \text{ } \mu\text{L}$). The total volume of blood collected from a single rat was $800 \text{ } \mu\text{L}$. Before the terminal blood drawing procedure, all rats were subjected to a gas mixture comprising 75% CO_2 and 25% O_2 within an induction chamber to induce unconsciousness [15]. After blood collection, cervical dislocation was performed to ensure humane euthanasia of the animal.

Assays

Determination of platelet activity in whole blood

The formation of PLAs (i.e., platelet and monocyte aggregates [Mo_PLTag], platelet and neutrophil aggregates [Gra_PLTag], and platelet and lymphocyte aggregates [Ly_PLTag]) was determined using flow cytometry (BD FACS Canto, BD Biosciences, USA). Whole blood samples were delivered to the laboratory within 10 min of blood collection.

Monoclonal antibodies were used to evaluate PLAs: BD Pharmingen™ monoclonal antibody against rat CD45 (clone: OX-1), labelled PE (CD45 PE anti-rat, clone: OX-1), and BioLegend monoclonal antibody against mouse/rat CD61 (clone: 2C9.G2 (HMβ3-1)), labelled with APC (CD61 APC anti-mouse/rat, clone: 2C9.G2 (HMβ3-1)).

The identification of PLAs was based on the expression characteristics of fluorescein-labelled antibodies. Leukocyte subpopulations (i.e., monocytes, neutrophils, and lymphocytes) were gated based on the CD45 and side scatter light (SSC) scatterplot, depending on the expression of CD45/SSC: neutrophils (CD45 +, SSC – high side scatter light), monocytes (CD45 +, SSC – moderate side scatter light), and lymphocytes (CD45 ++, SSC – low side scatter light). The expression of the platelet marker CD61 in these leukocyte subpopulations was measured, and the percentage of positive cells in the population was calculated.

Data analysis was performed using BD FACS Diva software (version 6.1.2). The results of the experimental study were presented as percentages (estimating the proportion of the population that expressed the marker) and mean fluorescence intensity (how strongly the platelet population expressed the marker).

Determination of platelet membrane fatty acids

For platelet isolation, a microvette containing sodium heparin was centrifuged at $3000 \times g$ for 10 min. Three-quarters of the plasma was removed without touching the cell and buffy coat layers. The remaining platelet-rich portion (i.e., one quarter) was separated and mixed with freezing medium (NutriFreez® D10, Biological Industries, Israel Beit Haemek Ltd.) in a ratio of 2:1 and frozen at -80°C .

Flow cytometry and anti-CD31 and anti-CD42a antibodies were used to verify the platelet-rich fraction. The results showed that over 90% of the captured cells were of platelet origin.

After thawing at room temperature, platelet membrane lipids were extracted using the Folch method [16]. To obtain platelet membrane phospholipids, one-dimensional thin-layer chromatography (Sil G-25 UV 254, MACHEREY-NAGEL GmbH & Co. KG, Germany) was performed [17] using standards for phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (16:0 PC, Avanti Polar Lipids, Inc., USA; 16:0 PE, Avanti Polar Lipids, Inc., USA; 18:1 PS, Avanti Polar Lipids, Inc., USA). After transesterification according to the Folch method, gas chromatography-mass spectrometry (GC-MS) was performed using a GC-MS-QP2010 Ultra (Shimadzu) analyser to identify the FA methyl esters. The GC oven temperature program was as follows: the initial temperature was set at 80°C and held for 1.0 min, followed by a ramp of $6^\circ\text{C}/\text{min}$ to 310°C , where it was held for 5.0 min. The obtained data were collected and processed using LabSolutions software (Shimadzu, version 4.52). FAs were identified through comparative analysis with a certified standard mixture (Supelco 37 Component FAME Mix, Sigma-Aldrich Chemie GmbH, Germany). This process involved mass spectral matching utilizing the NIST Mass Spectral Library, complemented by manual spectrum confirmation using reference data from

LipidWeb (<https://www.lipidmaps.org/resources/lipidweb/index.php?page=ms/methylesters.htm>).

In this experimental study, 13 FAs were detected and identified in the rat platelet phospholipid membranes. The percentage of each FA was calculated from the total FAs (on a 100% basis). The study calculated total sums of saturated FAs (Sum SFAs%) in percentage (i.e., C14:0, C16:0, C18:0), monounsaturated FAs (Sum MUFAs%) (i.e., C16:1 ω -7, C18:1 ω -9, C18:1 ω -7, C20:1 ω 9), PUFAs (Sum PUFAs%), as well as total ω -3 (i.e., C18:3 ω -3, C20:5 ω -3, C22:5 ω -3, C22:6 ω -3) and ω -6 (i.e., C18:2 ω -6, C20:4 ω -6) FAs separately. FA ratios were also calculated: ω -3/ ω -6, C18:2 ω -6/C20:4 ω -6, C18:3 ω -3/C20:5 ω -3, C20:4 ω -6/C20:5 ω -3.

