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Murine hepatic proteome adaptation to high-fat diets with different contents of saturated fatty acids and linoleic acid:α-linolenic acid polyunsaturated fatty acid ratios

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Abstract

Introduction: Some health disorders, such as obesity and type 2 diabetes, are associated with a poor diet and low quality of the fat in it. The type and duration of the diet have an impact on the liver. This investigation uses the proteomic approach to identify changes in the mouse liver protein profile in adaptation to high-fat diets with different saturated fatty acid contents and linoleic acid (18:2*n*-6) to α-linolenic acid (18:3*n*-3) fatty acid ratios. **Material and Methods:** Four groups of male mice were fed different diets: one standard diet and three high-fat diets were investigated. After six months on these diets, the animals were sacrificed for liver dissection. Two-dimensional electrophoresis was used to separate the complex liver protein mixture, which enabled the separation of proteins against a wide, 3–10 range of pH and molecular weights of 15–250 kDa. Protein profiles were analysed in the PDQuest Advanced 8.0.1 program. Differentially expressed spots were identified using matrix-assisted laser desorption/ionisation–time-of-flight tandem mass spectrometry and peptide mass fingerprinting. The levels of identified proteins were validated using Western blotting. Transcript levels were evaluated using a real-time quantitative PCR. **Results:** The analysis of mouse liver protein profiles enabled the identification of 32 protein spots differing between nutritional groups. **Conclusion:** A diet high in polyunsaturated fatty acids modulated the levels of liver proteins involved in critical metabolic pathways, including amino acid metabolism, carbohydrate metabolism and cellular response to oxidative stress.

Keywords: high-fat diet, PUFA, liver, two-dimensional electrophoresis, MALDI-TOF/TOF.

Introduction

Dietary guidelines recommend reducing saturated fatty acids (SFA) in diet and replacing them with omega-3 and omega-6 polyunsaturated fatty acids (*n*-3 and *n*-6 PUFAs) to decrease the risk of metabolic disturbances, diabetes and coronary heart disease (27). However, the influence of dietary *n*-6 and *n*-3 PUFAs and their ratio on the organism is not fully characterised. There is no consensus on the optimal ratio of omega-6 to omega-3

PUFAs in a healthy diet to reduce inflammation and positively affect other metabolic processes (10). There is also a recommendation to avoid simply dividing *n*-3 and *n*-6 FAs into "good" and "bad" PUFAs (4). The optimal ratio of acids from one series of PUFAs to acids of another may also differ between individuals because of the genetic and epigenetic variation in genes encoding desaturase and elongase enzymes (8).

The liver plays a central role in mammalian fatty acid metabolism. Fatty acids in the liver partially originate from dietary triacylglycerols and *de novo* lipogenesis (2). Because mammals lack Δ12 desaturase and Δ15 desaturase, they are unable to synthesise linoleic acid (LA, 18:2*n*-6) from oleic acid (18:1*n*-9), or α-linolenic acid (ALA, 18:3*n*-3) from LA. As a result, ALA and LA are defined as essential fatty acids and must be provided in the diet. Alpha-linolenic acid is the simplest *n*-3 PUFA, and it undergoes a series of desaturations and elongations, generating new longer-chain *n*-3 PUFAs such as eicosapentaenoic acid (EPA, 20:5*n*-3), docosapentaenoic acid (22:5*n*-3) and docosahexaenoic acid (22:6*n*-3). Linoleic acid is converted into longer products, including γ-linolenic acid, dihomo-γ-linolenic acid (20:3*n*-6, DGLA), arachidonic acid (20:4*n*-6, AA) and adrenic acid (22:4*n*-6, AdA) (26). It is worth underlining that the same set of desaturase and elongase enzymes metabolises LA and ALA; therefore, high levels of LA in the diet can potentially reduce the efficiency of *n*-3 PUFA synthesis (44).

Polyunsaturated fatty acids and their derivatives have the ability to regulate the action of transcription factors like sterol regulatory element-binding protein 1 (SREBP-1), carbohydrate-responsive element-binding protein (ChREBP), peroxisome proliferator–activated receptor α (PPARα), hepatocyte nuclear factors 4α and -γ and liver and retinoid X receptors α. As a result, PUFAs have an impact on many metabolic processes, including lipogenesis (20). The physiological effects of *n*-3 and *n*-6 PUFAs are also mediated by their oxygenated products (oxylipins), *i.e.* octadecanoids, eicosanoids and docosanoids, which regulate the immune response. Most oxylipins derived from *n*-3 PUFAs tend to have anti-inflammatory and proresolving effects. Most oxylipins derived from *n*-6 PUFAs, in contrast, are characterised as having more inflammatory and proliferative functions (12).

To express the effect of dietary PUFA status on liver proteomics, the LA:ALA ratio is used, which is defined with LA as the numerator and ALA as the denominator and is a more specific approach than the *n*-6:*n*-3 ratio. Diets known to have different LA:ALA ratios should reflect this in different liver proteome profiles. They can be investigated with two-dimensional gel electrophoresis (2DE), a gel-based proteomic technique that achieves the separation of individual proteins and their proteoforms from a complex protein mixture according to their isoelectric point (the first dimension) and molecular weight (the second dimension). It should be emphasised that gel-free approaches are bereft of these advantages of 2DE. The changes in a particular protein and its isoforms in the liver proteome represent the biological activity of different LA:ALA ratios in the diet. Proteomics analysis helps to monitor potential biomarkers for liver metabolism alterations brought about by these groups of essential PUFAs. The aim of the study was to conduct an analysis of mouse liver proteome profiles *via* 2DE for identification of changes in response to diets with varied LA:ALA ratios. In addition, the study's design was intended to show the relationship between the duration of the dietary intervention and the change in proteomic profiles.

Material and Methods

Experimental design: animals, diets and sampling. The nutritional experiment was carried out in the animal house of the Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences in Jastrzębiec. The experimental procedures were approved by the Second Warsaw Local Ethics Committee for Animal Experimentation under resolution WAW2_22/2016. The animals were maintained in standard cages under temperature- and humidity-controlled conditions with a 12-h light/dark cycle. Animals received water and food *ad libitum*.

Male Swiss Webster mice (n = 40) were fed standard growth diets for eight weeks after weaning. Next, the animals were divided into four groups (10 mice per group). The mice were fed one of four types of feed differing in fat contents: the total fat in the standard diet (provided to the STD group) was approximately 2%, while this constituent of the other diets was approximately 22%. A diet high in saturated fats was fed to the SFA group, and diets high in polyunsaturated fats were given to the two remaining groups, distinguished by different linoleic acid (C18:2*n*-6) to α-linolenic acid (C18:3*n*-3) ratios. One group's diet had this ratio fixed at 14:1 and the other was configured for it to be 5:1 (30). The amounts of addition of each vegetable oil had been determined previously by analysing the fatty acid profile. The four feeds were produced manually, portioned, vacuum packed and stored in the dark. They were given to the animals twice daily to avoid fat oxidation.

For 24 weeks (168 days), ten mice in each group were monitored for body weight gain. After six months of being fed the various diets, the mice were fasted overnight and then sacrificed in a UNO Euthanasia Unit (Uno Roestvaststaal BV, Zevenaar, the Netherlands) with two-step procedures to minimise stress. Firstly, the mice were exposed to carbogen (a mixture of 95% O_2 and 5% CO_2). Then, the flow of carbogen was stopped, and 100% CO_2 was introduced into the chamber to slowly replace oxygen with carbon dioxide. After sacrifice, the livers of the mice were dissected and perfused with cold phosphate-buffered saline, and the median lobes were frozen in liquid nitrogen and stored

at −70°C for further analyses. In addition, blood was collected from the tail for blood glucose measurement using an ABRA glucose meter (Diagnosis S.A., Białystok, Poland). Blood from the heart ventricle was collected for morphological tests using Abacus Junior Vet 5 analyser (Diatron MI Zrt., Budapest, Hungary). The workflow of this experiment is presented in Fig. 1.