Determination of malondialdehyde in blood serum

The concentration of MDA ($\mu\text{g}/\text{L}$) in rat blood serum was determined according to the methodology published by Khoschsorur *et al.* [18] for forming MDA and thiobarbituric acid derivatives (TBA-MDA). The TBA-MDA concentration was measured using a Shimadzu Nexera X2 ultra-high-performance liquid chromatography system (Shimadzu, Japan). A calibrator (ClinCal, RECIPE, Germany) and controls (ClinCheck levels 1 and 2, RECIPE, Germany) were used for the MDA analysis. Chromatographic separation was achieved using an Ascentis® Express C18 (4.6×150 mm, 90°A , $5 \mu\text{L}$) analytical column (Supelco, USA). Fluorimetric detection was performed by excitation at 515 nm and emission at 551 nm. The retention time of the MDA-TBA adduct was 4.77 min. The collected data were processed using the LabSolutions software (Shimadzu, Japan).

Statistical analysis

Statistical analyses were performed using the R Studio and R Commander software packages. MS Excel 2019 was used for the graphical representation of the analysis. As the sample size in each group was 10 rats and the total number of subjects was 30, non-parametric tests were applied. Shapiro–Wilk's test was also applied for normality, and Fligner–Killeen's test was used for homogeneity of variance. The dataset was not normally distributed, but the variances were homogeneous.

The Kruskal–Wallis test was used to determine the differences in median values among the three experimental groups. It served as a nonparametric alternative to the one-way analysis of variance because of the non-normal distribution of the data. The Wilcoxon rank-sum test was used as a post-hoc test to determine the differences between groups. Correlation analysis was performed using a non-parametric Spearman correlation coefficient to obtain more accurate correlations between the platelet membrane FA composition, percentage of PLA formation, and blood serum MDA concentration. A 0.6 (–0.6) correlation indicates a moderate positive (negative) correlation. A correlation of 0.7 (–0.7) indicates a strong positive (negative) correlation [19].

Data in this study are presented as median, minimum, and maximum values, and as an interquartile range (or interquartile range/2, where specified for visual clarity). Differences were considered statistically significant if the p-value was less than 0.05.

RESULTS

In analysing the results of SFAs in rat platelet phospholipid membranes, regardless of the obtained PUFA supplements, more C16:0 was observed in the first group than in

the second and third groups, where animals were subjected to psychological stress ($p = 0.020$; $p = 0.004$). A tendency of decreasing Sum SFAs% was observed when comparing the first group with the third (median 82.120 vs. median 74.060, $p = 0.064$) (Table 1).

Table 1. Levels of saturated fatty acids in rat platelet phospholipid membranes under different experimental conditions

Fatty acids (percentage of the total amount)	Median, minimum, maximum, interquartile range	I group ω -3 and ω -6 Stress-free (n=10)	II group ω -9 Stress (n=10)	III group ω -3 and ω -6 Stress (n=10)	P value
C14:0*	Med.	5.845	4.625	5.485	& 0.152
	Min.	4.020	2.220	4.220	
	Max.	9.230	6.420	7.440	
	IQR	1.855	1.680	1.545	
C16:0	Med.	52.850	47.560	46.660	§ I vs. II = 0.020 I vs. III = 0.004 II vs. III = 0.700
	Min.	46.820	22.520	41.130	
	Max.	57.490	53.140	56.210	
	IQR	1.038	11.478	6.285	
C18:0	Med.	22.880	25.460	21.840	& 0.185
	Min.	16.240	14.470	16.070	
	Max.	26.160	31.020	25.790	
	IQR	1.205	3.823	6.628	
Sum SFAs%	Med.	82.120	79.620	74.060	§ I vs. II = 0.322 I vs. III = 0.064 II vs. III = 1.000
	Min.	72.910	39.970	63.300	
	Max.	85.460	85.030	88.320	
	IQR	3.438	13.763	11.275	

Med. – median, Min. – minimum, Max. – maximum, IQR – interquartile range. *CX: Y – CX indicates the number of carbon atoms, Y indicates the number of double bonds. &Kruskal-Wallis test. §Wilcoxon rank-sum test. Sum SFAs% – the sum of saturated fatty acids.

There was no statistically significant difference in the levels of MUFAs in the platelet phospholipid membrane among the groups where rats were fed ω -3, ω -6, or ω -9 supplements, regardless of whether psychological stress was applied (Table 2). However, the Sum MUFAs% was higher

in the third group, where the animals received additional ω -3 and ω -6 and were subjected to psychological stress, than in the first group, where the rats received ω -3 and ω -6 without stress ($p = 0.037$). A similar trend was observed for C18:1 ω -9 ($p = 0.065$).

Table 2. Levels of monounsaturated fatty acids in rat platelet phospholipid membranes under different experimental conditions

Fatty acids (percentage of the total amount)	Median, minimum, maximum, interquartile range	I group ω -3 and ω -6 Stress-free (n=10)	II group ω -9 Stress (n=10)	III group ω -3 and ω -6 Stress (n=10)	P value
C16:1 ω -7*	Med.	2.005	1.550	2.650	& 0.107
	Min.	1.210	0.750	1.340	
	Max.	4.600	3.980	5.790	
	IQR	1.150	1.343	1.665	
C18:1 ω -7	Med.	1.320	1.300	2.235	& 0.168
	Min.	0.310	0.610	0.580	
	Max.	1.960	3.450	4.290	
	IQR	0.748	1.368	1.853	
C18:1 ω -9	Med.	3.435	5.165	7.585	§ I vs. II = 0.160 I vs. III = 0.065 II vs. III = 0.846
	Min.	1.670	2.260	4.030	
	Max.	11.100	19.750	16.980	
	IQR	3.358	4.490	5.668	
C20:1 ω -9	Med.	1.410	1.660	1.335	& 0.251
	Min.	0.590	0.700	0.150	
	Max.	3.670	21.010	14.020	
	IQR	1.138	5.423	1.268	
Sum MUFAs%	Med.	9.145	11.220	14.810	§ I vs. II = 0.322 I vs. III = 0.037 II vs. III = 0.922
	Min.	6.970	5.570	6.100	
	Max.	17.200	42.630	26.250	
	IQR	1.715	9.690	9.015	

Med. – median, Min. – minimum, Max. – maximum, IQR – interquartile range. *CX: Y ω -Z – CX indicates the number of carbon atoms, Y indicates the number of double bonds, and ω -Z indicates the fatty acid of the omega family. &Kruskal-Wallis test. §Wilcoxon rank-sum test. Sum MUFAs% – the sum of monounsaturated fatty acids.

In the PUFA analysis, a statistically significant increase in C22:6 ω -3 was observed in the platelet phospholipid membranes of the first group, where experimental animals received additional ω -3 and ω -6 FAs without exposure to stress, compared to the third group, which received the same supplementation but was also subjected to psychological stress ($p = 0.037$) (Table 3). The levels of C20:5 ω -3 and C22:5 ω -3 decreased in the platelet membranes under stress ($p = 0.084$). In the second group, where the animals received ω -9 FAs and were subjected

to psychological stress, C20:5 ω -3 levels were comparable to those in the first group and higher than those in the third group ($p = 0.084$), with a similar tendency observed for C22:5 ω -3 ($p = 0.081$). Additionally, ω -6 FA levels were higher in the second group than in the first and third groups ($p = 0.065$ and $p = 0.322$, respectively) (Table 3). Furthermore, the ratio of C18:3 ω -3 to C20:5 ω -3 was significantly higher in the third group than in the second group, which also received ω -3 and ω -6 supplementation under psychological stress ($p = 0.049$) (Fig. 1).

Table 3. Levels of polyunsaturated fatty acids in rat platelet phospholipid membranes under different experimental conditions