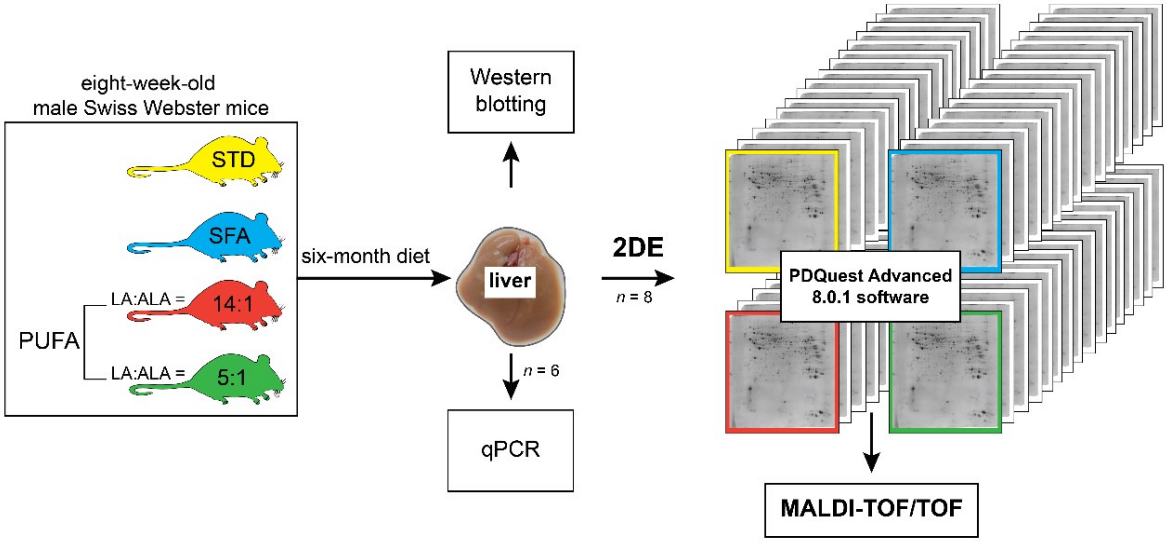

Fig. 1. Experimental design to elucidate liver proteomic profile in mice fed four fat-varied diets. PUFA – polyunsaturated fatty acids; STD – standard diet; SFA – diet high in saturated fatty acids; 14 :1 – diet high in PUFA with this linoleic acid to α-linolenic acid ratio; 5 :1 – diet high in PUFA with this linoleic acid to α-linolenic acid ratio; qPCR – real-time quantitative polymerase chain reaction; 2DE – two-dimensional electrophoresis. PDQuest Advanced 8.0.1 (Bio-Rad Laboratories, Hercules, CA, USA) software used for analysis of protein profiles. MALDI-TOF/TOF – matrix-assisted laser desorption/ionisation–time-of-flight tandem mass spectrometry

Table 1. Western blot antibodies for identification of liver proteins in mice fed four fat-varied diets

Target protein	UniProtKB accession number	Gene symbol	Molecular weight	Dilution	Host species	Conjugate	Supplier	Catalogue number
				Primary antibody				
Ornithine aminotransferase	P29758	*Oat*	49 kDa	1 :500	mouse	monoclonal	Santa Cruz Biotechnology Dallas, TX, USA	sc-376050
Glyceraldehyde-3-phosphate dehydrogenase	P00355	*GAPDH*	36 kDa	1 : 10,000	rabbit	polyclonal	Abcam, Cambridge UK	ab190304
Peroxiredoxin 6	P30041	*PRDX6*	25 kDa	1 : 400	mouse	monoclonal	Santa Cruz Biotechnology, Dallas, TX, USA	sc-101522
Ferritin light chain	P02792	*FTL*	21 kDa	1 :1000	rabbit	polyclonal	Abcam, Cambridge UK	ab69090
				Secondary antibody				
m-IgGκ BP-HRP					mouse	IgGκ light chain–binding protein conjugated to horseradish peroxidase (HRP)	Santa Cruz Biotechnology, Dallas, TX, USA	sc-516102
Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)					goat	polyclonal horseradish peroxidase (HRP) conjugates	Jackson ImmunoResearch, Ely, UK	111-035-003

IgG – immunoglobulin G; H+L – heavy and light chains

Two-dimensional electrophoresis coupled with matrix-assisted laser desorption/ionisation–time-of-flight tandem mass spectrometry (MALDI-TOF/TOF). Due to the capacity of the 2DE technique, only eight mouse livers from each group were used for proteomic analysis. Sample preparation, isoelectrofocusing, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), image staining and protein profiles analysis in the PDQuest Advanced 8.0.1 software (Bio-Rad Laboratories, Inc Hercules, CA, USA) were carried out as previously described (30). Protein spots of intensity which differed between diet groups were identified using MALDI-TOF/TOF (ultrafleXtreme; Bruker Daltonics, Bremen, Germany). Data were captured 2,000 times to generate the mass spectra. In addition, several areas at the edge of a 2DE gel without protein spots were analysed in parallel to generate a list of contaminants, which was used to filter the peak lists of the samples. Mass spectra were analysed in FlexAnalysis 3.4 and BioTools 3.2 (Bruker Daltonics). Protein identification was performed using the peptide mass fingerprinting (PMF) technique, in which the experimental data were compared with SwissProt database records for the taxon Mammalia using the MASCOT application (Matrix Science, London, UK). The following parameters were set: trypsin digestion; carbamidomethylation of cysteine as the fixed modification; protein N-terminal acetylation and methionine oxidation as the variable modifications; 150 ppm as the theoretical-to-experimental ion mass tolerance; and a maximum of one missed cleavage site.

Building protein–protein interaction networks. The interaction between differentially expressed proteins and their participation in biological processes were visualised and analysed using the Cytoscape application v. 3.9.0 and its ClueGO plugin v. 2.5.8 in combination with CluePedia v. 1.5.8, annotating with GO-Biological Process (GO – Gene Ontology), KEGG (Kyoto Encyclopaedia of Genes and Genomes), and Reactome Pathways (5).

Protein–protein interaction networks were built using proteins differentiated in the 14:1, 5:1 and SFA groups compared to the STD group and proteins differentiated in the 14:1 and 5:1 groups compared to the SFA group. The ClueGO criteria included a 2–8 GO tree interval; extraction of GO terms/pathways with at least two genes from one gene cluster which represented at least 3% of the total number of genes of the term; a GO term/pathway qualifying as specific when more than 60% of the genes were from one cluster; 0.4 kappa score threshold; and subjection to a two-sided hypergeometric test for enrichment or depletion with Bonferroni step down correction.

For proteins differentiated between the 14:1 and 5:1 groups, the protein–protein interaction network was built using these ClueGO criteria: a 7–8 GO tree interval; extraction of GO terms/pathways with at least one gene from one gene cluster which represented at least 50% of the total number of genes of the term; a GO term/pathway qualifying as specific when more than 60% of the genes were from one cluster; 0.4 kappa score threshold; and subjection to a two-sided hypergeometric test for enrichment or depletion with Bonferroni step down correction.

RNA extraction, reverse transcription and quantitative PCR. Isolation of RNA, reverse transcription, primer design, amplified product verification and real-time PCR analysis were conducted for six mice per group as described earlier (30). The list of primer sequences is presented in Supplementary Table S1.

Western blotting. Sample disintegration was performed by solubilising an approximately 50 mg piece of frozen liver median lobe in RIPA (radioimmunoprecipitation assay) Lysis and Extraction Buffer (Thermo Fisher Scientific, Rockford, IL, USA) containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% nonyl phenoxypolyethoxylethanol (NP-40), 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate and adding protease inhibitors to the solution (Complete, Mini Protease Inhibitor Cocktail; Roche Diagnostics, Mannheim, Germany). Samples were homogenised using a MagNA Lyser instrument (Roche Diagnostics). The homogenisation began with two runs of 20 s at 5,000 rpm, and between them the samples were put in a cooling block for 3 min. Subsequently, the homogenates were centrifuged at 12,000 × *g* for 25 min at 4°C. The supernatants were collected and stored at −70°C. Protein Assay Dye Reagent Concentrate and Quick Start Bovine Serum Albumin Standard (Bio-Rad Laboratories) were used to determine the protein concentration in extracts according to the manufacturer's instructions. First, 50 µg of protein extract per sample was separated in SDS-PAGE with 12% polyacrylamide at 40 V for 30 min and 100 V for 3 h. The proteins were then transferred to a polyvinylidene fluoride membrane (Merck, Darmstadt, Germany). Next, the membrane was blocked in 5% non-fat dry milk in 25 mM Tris, 150 mM NaCl and 0.05% (v/v) Tween-20 at pH = 7.5 for 1 h at room temperature and incubated overnight with primary antibodies at 4°C. A list of the antibodies used in the study is presented in Table 1.