Fatty acids (percentage of the total amount)	Median, minimum, maxi- mum, interquartile range	I group ω -3 and ω -6 Stress-free (n=10)	II group ω -9 Stress (n=10)	III group ω -3 and ω -6 Stress (n=10)	P value
C20:4 ω -6*	Med.	1.005	1.250	0.935	& 0.603
	Min.	0.580	0.290	0.300	
	Max.	2.560	7.550	3.140	
	IQR	0.615	0.915	0.945	
C20:5 ω -3	Med.	0.690	0.740	0.285	§ I vs. II = 0.557 I vs. III = 0.084 II vs. III = 0.084
	Min.	0.230	0.230	0.140	
	Max.	1.960	2.150	1.250	
	IQR	0.405	0.503	0.115	
C22:5 ω -3	Med.	0.605	0.350	0.455	§ I vs. II = 0.081 I vs. III = 0.286 II vs. III = 0.770
	Min.	0.170	0.060	0.110	
	Max.	1.870	1.010	0.990	
	IQR	0.628	0.393	0.450	
C22:6 ω -3	Med.	0.835	0.425	0.320	§ I vs. II = 0.103 I vs. III = 0.049 II vs. III = 0.813
	Min.	0.280	0.080	0.150	
	Max.	1.440	0.810	1.160	
	IQR	0.660	0.315	0.408	
ω -3	Med.	3.745	2.735	2.660	& 0.3944
	Min.	1.480	1.410	0.760	
	Max.	4.720	7.880	13.070	
	IQR	0.723	1.770	2.388	
ω -6	Med.	5.300	7.115	5.760	§ I vs. II = 0.065 I vs. III = 0.275 II vs. III = 0.322
	Min.	2.860	2.590	3.940	
	Max.	9.820	13.610	10.250	
	IQR	1.183	2.468	3.555	
Sum PUFAs%	Med.	8.900	10.855	9.300	& 0.801
	Min.	7.570	5.070	5.580	
	Max.	11.300	17.400	18.670	
	IQR	1.373	7.013	3.830	

Med. – median, Min. – minimum, Max. – maximum, IQR – interquartile range. *CX: Y ω -Z – CX indicates the number of carbon atoms, Y indicates the number of double bonds, and ω -Z indicates the fatty acid of the omega family. &Kruskal–Wallis test. §Wilcoxon rank-sum test. Sum PUFAs% – the sum of polyunsaturated fatty acids.

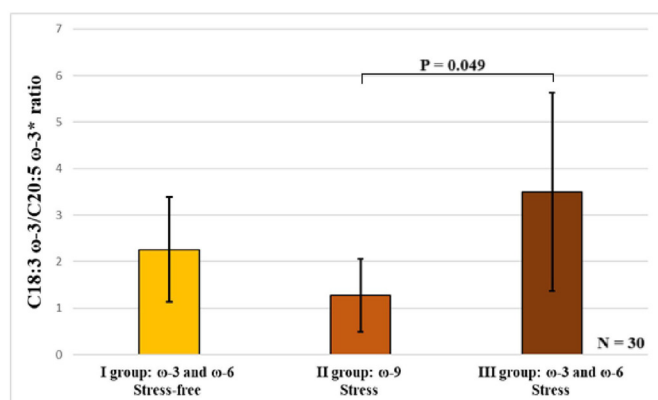


Fig. 1. Bar plots show the C18:3 ω -3/C20:5 ω -3 ratio in rat platelet phospholipid membranes under different experimental conditions. Data are presented as medians $\pm$ half the interquartile range. II group (ω -9 + stress) vs. III group (ω -3 and ω -6 + stress), $p = 0.049$ §. N = 30. *CX: Y ω -Z – CX indicates the number of carbon atoms, Y the number of double bonds, and ω -Z the omega family to which the fatty acid belongs. §Wilcoxon rank-sum test

The PLA formation results showed statistically significant differences between the first and second experimental groups and the second and third groups of Ly_PLTag and Gra_PLTag ($p = 0.002$ and $p = 0.013$; $p = 0.002$ and $p = 0.037$). The formation of Mo_PLTags

differed significantly only between the first and second groups ($p = 0.002$) (Table 4). According to these results, a tendency was observed in which aggregate formation differed significantly among the groups fed various PUFA diets.

Table 4. The level of platelet-leukocyte aggregate formation in rat blood under different experimental conditions

Platelet-leukocyte aggregates (percentage of formation)	Median, minimum, maximum, interquartile range	I group ω -3 and ω -6 Stress-free (n=10)	II group ω -9 Stress (n=10)	III group ω -3 and ω -6 Stress (n=10)	P value
Ly_PLTag	Med.	7.200	5.000	7.300	\$ I vs. II = 0.002 I vs. III = 0.838 II vs. III = 0.013
	Min.	5.600	3.200	4.000	
	Max.	10.400	7.000	10.100	
	IQR	1.230	1.600	1.830	
Mo_PLTag	Med.	2.650	1.250	2.000	\$ I vs. II = 0.002 I vs. III = 0.878 II vs. III = 0.103
	Min.	1.100	0.300	0.600	
	Max.	4.300	2.300	6.500	
	IQR	1.350	1.250	1.900	
Gra_PLTag	Med.	6.100	2.950	5.900	\$ I vs. II = 0.002 I vs. III = 0.922 II vs. III = 0.037
	Min.	3.900	1.800	2.300	
	Max.	9.200	5.300	10.600	
	IQR	1.850	1.880	2.850	

Med. – median, Min. – minimum, Max. – maximum, IQR – interquartile range. Ly_PLTag – platelet and lymphocyte aggregates, Mo_PLTag – platelet and monocyte aggregates, Gra_PLTag – platelet and neutrophil aggregates. \$Wilcoxon rank-sum test.