Statistical analysis. The comparative densitometric analysis of protein spots included liver samples isolated from 32 mice after a six-month diet. The data consisted of a set of 510 spots that occurred on each 2DE gel. The values of each spot's intensity in the four groups in duplicate for each individual were used in the multiple linear regression analysis. The formula used was the one with a precedent in the model of Liput *et al.* (30):

$$y_{ijkl} = \mu + O_i + M_j(O_i) + G_k + e_{ijkl}$$

where y is the response variable, O_i is the i-paternal genetic effect, $M_j(O_i)$ is the j-maternal genetic effect nested into i-paternal genetic effect, G_k is the effect of diet type and e is the error term.

The same model was used to analyse relative expression levels of selected target genes. The analysis was performed in the SAS 9.4 system. (SAS Institute,

Cary, NC, USA). The differences between diet groups were estimated using Tukey's multiple comparisons test. For the observations that presented data with non-normal distribution, the Wilcoxon–Mann–Whitney test was used to compare the differences between diet groups.

The significance of body weight gain differences between groups was analysed by two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Statistically significant differences between groups in final body weight, liver weight, a percentage of liver weight to total body weight, blood glucose, and haematological data were determined by one-way ANOVA with Tukey's multiple comparisons test. Mean values, standard deviations and standard errors of the mean were calculated and bar plots were created in GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA).

Results

Body weight and blood parameter analysis. The four groups of mice containing 10 mice per group started with similar mean body weights: 32.61 ± 1.62 g in the STD group, 32.08 ± 1.31 g in the SFA group, 32.24 ± 2.60 g in the 14:1 group and 30.93 ± 2.12 g in the 5:1 group. Body weight gain showed the first difference between the STD and SFA groups after 84 days of diets (P-value < 0.05). After 24 weeks, the SFA group at 56.37 ± 6.51 g weighed significantly more than the STD group at 43.16 ± 2.78g (P-value < 0.001). Similarly, the 14:1 group at 52.18 ± 8.26 g and the 5:1 group at 49.85 ± 5.31 g weighted more than the STD group (P-value < 0.05) (Fig. 2).

After 24 weeks of dietary intervention and overnight fasting, the mice fed high-fat diets (the SFA, 14:1 and 5:1 groups) had significantly higher body weight than mice fed the standard diet (P-value < 0.001 and P-value < 0.05), (Fig. 3A). Liver weights were not significantly different between experimental groups (Fig. 3B), but the liver weight as a percentage of total body weight was lower in the SFA group than in the STD and 5:1 groups (P-value < 0.01 and P-value < 0.05, respectively) (Fig. 3C).

Figure 4A shows that groups given high-fat diets for six months showed significantly higher blood glucose levels than the 6.41 ± 1.27 mmol/L of the STD group: the level in the SFA group was 10.51 ± 2.25 mmol/L (P-value < 0.001), in the 14:1 group it was 9.05 ± 0.99 mmol/L (P-value < 0.05) and in the 5:1 group it was 10.06 ± 1.55 mmol/L (P-value < 0.001). The mean red blood cell (RBC) count in mice fed the 14:1 diet was lower than that in mice fed the STD diet (P-value < 0.05) (Fig. 4B). In addition, the mean platelet volume (MPV) of mice from the 5:1 group of 9.08 ± 0.71 fL was higher than that of mice from the SFA group of 8.20 ± 0.32 fL (P-value < 0.05) (Fig. 4C). The other morphological parameters, including white blood cells; lymphocytes; monocytes; neutrophils; platelet contents and mean corpuscular volume; mean corpuscular haemoglobin concentration and red cell distribution width related to corpuscular volume showed no significant differences between groups (Supplementary Table S2).

Proteomics analysis. The hepatic protein expression of mice fed high-fat diets enriched with LA and ALA at different ratios (the 14:1 and 5:1 groups), diets enriched with saturated fatty acids (the SFA group) and a standard diet (the STD group) was analysed using 2DE coupled with mass spectrometry to identify differentially expressed proteins. Image analyses of 64 2DE gels revealed 877–1,066 protein spots per gel. The mean number of spots per 2DE gel was 954. The mean coefficients of variance of the STD, SFA, 14:1 and 5:1 groups were estimated at 48.33%, 43.23%, 47.50% and 46.07%, respectively. Spots totalling 510 were matched to every member subjected to statistical analysis between the groups. Thirty-two protein spots were identified across the four groups that were significantly varied in expression in a comparison of the results of the 2DE (Fig. 5). Mass spectrometry identification results and the ratios of the mean values of protein spot intensity signals between the groups are presented in Supplementary Table S3. The resolving of the 2-D SDS-PAGE Standards (Bio-Rad Laboratories) in the same parameters is presented in Supplementary Fig. S1.

The set of 32 protein spots which significantly varied in relative expression between groups was excised from the 2DE gels and an average three biological replicates per spot were identified using the PMF technique. The identification results of protein spots are presented in Supplementary Table S3. The MASCOT score ranged from 61 to 288 and averaged 148.53, approximately 75% of the scores being above 94. The amino acid sequence coverage varied from 20% to 81% and averaged 47%, 75% of the spots having coverage greater than 31%. The method resulted in the average number of matching peptides being 15. All proteins were identified as *Mus musculus* proteins based on the SwissProt database. Two proteins (acetyl-CoA acyltransferase 2 – ACAA2; and albumin – ALB) were present in several spots (SSP 8207 and 8211; and SSP 3605 and 3610, respectively).

The hepatic proteins which were significantly altered after six months of diets were categorised according to their biological function based on the STRING database (41). Their expression change patterns were visualised using heatmaps. Proteins associated with lipid metabolism are presented in Fig. 6A. This group includes proteins responsible for high-density lipoprotein remodelling (albumin – ALB), the cholesterol biosynthesis process (hydroxymethylglutaryl-CoA synthase, mitochondrial – HMGCS2) and fatty acid β-oxidation (3-ketoacyl-CoA thiolase, mitochondrial – ACAA2). The group of regulated proteins was responsible for amino-acid metabolism (Fig. 6B). Functional annotation by STRING assigned the proteins to the alpha-amino-acid biosynthetic process (betaine-homocysteine S-methyltransferase 1 – BHMT; mitochondrial ornithine aminotransferase – OAT; S-adenosylmethionine synthase

isoform type-1 – MAT1A; formimidoyltransferase cyclodeaminase – FTCD; aldehyde dehydrogenase family 6 member A1 – ALDH6A1; and homogentisate 1,2-dioxygenase – HGD) as well as to the tryptophan metabolism pathway (indolethylamine N-methyltransferase – INMT).

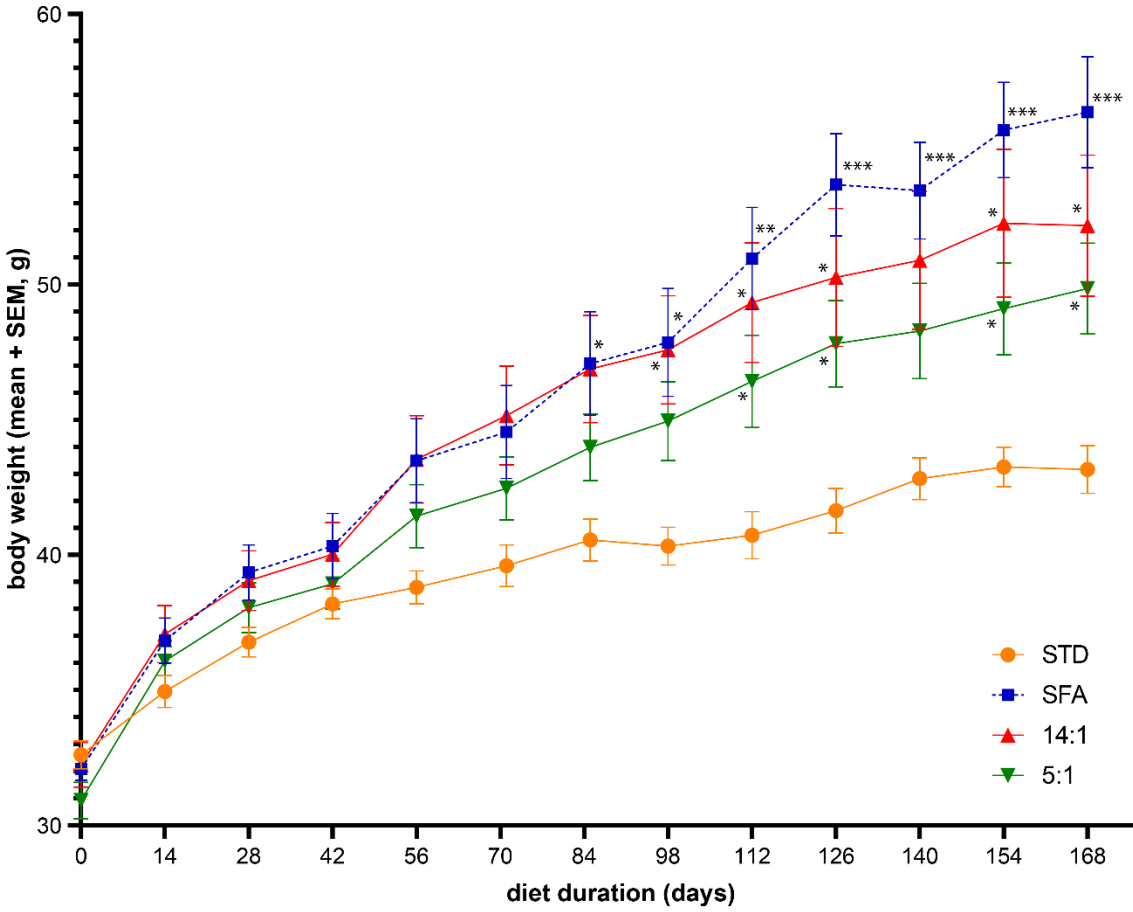

Fig. 2. Effect on mice body weight of six months' provision of experimental diets varied in fat content. Data are expressed as mean ± standard error of the mean, n = 10 mice per group. SEM – standard error of the mean; STD – standard diet; SFA – diet high in saturated fatty acids; 14:1 – diet high in polyunsaturated fatty acids (PUFA) with this linoleic acid to α-linolenic acid ratio; 5:1 – diet high in PUFA with this linoleic acid to α-linolenic acid ratio; * – significance difference at P-value < 0.05; ** – significant difference at P-value < 0.01; *** – significant difference at P-value < 0.001

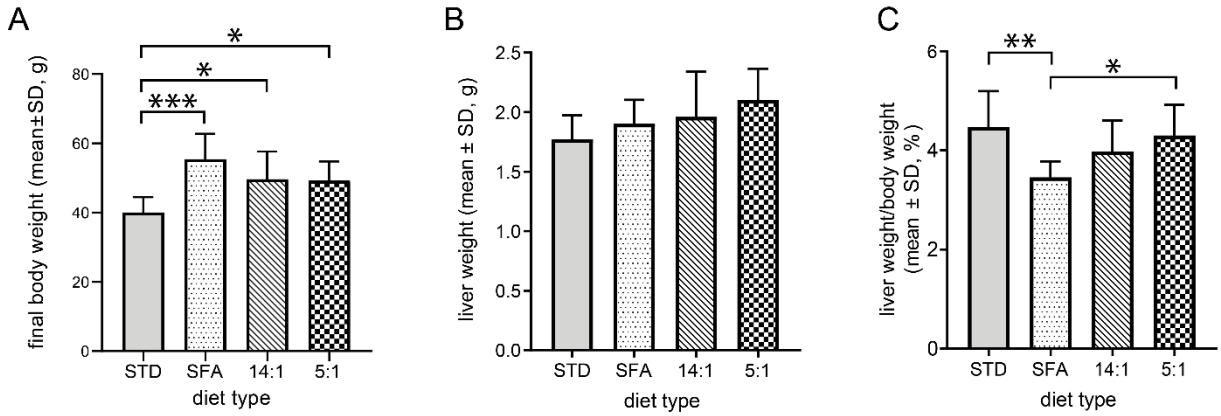

Fig. 3. Final body weights of mice measured after six months' provision of experimental diets varied in fat content and overnight fasting (A). Liver weights at the time of dissection (B). Ratios of liver weight to body weight (C). Data are expressed as mean ± standard deviation, n = 8 mice per group. STD – standard diet; SFA – diet high in saturated fatty acids; 14:1 – diet high in polyunsaturated fatty acids (PUFA) with this linoleic acid to α-linolenic acid ratio; 5:1 – diet high in PUFA with this linoleic acid to α-linolenic acid ratio; * – significant difference at P-value < 0.05; ** – significant difference at P-value < 0.01; *** – significant difference at P-value < 0.001

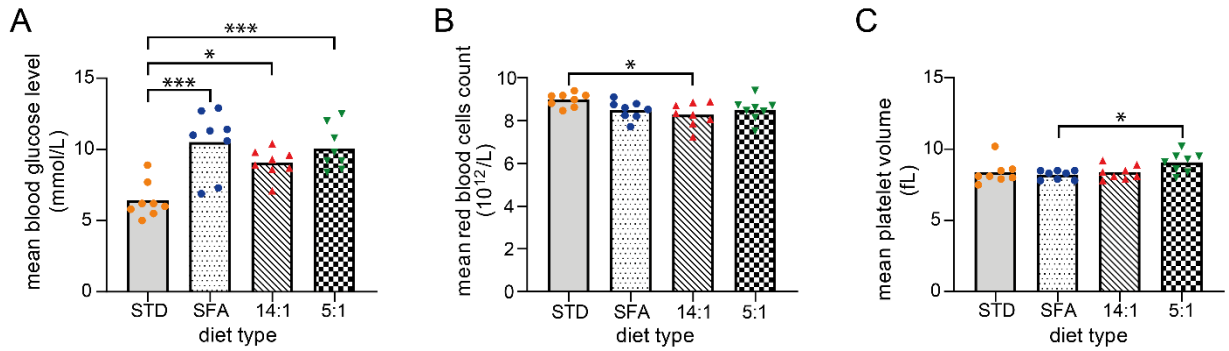

Fig. 4. Blood parameters in mice after six months' provision of experimental diets varied in fat content. Blood glucose level measured from tail blood (A). Mean red blood cell count measured from heart blood (B). Mean platelet volume measured in heart blood (C). STD – standard diet; SFA – diet high in saturated fatty acids; 14:1 – diet high in polyunsaturated fatty acids (PUFA) with this linoleic acid to α-linolenic acid ratio; 5:1 – diet high in PUFA with this linoleic acid to α-linolenic acid ratio; The plot shows the mean value, and the individual data points are presented in colours and shapes specific to each group; * – P-value < 0.05; ** – P-value < 0.01; *** – P-value < 0.001