In the analysis of the MDA results ($\mu\text{g/L}$), statistically significantly higher concentrations were found in samples from the third group, where animals were under psychological stress in addition to extra ω -3 and ω -6, than in samples from the first group, where rats were not stressed. Still, they were given additional ω -3 and ω -6 (median 563.69 (IQR 180.60) vs. median 493.42 (IQR 73.86), $p = 0.049$) (Fig. 2).

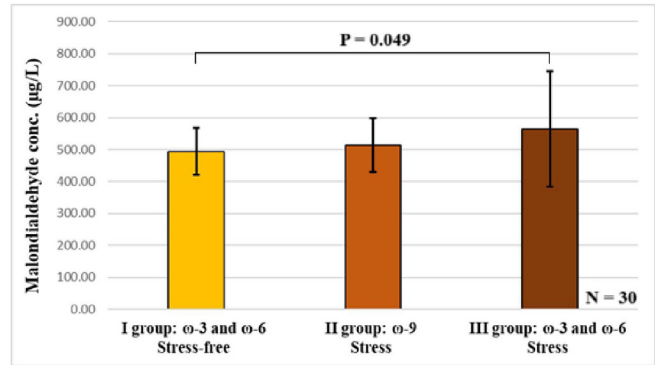
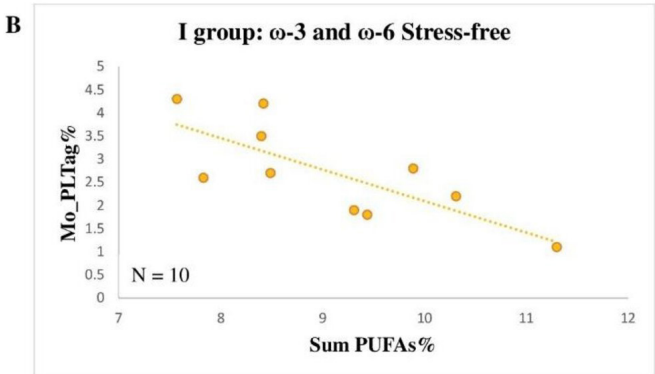
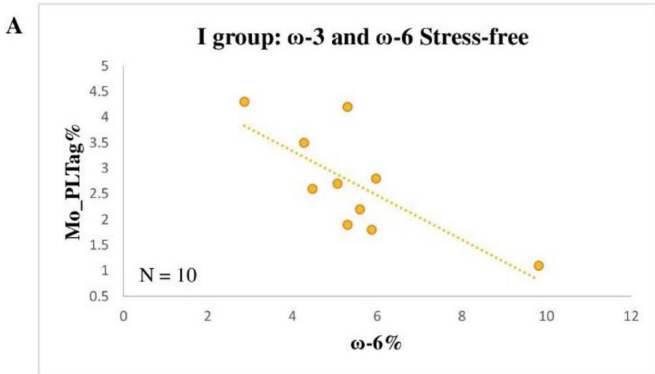


Fig. 2. Bar plots display malondialdehyde concentrations ($\mu\text{g/L}$) in rat blood serum under different experimental conditions. Data are presented as median $\pm$ interquartile range. I group (ω -3 and ω -6 + stress-free) vs. III group (ω -3 and ω -6 + stress), $p = 0.049^{\$}$. $N = 30$. $^{\$}$ Wilcoxon rank-sum test

After evaluating these study correlation results, a statistically significant moderate inverse correlation was found in the first group between Mo_PLTag and ω -6 ($R = -0.6626$, $p = 0.037$) and between Mo_PLTag and Sum PUFA% ($R =$

-0.7091 , $p = 0.022$). In the second group, a strong and statistically significant direct correlation was found between Gra_PLTag and ω -3 ($R = 0.7455$, $p = 0.013$). In the third group, a moderate statistically significant inverse correlation between Ly_PLTag and ω -6 was observed ($R = -0.6442$, $p = 0.044$) (Fig. 3).