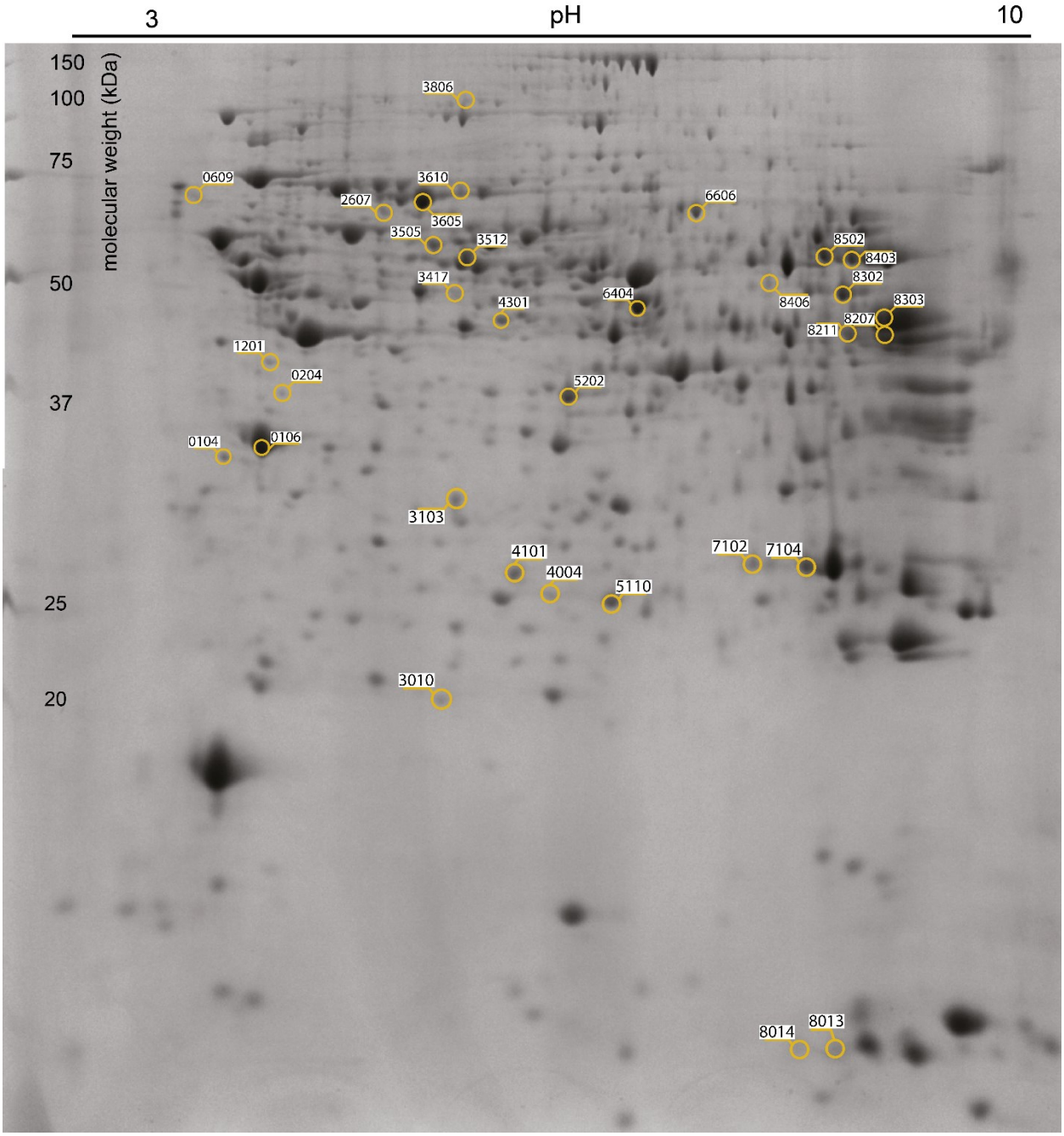

Fig. 5. Differential murine liver protein spots after six months' provision of experimental diets varied in fat contents. Spot numbers (SSP) and areas of protein spots with different expression levels between experimental groups correspond to the SSP number in Supplementary Table S3

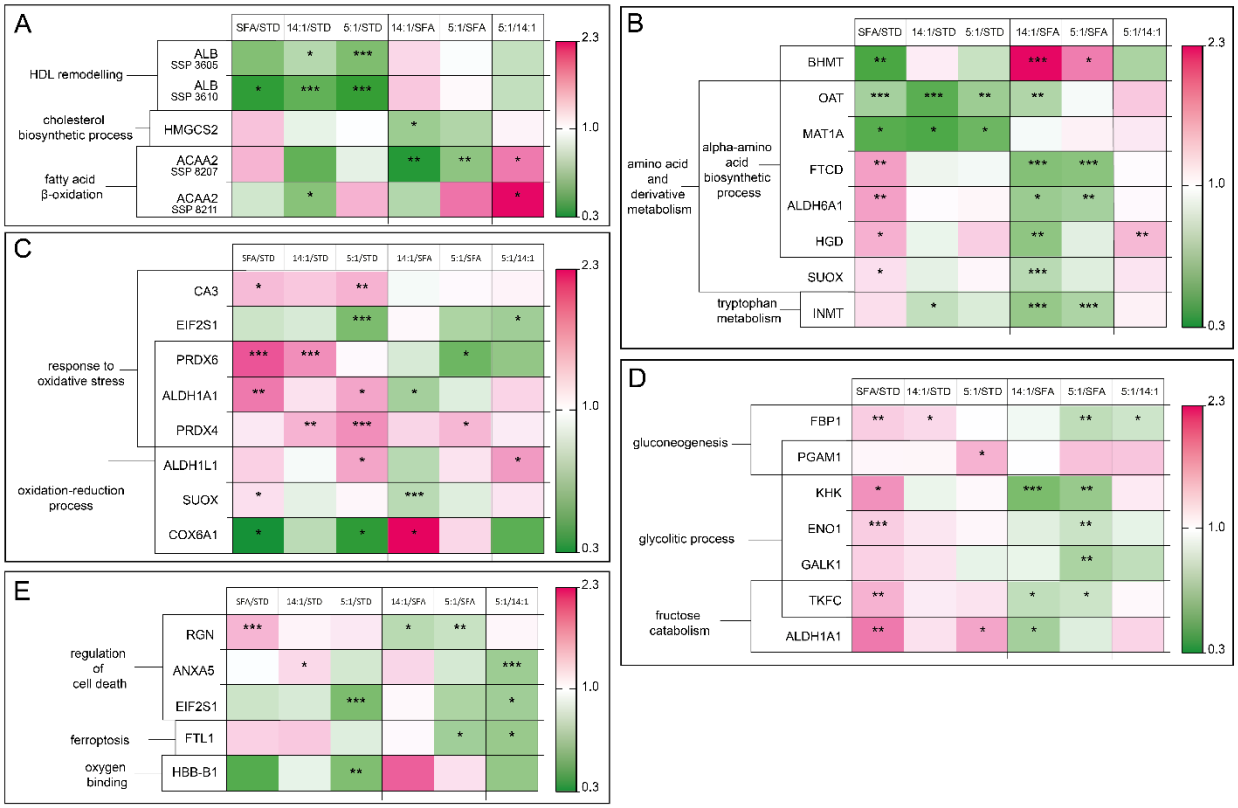

Fig. 6. Differentially expressed murine liver proteins after six months' provision of experimental diets varied in fat contents. The heatmap shows the increase (red) and the decrease (green) based on the comparison of the average spot intensity of the standard diet group (STD), group on a diet high in polyunsaturated fatty acids (PUFA) with 14:1 linoleic acid to α-linolenic acid ratio (14:1), group on a diet high in polyunsaturated fatty acids (PUFA) with 5:1 linoleic acid to α-linolenic acid ratio (5:1) and the saturated fatty acid diet (SFA) group. Lipid metabolism proteins (A); amino acid metabolism proteins (B); oxidative stress proteins (C); carbohydrate metabolism proteins (D); proteins regulating cell death pathway (E). HDL – high-density lipoprotein; ALB – albumin; HMGCS2 – 3-hydroxy-3-methylglutaryl-CoA synthase 2; ACAA2 – acetyl-CoA acyltransferase 2; BHMT – betaine-homocysteine S-methyltransferase; OAT – ornithine aminotransferase, mitochondrial; MAT1A – S-adenosylmethionine synthase isoform type-1; FTCD – formimidoyltransferase-cyclodeaminase; ALDH6A1 – aldehyde dehydrogenase family 6 member A1; HGD – homogentisate 1,2-dioxygenase; SUOX – sulfite oxidase, mitochondrial; INMT – indolethylamine N-methyltransferase; CA3 – carbonic anhydrase 3; EIF2S1 – eukaryotic translation initiation factor 2 subunit 1; PRDX6 – peroxiredoxin-6; ALDH1A1 – aldehyde dehydrogenase 1A1; COX6A1 – cytochrome c oxidase subunit 6A; FBP1 – fructose-1,6-bisphosphatase 1; PGAM1 – phosphoglycerate mutase 1; KHK – ketohexokinase; ENO1 – alpha-enolase; GALK1 – galactokinase; TKFC – triokinase/FMN cyclase; RGN – regucalcin; ANXA5 – annexin A5; FTL1 – ferritin light chain 1; HBB-B1 – haemoglobin subunit beta-1; * – significant difference at P-value < 0.05; ** – significant difference at P-value < 0.01; *** – significant difference at P-value < 0.001

The next group of altered proteins in the mouse liver were related to oxidative stress (Fig. 6C). These proteins were defined as participating in the oxidative-stress response (carbonic anhydrase 3 – CA3; eukaryotic translation initiation factor 2 subunit 1 – EIF2S1; peroxiredoxin-6 – PRDX6; aldehyde dehydrogenase 1A1 – ALDH1A1; and peroxiredoxin-4 – PRDX4) and the oxidation-reduction process (PRDX6; ALDH1A1; PRDX4; cytosolic 10-formyltetrahydrofolate dehydrogenase – ALDH1L1; mitochondrial sulfite oxidase – SUOX; and mitochondrial cytochrome c oxidase subunit 6A1 – COX6A1). Proteins related to carbohydrate metabolism are also regulated by diets with different PUFA and SFA contents (Fig. 6D). The target proteins were involved in gluconeogenesis (fructose-1,6-bisphosphatase 1 – FBP1; and phosphoglycerate mutase 1– PGAM1), the glycolytic process (PGAM1; ketohexokinase – KHK; alpha-enolase – ENO1; galactokinase – GALK1; and triokinase/FMN cyclase – TKFC) and fructose catabolism (TKFC and ALDH1A1). Figure 6E shows the regulation of cell death pathway proteins (regucalcin – RGN; annexin A5 – ANXA5; and EIF2S1). One protein (ferritin light chain 1 – FTL1), which is essential for storing iron in a non-toxic form, was downregulated in the 5:1 group compared to the SFA and 14:1 groups (P-value < 0.05). This study indicated that the oxygen-binding–protein haemoglobin subunit beta-1 (HBB-B1) also depended on the amount of polyunsaturated fatty acids in the diet.

The results of liver proteomic alterations observed after six months of specified-fat-level diets were also compared with previously published outcomes after three months of these diets (30). After six months, five proteins were significantly upregulated in the STD group compared to the SFA group. Three proteins (ALB, MAT1A and OAT) were expressed at higher levels after three as well as six months of the experiment in the STD group than in the SFA group (Supplementary Fig. S2A). Twelve proteins were expressed at lower levels in the STD group than in the SFA group after six months. Two proteins were more weakly expressed after three and six months versus the STD group (KHK and TKFC) (Supplementary Fig. S2B). Differentially expressed

proteins from the 14:1 groups were compared to the SFA group after both diet provision periods. There were not many common tendencies among these changed protein spots. We identified five proteins overexpressed after three months but not after six months and another two proteins overexpressed after six months but not after three months, all being overexpressed in the 14:1 group (Supplementary Fig. S3A). More proteins were downregulated only after six months of the 14:1 diet compared to the SFA diet (thirteen proteins) than only after three months (six proteins), two proteins (HGD and HMGCS2) being downregulated after both diet provision periods (Supplementary Fig. S3B). Comparing the proteins differentially expressed in the 5:1 group to in the SFA group, three proteins were overexpressed in the 5:1 group after three months of this diet, and two were after six months (BHMT, PRDX4) (Supplementary Fig. S4A). Three proteins (FTL1, GALK1 and TKFC) were expressed less after three and six months of the 5:1 diet than after these periods of the SFA diet (Supplementary Fig. S4B). Carbonic anhydrase 3 and BHMT were seen to be upregulated in both high-PUFA diet groups compared to the SFA-diet group, but in a time-specific manner. Regarding downregulated proteins, ACAA2, ALDH6A1, FTCD, INMT, KHK and RGN were less abundant in both high-PUFA diets compared to the SFA diet within a particular provision period. The results of the comparison of the upregulated hepatic proteins in the 5:1 group to those in the 14:1 group offered no proteins common to both timeframes (Supplementary Fig. S5A). Similarly, no protein was downregulated in the 5:1 group after both three months and six months of diet (Supplementary Fig. S5B).

The functional clustering of proteins differentially expressed between groups was performed using the Cytoscape software with the ClueGO plug-in. The representative biological terms and pathways for these differentially expressed proteins in the SFA, 14:1 and 5:1 groups compared to the STD group are presented in Supplementary Fig. S6. Among the most probably affected biological functions revealed by Cytoscape-ClueGO software were fructose metabolism and catabolism. Supplementary Fig. S7 presents the network analysis of differentially expressed proteins in the livers of mice provided the high-PUFA diets (14:1 and 5:1) compared to these proteins in the livers of mice provided the SFA diet. The proteins at changed levels in both PUFA diets were involved in several biological pathways, such as gluconeogenesis and fructose catabolism. These were downregulated in the 5:1 group. Proteins which the comparison between the 14:1 and 5:1 groups indicated to be differentially expressed showed their involvement in the regulation of the translational initiation process. Their networks are presented in Supplementary Fig. S8.

The different protein level of FBP1 was visible after six months of diet (Supplementary Fig. S9A and B). However, after the three-month exposure to high-fat diets investigated in previous research by the present authors (30), the FBP1 level was not different between groups. The FBP1 messenger RNA (mRNA) level was comparable in the experimental groups at both time points (Supplementary Fig. S9C). After six months, the OAT protein level was decreased in all high-fat groups (Supplementary Fig. S10A and B). The fall in this protein's level from its level in the STD group was confirmed by Western blotting and qPCR only in the 5:1 group (Supplementary Fig. S10C–E). Peroxiredoxin-6, identified in spot 5110, was differentially expressed in the SFA and 14:1 groups compared to the STD group (P-value < 0.001). Its spot intensity was reduced in the 5:1 group compared to its intensity in the SFA group (P-value < 0.05) (Supplementary Fig. S11A and B). On the other hand, its abundance as determined by immunoblotting was not significantly changed between groups (Supplementary Fig. S11C and D). The expression of the *Prdx6* gene was similar in all groups (Supplementary Fig. S11E). For comparison, the protein signal intensity of FTL1 after three months of provision of these four diets presented here came from the previous study by Liput *et al.* (30). After six months, differences between SFA group and 5:1 group FTL1 levels were still detectable (P-value < 0.05), but no differences were discernible between the SFA and 14:1 group levels (Supplementary Fig. S12A and B). Differences were not detected by immunoblotting (Supplementary Fig. S12C and D). The relative expression level of the mRNA of *Srebf1*, the sterol regulatory element-binding transcription factor 1 gene, was affected by high-fat diets and was higher than that in the STD group (Supplementary Fig. S13A). The expression of *Fasn*, the fatty acid synthase gene, was higher in the SFA, 14:1 and 5:1 groups than in the STD group (Supplementary Fig. S13B). In the liver, all high-fat diets augmented the expression of fatty acid desaturase 1 *Fads1* mRNA compared to its expression sustained by the standard diet. However, among the high-fat diets, no significant difference was observed between the PUFA-rich diets and the SFA-rich diet (Supplementary Fig. S13C). There was a higher expression of fatty acid desaturase 2 *Fads2* mRNA in the high-PUFA groups than in the STD group. Moreover, after six months of diet provision, increased *Fads2* mRNA was noted in the high-PUFA groups compared to the SFA group (Supplementary Fig. S13D).

Discussion

Diet is one of the factors affecting the body's metabolic activity. The type of dietary fat can also be a significant factor in human health. Nutritional recommendations promote the reduction of saturated fatty acids in favour of unsaturated fatty acids (27). Replacing them with polyunsaturated acids reduces the risk of cardiovascular events (16). Partial replacement of dietary saturated fat with PUFAs also alleviates the lipopolysaccharide-induced insulin resistance, hepatic

steatosis and hepatic inflammation that may accompany high-fat diet consumption (40). However, the most desirable type of unsaturated fat and the appropriate proportion of a diet which should comprise unsaturated fats is still not fully clear. Nevertheless, there is evidence for the importance of a low *n*-6 : *n*-3 ratio in the diet to prevent chronic disease development (22). Moreover, a complex meta-analysis indicated that higher intakes of *n*-3 PUFAs, but not *n*-6 PUFAs, were associated with a lower risk of metabolic syndrome (18). This proportional relationship may be a crucial factor in contemporary diets because a high content of *n*-6 PUFAs together with a high *n*-6 : *n*-3 ratio are often found in the Western diet (9).

On the other hand, based on advanced lipidomics, the *n*-6 : *n*-3 ratio may not explain or represent the complex potential of PUFAs to shape the inflammatory response. It has to be emphasised that the *n*-6 : *n*-3 ratio is often imprecise and non-specific because of the lack of a standardised definition of which FAs constitute the numerator and which the denominator of the ratio (15). In the present study, the effect of the major groups of PUFAs was investigated using diets characterised by different LA : ALA ratios and different diet durations. The experiment used mice because of their low maintenance costs, rapid maturation and well-known genome, in which approximately 99% of genes have homologues in the human genome. The proteomic results of three months of diet provision were published previously and indicated 37 protein spots as significantly altered (30).

Two-dimensional electrophoresis is still a valuable, robust technique for separation of a complex mixture of proteins in biological samples. The conditions of mouse liver protein separation used in this study using 2DE in an extensive pH range from 3 to 10 and molecular weight from approximately 250 kDa to 15 kDa were sufficient for an average collection of over 900 protein spots on each gel to be obtained. The number of protein spots was higher than in the previous studies using pig and goat livers, in which respectively 399 and 520 spots were observed (19, 48). However, the number of protein spots was smaller than that obtained by Sanchez *et al.* (39), who, by separating mouse liver proteins in the same pH range, obtained 2,836 spots. The reason for such a difference in spot number is the use of different methods of protein detection – the Coomassie Brilliant Blue G-250 blue staining used in our research has a lower sensitivity than the silver staining used by Sanchez *et al.* (39). In the densitometric analysis of 64 2DE images, the average coefficients of variation for individual groups ranged from 43.23% to 48.33% and were lower than those obtained by Lepczyński *et al.* (28), which were in the range of 48.42–53.33%.