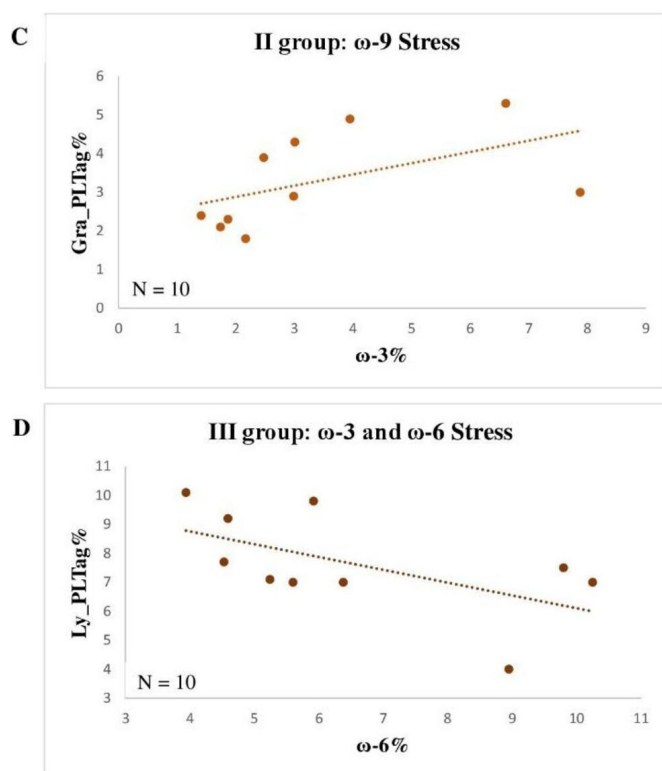


Fig. 3. Scatter plots show the Spearman correlation results between PLA formation and platelet phospholipid membrane FAs in different experimental conditions.

(A) I group: Mo_PLTag vs ω -6%, $R = -0.6626$, $p = 0.037$. $N = 10$. (B) I group: Mo_PLTag vs Sum PUFA%, $R = -0.7091$, $p = 0.022$. $N = 10$. (C) II group: Gra_PLTag vs ω -3%, $R = 0.7455$, $p = 0.013$. $N = 10$. (D) III group: Ly_PLTag vs ω -6%, $R = -0.6442$, $p = 0.044$. $N = 10$

No statistically significant differences were found in any experimental group after analysing the correlation between MDA and platelet membrane FAs (data not shown). Therefore, we did not observe a clear relationship between these indicators, regardless of dietary supplements and the application of psychological stress.

DISCUSSION

This study demonstrates that psychological stress affects the FA profile of platelets, specifically reducing the integration of C20:5 ω -3 and C22:6 ω -3 into the phospholipid membranes, potentially diminishing their anti-inflammatory properties. Moreover, when ω -3 and ω -6 were supplemented during stress, there was an increase in the formation of PLAs and higher MDA levels, indicating greater platelet activation and OS. These observations suggest that targeted PUFA supplementation may help counteract stress's prothrombotic and pro-oxidative effects.

According to scientific studies, ω -3 PUFAs, such as C20:5 ω -3 and C22:6 ω -3, are among the main FAs influencing an individual's psychological state [20]. In addition, C20:5 ω -3 and C22:6 ω -3 are thought to prevent depression and other diseases caused by psychological stress [21]. Animal studies have also shown a negative correlation between ω -3 PUFAs levels and disorders associated with stressful environmental

conditions [22]. Similar results were obtained in the present study. Under psychological stress, the platelet membranes of experimental animals showed a reduction in SFAs (i.e., C16:0), regardless of the additional ω family PUFAs. The sum of MUFAs% increased under stress and extra ω -3 and ω -6 feeding compared to the stressed ω -9 group. This finding suggests a stress-related compensatory mechanism in the ω -3+ ω -6 stress group, wherein MUFAs, particularly C18:1 ω -9, are upregulated to maintain membrane integrity under oxidative conditions. It is possible that the limited incorporation or accelerated peroxidation of supplemented ω -3 and ω -6 PUFAs during stress prompted a shift toward MUFA biosynthesis or retention. Alternatively, altered enzymatic activity under stress conditions may have favoured MUFA accumulation. However, the analysis of PUFA content showed the opposite trend. C22:6 ω -3 in the platelet phospholipid membrane was higher when rats were not under psychological stress but received ω -3 and ω -6. C20:5 ω -3 levels decreased under psychological stress, with additional ω -3 and ω -6 intake. These results confirm the data reported in scientific publications, where higher levels of C20:5 ω -3 and C22:6 ω -3 are associated with decreased psychological stress [23, 24]. Thus, our results show that C20:5 ω -3 and C22:6 ω -3 in the platelet phospholipid membrane were markedly reduced, especially when the experimental animals were subjected to psychological stress.

Possible mechanisms for this include anti-inflammatory effects, cell membrane normalisation, regulation of the hypothalamic-pituitary-adrenal (HPA) axis, and enhancement of serotonin neurotransmission [25]. PUFAs are precursors of oxidised mediators, eicosanoids, and docosanoids. These biologically active compounds are involved in the inflammatory process [22]. C20:5 ω -3 is one of the most important PUFAs in regulating immune function, as it competes with C20:4 ω -6 in the cell membrane, thereby reducing prostaglandin E2 synthesis and preventing inflammation [26]. An increased ω -6/ ω -3 ratio has been reported in patients with depression [27]. Psychological disorders have also been associated with increased production of pro-inflammatory cytokines; therefore, it can be suspected that a higher ω -6/ ω -3 ratio, mild inflammation, and psychological stress are interrelated.

Although ω -3 and ω -6 PUFAs are known to compete with each other for the enzyme metabolic centers of biologically active compounds and are considered antagonists, this is not sufficient to evaluate ω -3 or ω -6 alone during inflammation. The balance of ω -3 and ω -6 in organisms must be considered. An optimal balance of ω -3 and ω -6 intake may prevent the consequences of psychological stress by promoting the uptake of C20:5 ω -3 and C22:6 ω -3 into the cell membrane, which also depends on ω -6 PUFAs intake. Clinical studies have shown that a low C18:3 ω -3 diet increases ω -3 levels in the cell membrane, although ω -3 intake remains unchanged. However, reducing the intake of ω -6 PUFAs does not necessarily decrease psychological stress [25]. Some scientists have confirmed this insight, but some publications refute this idea [28]. The results of our study are also similar to those of the latter studies. According to

our data, the ω -3/ ω -6 ratio varied depending on the housing conditions of the experimental animals, but no statistically significant differences were found. The results also showed that only the composition of solitary PUFAs in the platelet membrane changed under stress. Still, no general tendency was observed in the analysis of the ratios of different ω families. Similar results were observed when the FAs were grouped into ω -3 and ω -6 families. Thus, in applying psychological stress to rats, the increase in platelet activation and promotion of inflammatory response were influenced only by individual FAs, not by a particular ω family of FAs.

Another sensitive biomarker of platelet activation that contributes to the risk assessment of atherothrombosis is PLA formation in whole blood [29]. Scientific data often show an inverse correlation between Mo_PLTag and PUFA levels in the blood [30]. The influence of different ω family FAs on PLA formation was also observed in our study. Our results showed that more PLAs were formed in the rat blood samples of experimental animals supplemented with ω -3 and ω -6 PUFAs. Such data suggest that ω -3 and ω -6 PUFAs antagonise each other; ω -3 PUFAs correlate directly with PLA formation, whereas ω -6 PUFAs have an inverse correlation. These results were observed between different experimental groups dominated by different PUFAs. Thus, PLA formation may have been influenced by both dietary PUFAs supplements and applied psychological stress.

Epidemiological studies also suggest that psychological stress plays a vital role in the development and progression of CVDs. Acute psychological stress promotes circulating PLAs in healthy people [31]. Therefore, increased platelet activity is considered one reason for the association between psychosocial stress and elevated CAD risk [32]. Steptoe and co-authors indicated in their study that there was a significant increase in Ly_PLTag, Mo_PLTag, and Gra_PLTag, as well as an overall increase in PLA formation after the experienced stress. The most significant shift was observed in the Mo_PLTag formation [33]. Our data also showed a substantial change in the percentage of Mo_PLTag formation; Mo_PLTag formation increased, but C20:5 ω -3 and C22:6 ω -3 PUFAs decreased under psychological stress. The composition of platelet phospholipid membrane FAs changes due to the reduction of C20:5 ω -3 and C22:6 ω -3, PUFAs in the cell membrane, and psychological stress. Increased platelet activation and aggregation, which lead to intensified PLA formation in the blood, contribute to the development and progression of CAD. Therefore, ω -3 PUFAs supplements (i.e., C20:5 ω -3 and C22:6 ω -3) could contribute to reducing the damage caused by stress and the formation of circulating PLAs in whole blood, which are risk factors for future thrombosis.