In the presented study, the analysis of these 64 two-dimensional protein maps of the livers collected from 32 mice showed 32 spots with significantly different expression between dietary groups, and these were identified by mass spectrometry. Two proteins were identified in more than one spot. The presence of the same gene expression products in two spots with different coordinates on the two-dimensional protein maps could be due to post-translational modifications of the analysed protein, including phosphorylations and methylations. According to Veredas *et al.* (43), almost all proteins (98%) containing oxidised methionine are phosphoproteins. The differences between the isoforms in the amount and types of modifications contribute to the change of the isoelectric point and molecular mass, which results in a shift of protein coordinates in the 2DE gel.

Increasing consumption of more energy-dense foods, which are high in fat, is considered the primary cause of the increase in obesity. Generally, high-fat diets significantly increase body and liver weights, leading to obesity, hyperlipidaemia and fatty liver. An approximate 22% difference in final body weight was observed between the mice from the group fed high-fat diets (the SFA, 14 : 1 and 5 : 1 groups) and the mice from the STD group. It may be the result of the increased caloric content of the high-fat feeds and a difference in the amount of feed consumed because, as demonstrated by Licholai *et al.* (29), *ad libitum* access to high-fat feed causes that it is consumed in excess, even during the light phase, *i.e.* outside the standard period of food intake by mice. According to the authors, this may be associated with the better palatability of high-fat food. The present study did not show any significant differences between the body weights of animals from the high-PUFA content diet groups and the body weights of animals from the SFA-diet group (P-value > 0.05). However, mice fed the diet with an LA : ALA 14 : 1 ratio achieved a higher final body weight than mice from the 5 : 1 group. This observation is similar to the results obtained by Ikemoto *et al.* (17), in which the final body weights of mice fed with soybean oil (rich in LA) were higher than the body weights of mice fed with perilla and fish oils with a predominant content of *n*-3 acids.

In the present studies, the RBC count decreased after six months in the 14 : 1 group compared to the STD group. A similar effect was demonstrated for the intravenous administration of LA to rats, which reduced the total RBC counts (49). A falling number of red blood cells may indicate increased haemolysis. The effect of PUFAs on the erythrocyte parameters may be associated with their effect on the cell membrane. Rises in MPV may indicate platelet activation and aggregation, which larger platelets have to a higher degree. In this study, the 5 : 1 high-PUFA diet may have been responsible for increased platelet aggregation compared to the SFA diet.

In the presented studies, under the influence of high-fat diets (SFA, 14 : 1 and 5 : 1), the relative expression of the *Srebf1* gene at the level of mRNA coding for the SREBP-1 transcription factor was increased. Helpful information in determining the impact of the tested diets would be detailed elucidations of the level of SREBP-1 in the cytoplasm and the nucleus, because an increase in the SREBP-1 transcript is not a definitive result. It may indicate an increase in the mature form of SREBP-1c, which can activate

transcription of genes associated with *de novo* lipogenesis, or an increase in the precursor form of SREBP-1c in the cytoplasm, indicating impairment of proteolytic cutting to the mature form. The presented results show that high-fat diets probably increased the level of SREBP-1 in the nucleus, as evidenced by the increase in expression of the gene activated by SREBP-1 – the gene encoding fatty acid synthase (FASN). The *Fasn* mRNA level was increased after both periods of high-fat diets compared to the level in the liver tissue of mice on the standard diet over these periods. These results prove the increase in *de novo* lipogenesis as an effect of a high-fat diet. The changes are consistent with the study of Khateeb *et al.* (25).

In the liver of mice, the expression of PPARα-regulated genes increases under the influence of a high-fat diet. The transcription factor PPARα regulates gene expression in the liver, including expression of *Fads1* and *Fads2*, key enzymes of long-chain polyunsaturated fatty acid biosynthesis. The present results indicate increased *Fads1* transcript levels in all high-fat groups compared to the standard diet group but no change in *Fads1* between the SFA and 14:1 and 5:1 groups, although the SFA feed contained almost eight times less PUFA content. The increase in *Fads1* mRNA in the SFA group supports the novel enzymatic activity of fatty-acid desaturase 1 (FADS1) in oleic acid metabolism (37).

In the present study, the lack of difference in the *Fads1* expression between the two types of PUFA diet is in line with literature data, because both the LA and the ALA conversion pathways use Δ-5 desaturase. This may be due to FADS1 not only fulfilling the function of DGLA and eicosatetraenoic acid desaturase but also regulating the initiation and suppression of inflammation (14). Differences in the expression of the gene encoding desaturase Δ-6 (FADS2) in the liver were observed after six months of provision of experimental diets. They were shown between the groups of mice fed diets with a high PUFA content (14:1 and 5:1) and the group fed the diet with high SFA content. It may indicate that diets with different LA:ALA ratios similarly affected the regulation of PPARα despite the difference, and brought about the induction of *Fads1* and *Fads2* transcription, while the diet with saturated fatty acids affected PPARα in a different way.

The experiment showed that diets with different proportions of saturated and polyunsaturated fatty acids differently affected the levels of proteins associated with carbohydrate metabolism, one difference being notable in the induction of glycolytic proteins by the saturated fatty acid diet. Given the high rate of glycolysis in cancer cells (34), a diet with a low LA:ALA ratio has a higher potential to limit glycolytic processes and may have anti-cancer effects. Six months sustaining mice on a diet rich in SFA induced higher expression of the fructose-1,6-bisphosphatase gene (*Fbp1*) than this period on the STD and 5:1 PUFA diets. Fructose-1,6-bisphosphatase 1 is a key enzyme in gluconeogenesis (50). The stronger secretion of FBP1 caused by the saturated fatty acid and 14:1 diets may be a reason for higher blood glucose. This result is in line with that obtained by Kappe *et al.* (23), who showed a rise in fasting blood glucose in mice fed a high-fat diet. However, the increased glucose level in the mice eating the 5:1 PUFA diets is not related to FBP1. Changes in glucose may also result from the different regulations of the ChREBP transcription factor.

In the liver, OAT is a key enzyme in the urea cycle that metabolises ornithine to glutamate semialdehyde. Our research showed a reduction in the expression of the *Oat* gene at the transcript and protein levels in groups fed high-fat diets compared to the gene's expression in the group fed the STD diet after six months. These results are different from those obtained by Luo *et al.* (32). Those authors showed increased expression of the *Oat* gene at the protein level in the livers of mice kept on a high-fat diet compared to its expression in the livers of a group of animals kept on a low-fat diet. The discrepancy in results between studies could be due to the difference in the total fat content of the feed used by Luo *et al.* (32), who used feeds with higher fat contents – 10% and 60%.

It should be emphasised that excessive intake of PUFA is associated with intense oxidation of fatty acids, which promotes an increase in the concentration of very reactive free radicals. Reactive oxygen species production mainly arises in mitochondria (33). When the mechanisms responsible for the prevention of oxidative stress or increased oxidation are less active, reactive oxygen species accumulate. This causes oxidative stress which can lead to damage to the structure of lipids, proteins, carbohydrates and nucleic acids, consequently leading to dysfunction and even neoplastic transformation of cells. Biological membranes are susceptible to oxidative stress because electrons near the double bonds in the structure of phospholipids are unstable. Because double bonds are present in the carbon backbone, PUFAs are more sensitive to free radicals than SFAs (42). Polyunsaturated fatty acids located in the phospholipids of cell membranes are particularly sensitive to free radicals, *i.e.* $OH^{\bullet}$, $O_2^{\bullet-}$, during contact with which they may undergo an oxidation process called peroxidation. Increased PUFA content in the diet may increase the fraction of PUFA-rich membrane phospholipids, which are very easily peroxidated by free radicals. Peroxidation damages cell membranes, changes their properties, and causes harmful compounds to form, including some formed from *n*-3 acids, *e.g.* 4-hydroxy-2-hexenal, and some formed from *n*-6 acids, *e.g.* 4-hydroxy-2-nonenal and malondialdehyde (46). Peroxiredoxin 6 is a multifunctional enzyme with glutathione peroxidase, phospholipase A2 and lysophosphatidylcholine acyltransferase activities, which plays a protective role against oxidative damage. Oxidative stress is a potential inducer of *Prdx6* expression. Peroxiredoxin-6 reduces short-chain hydroperoxides and phospholipid hydroperoxides. It has been shown to play a major role in the repair of oxidised membrane phospholipids (11). It also acts as a cellular indicator of oxidative stress and is involved in the biosynthesis of fatty acid esters of hydroxy fatty acids. The level of

PRDX6 protein was increased in mouse liver after 6 months of high saturated diet and high-polyunsaturated diet 14:1. The mRNA level for *Prdx6* did not differ between groups.

Changes in the relative concentration of protein spots representing HBB-B1 were observed in the liver under the influence of nutrition. The decrease in HBB-B1 expression level occurred only as an effect of a six-month diet rich in PUFAs with the LA:ALA ratio of 5:1. The change in the level of HBB-B1 expression in the liver appeared to occur locally, because no significant changes in mean corpuscular haemoglobin concentration were detected in the haematological analysis. The main function of haemoglobin found in erythrocytes is the transport of oxygen from the lungs to tissues and carbon dioxide from the tissues to the lungs. The function of haemoglobin in other cells is not fully understood (31). Liu *et al.* (31) noted that both haemoglobin subunits were expressed in hepatocytes from patient biopsies as well as in the HepG2 cell line, and that the mRNA of HBB significantly increased in the livers of nonalcoholic steatohepatitis patients. Haemoglobin beta-1 subunit expression was induced by oxidative stress, because treatment of HepG2 cells with H_2O_2 increased HBB haemoglobin expression at both transcript and protein levels. Upregulation of haemoglobin subunit beta may also have been associated with the protective effect of HBB on cells, because overexpression of both HBA and HBB subunits was shown to reduce the oxidative stress induced by H_2O_2 (31).

Hepatocytes and Browicz–Kupffer cells are the main cell types responsible for iron storage. In hepatocytes, iron is stored in the cytoplasm, endoplasmic reticulum, mitochondria and lysosomes and is mainly bound to ferritin in the form of Fe^{3+} (35). Ferritin is a multimeric protein with a molecular weight of over 450 kDa, composed of heavy (H) and light (L) subunits. Heavy chains have ferroxidase activity consisting of oxidation of Fe^{2+} to Fe^{3+} ions. The L subunits do not have such activity, and their role is based on the mineralisation of Fe^{3+} in the core of the ferritin molecule. The ratio of H to L ferritin chains is tissue specific. Ferritin reduces the concentration of Fe^{2+} ions in the cytosolic labile iron pool (CLIP). Ions of Fe^{2+} participate in the Fenton reaction, of which the product is a highly reactive hydroxyl radical. Therefore, the regulation of iron oxidation and storage processes, which can be determined by the ratio of heavy ferritin chains to light chains, plays an important role in protecting against oxidative stress (6). In our research, both three-month and six-month provision of a diet with a high content of PUFAs and an LA:ALA ratio of 5:1 resulted in downregulated ferritin light chain 1 expression at the protein level. The weakening of FTL1 expression can increase the concentration of Fe^{2+} ions in CLIP and cause oxidative stress. Cells showing reduced expression of L ferritin show increased apoptosis and inhibition of cell proliferation *in vitro* (3). Given that ferritin light chains predominate in the liver, a decrease in their levels may indicate a decrease in total liver ferritin levels. The decrease in the FTL1 level under the influence of the PUFA diet may indicate the occurrence of several possible mechanisms: 1) reduction of *Ftl1* transcription, 2) post-transcriptional regulation of *Ftl1* mRNA and 3) degradation of FTL1, *e.g.* in lysosomes. Changes in the expression level of FTL1 may also be associated with the effect of PUFAs on the process of ferroptosis. This may be theorised because Yang *et al.* (47) showed that LA and AA sensitise cells to induced ferroptosis, which is associated with the toxic accumulation of phospholipid peroxidation products containing PUFAs. Studies by Kagan *et al.* (21) indicated that phosphatidylethanolamines containing AA and AdA were a key class of phospholipids that undergo oxidation and promote ferroptosis. Significant changes in the expression of the FTL1 gene may also be associated with the different effects of PUFAs and SFAs on the ferritin molecule. Bu *et al.* (7) proved there were binding sites for free fatty acids in the ferritin molecule and demonstrated a higher affinity for ferritin by unsaturated AA 20:4*n*-6 acid than saturated 8:0 caprylic acid. As a result of AA binding to ferritin, increased iron mineralisation and protection of AA against oxidation have also been shown. The results of the present study on the effect of PUFAs on the level of FTL1 protein expression differ from those obtained at the transcript level by Sakai *et al.* (38), who showed that *n*-3 fatty acids increase the mRNA level for FTL. The differences between the influence of *n*-3 fatty acids on the transcript level and the FTL protein level may indicate the occurrence of post-transcriptional regulation, because the regulation of *Ftl1* expression occurs in two stages – transcription regulated by the transcription factor Nrf2 followed by translation through iron regulatory proteins binding to the iron response element located on *Ftl1* mRNA (24). Human studies confirm that serum ferritin concentration is associated with several phospholipids containing long-chain polyunsaturated fatty acids.

In the experiment, a reduction in the expression of the α subunit gene of the eukaryotic translation initiation factor 2 (*Eif2s1*) at the protein level was observed under the influence of the high-ALA (5:1) diet. This may indicate the influence of *n*-3 fatty acids on the translation initiation process. A similar relationship was proposed in studies of Aktas and Halperin (1), in which EPA increased the amount of the phosphorylated form of eIF2α by the release of Ca^{2+} from the endoplasmic reticulum, which consequently lowered the rate of translation initiation.

Significant increases in the expression of the apoptotic gene annexin 5 (*Anxa5*) on protein level were evident in the high-fat 14:1 diet over the expression of the gene in the STD diet group. A similar result for the mRNA level was obtained by Miller *et al.* (36), but the high-fat diet in that research was rich in SFAs. In the present study, there was also a difference in the level of the ANXA5 protein between the 5:1 and 14:1 groups. The role of ANXA5 is still not entirely known; the protein may contribute to fat deposition, storage or

mobilisation (13). Moreover, Xu *et al.* (45) identified that ANXA5 induces a hepatic macrophage phenotype shift from M1 to M2, as well as metabolic reprogramming from glycolysis to oxidative phosphorylation. The downregulating of ANXA5 can affect macrophage activity in regulating the inflammation process.

The presented proteomic analyses provide more profound insight into the differences in liver protein expression which arise from diets constituted with different FA contents and from provision of them for particular durations. Variations in the expression of proteins showed diet-duration association, some changes occurring after three months of the diet, and others being observed only after six months. For several differentiated proteins including OAT and FTL1, the expression pattern was similar at both time intervals, whereas for other proteins including FBP1, the pattern of changes was different for each interval. This may suggest that the diet duration is one of the factors in changes in the liver protein profile.

Conclusion

A high-PUFA diet modulated the level of liver proteins involved in critical metabolic pathways, including amino acid metabolism (*e.g.* ornithine aminotransferase), carbohydrate metabolism (*e.g.* fructose-1, 6-bisphosphatase 1) and the cellular response to oxidative stress (*e.g.* peroxiredoxin 4). High PUFA diets with LA:ALA ratios of 14:1 and 5:1 decreased the relative concentrations of TKFC and ALDH1A1 compared to the saturated fatty acid diet, which suggest that PUFAs influence hexose metabolism, especially fructose catabolism. The qPCR results showed increased liver *Fasn* and *Fads1* mRNA expression in groups given the high-fat diet compared with the standard-feed-receiving group, suggesting that high-fat diets accelerate fatty acid metabolisms in the liver. Long-term provision of the SFA diet decreased *Fads2* expression. In addition, high-PUFA and high-SFA diets induced distinct changes in the metabolism of long-chain PUFAs, possibly contributing to the fatty acid profile shift in the liver. Long-term consumption of high PUFA diet with LA:ALA ratio of 14:1 increased the production of the antioxidant proteins PRDX4 and PRDX6 that scavenge cellular reactive oxygen species. It suggests that a high-PUFA 14:1 diet may induce stronger oxidative stress than a 5:1 diet. The results obtained prove that *n*-6 and *n*-3 fatty acids play a vital role in liver function and affect the level of hepatic proteoforms differently. Thus, these presented results broaden the knowledge of the impact of low and high LA:ALA ratios in the diet as different regulators of gene expression, as well as confirm the importance of including those bioactive components in diets to improve homeostasis and health.

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