The additional biomarker measured in our study was MDA. Unstable and reactive free radicals interact with lipids in the cell membrane, causing lipid peroxidation. During lipid peroxidation, MDA is formed, one of OS's most commonly detected biomarkers [34]. ω -3 PUFAs can act as antioxidants (i.e., increasing the power of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase [35]) and inhibit lipid peroxidation. However,

the antioxidant effects of ω -3 PUFAs are influenced by ω -6 PUFAs [36]. By comparing our results with other scientific studies, an additional tendency was observed: the level of MDA varied not only depending on the obtained supplements of PUFAs but also on the housing conditions for experimental animals, including psychological stress. A higher concentration of MDA was found in the blood serum of rats subjected to psychological stress without additional ω -3 and ω -6 PUFAs. It can be assumed that during psychological stress, a reduction in C20:5 ω -3 and C22:6 ω -3 in the platelet phospholipid membrane is accompanied by a decrease in the antioxidant potential of ω -3 PUFAs, which may lead to higher blood serum MDA levels. However, no significant relationship was detected between MDA and platelet membrane FAs, regardless of the experimental animals' additional PUFAs and housing conditions. Therefore, further research is needed to confirm or reject the hypothesis that PUFAs affect lipid peroxidation and reduce OS during psychological stress.

Despite these findings, this study has limitations. A significant limitation is the small sample size, which may hinder the statistical power and generalizability of the findings. Since the rats were female, the study does not allow for investigating sex-related divergences in how dietary FAs and psychological stress affect the rats' behavior. In addition, the two-week intervention period may have been too short to capture the long-term impact of FA supplementation on platelet function.

CONCLUSION

This study demonstrates that psychological stress can significantly influence the incorporation of ω -3 FAs, particularly C20:5 ω -3 and C22:6 ω -3, into the platelet membranes. This alteration is associated with increased PLA formation and elevated MDA levels, indicating enhanced platelet activation and OS under stress. However, the conclusions are limited by the small sample size, use of only female participants, and short duration of the intervention. Future studies should involve more diverse populations and investigate extended supplementation periods to understand better the interplay between stress, FA metabolism, and platelet function. Additionally, research exploring how varying the balance of ω -3 and ω -6 intakes may influence these outcomes could provide further insight into optimizing dietary strategies under stress conditions.

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Santrauka

OMEGA-3, -6 IR -9 RIEBALŲ RŪGŠČIŲ POVEIKIS TROMBOCITŲ AKTYVUMUI PSICHOLOGINIO STRESO SĄLYGOMIS

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Įvadas. Šiame tyrime pasitelkiant žiurkių modelį buvo nagrinėjamas nesočiųjų riebalų rūgščių (RR) papildų ir psichologinio streso poveikis bendrajai fosfolipidų RR sudėčiai bei trombocitų aktyvacijos žymenims.

Tyrimo medžiaga ir metodai. Sveikos Wistar žiurkių patelės ($n = 30$) buvo suskirstytos į tris grupes: dvi savaites pirmoji grupė gavo $\omega-3 + \omega-6$ RR be papildomo streso, antroji gavo $\omega-9$ RR ir patyrė stresą, trečioji gavo $\omega-3 + \omega-6$ RR ir kartu patyrė stresą. Papildai buvo dozuojami taip: 1,05 mg C18:1 $\omega-9$, 0,62 mg C18:3 $\omega-6$, 5,74 mg C20:5 $\omega-3$ ir 1,79 mg C22:5 $\omega-3$ 100 g kūno svorio. Visi gyvūnai turėjo neribotą prieigą prie maisto ir vandens.

Rezultatai. Nustatyta, kad C22:6 $\omega-3$ RR kiekis (%) buvo statistiškai reikšmingai didesnis streso nepatyrusioje $\omega-3 + \omega-6$ grupėje nei stresą patyrusioje $\omega-3 + \omega-6$ grupėje (mediana 0,84 vs. 0,32, $p = 0,049$). Malono dialdehido koncentracija ($\mu\text{g/l}$) taip pat buvo didesnė stresą patyrusioje $\omega-3 + \omega-6$ grupėje, lyginant su streso nepatyrusia grupe (mediana 563,69 vs. 493,42, $p = 0,049$). Be to, trombocitų ir granulocitų agregatų susidarymas (%) buvo didesnis stresą patyrusioje $\omega-3 + \omega-6$ grupėje nei stresą patyrusioje $\omega-9$ grupėje (mediana 5,90 vs. 2,95, $p = 0,037$).

Išvados. Šie rezultatai rodo, kad psichologinis stresas keičia trombocitų membranų RR sudėtį ir gali skatinti trombocitų aktyvaciją bei oksidacinį stresą, nepriklausomai nuo vartojamų RR papildų.

Reikšminiai žodžiai: riebalų rūgštys, trombocitų aktyvacija, stresas.

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