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Original Paper

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Key Words
Tff3 deficient mice • Metabolism • Fatty acids profile • Oxidative stress

Abstract
Background/Aims: Tff3 protein plays a well recognized role in the protection of gastrointestinal mucosa. The role of Tff3 in the metabolism is a new aspect of its function. Tff3 is one of the most affected liver genes in early diabetes and fatty liver rodent models. The aim of this study was to investigate the effect of Tff3 deficiency on lipid and carbohydrate metabolism and on markers of oxidative stress that accompanies metabolic deregulation. ***Methods:*** Specific markers of health status were determined in sera of Tff3 deficient mice, including glucose level, functional glucose and insulin tolerance. Composition of fatty acids (FAs) was determined in liver and blood serum by using gas chromatography. Oxidative stress parameters were determined: lipid peroxidation level via determination of lipid hydroperoxide and thiobarbituric acid reactive substances (TBARS), antioxidative capacity (FRAP) and specific antioxidative enzyme activity. The expression of several genes and proteins related to the metabolism of lipids, carbohydrates and oxidative stress (CAT, GPx1, SOD2, PPARα, PPARγ, PPARδ, HNF4α and SIRT1) was determined. ***Results:*** Tff3 deficient mice showed better glucose utilization in the glucose and insulin test. Liver lipid metabolism is affected and increased formation of small lipid vesicles is noticed. Formation of lipid droplets is not accompanied by increased liver oxidative stress, although expression/activity of monitored enzymes is deregulated when compared with wild type mice. Tff3 deficient mice exhibit reduced expression of metabolism relevant SIRT1 and PPARγ genes. ***Conclusion:*** Tff3 deficiency affects the profile and accumulation of FAs in the liver, with no obvious oxidative stress increase, although expression/activity of monitored enzymes is changed as well as the level of SIRT1 and PPARγ protein. Considering the strong

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downregulation of liver Tff3 in diabetic/obese mice, presence in circulation and regulation by food/insulin, Tff3 is an interesting novel candidate in metabolism relevant conditions.

Introduction

Trefoil factor family proteins (Tff1, Tff2, Tff3) are small and compact peptides containing one (Tff1, Tff3) or two (Tff2) so called trefoil domains [1]. They are highly expressed in the gastrointestinal tract as secretory proteins involved in the maintenance of epithelial barrier integrity during the process of restitution upon mucosal injury [2]. Namely, mice with targeted disruption of intestinal trefoil factor (Tff3) exhibited impaired mucosal healing and died from extensive colitis after oral administration of dextran sulfate sodium, an agent that causes mild epithelial injury in wild type mice. Tff3 is present in human serum at a high concentration (200 pmol/L) but the origin of circulating Tff3 is unknown [3]. However, a body of evidence demonstrates that Tff proteins (Tffs) are widely distributed [4] and may play other important roles also in the immunological defense [5]. In addition, Tffs have been increasingly recognized as novel participants in complex metabolic interactions [6].

Tff3 protein expression in normal human liver is localized mainly to the large bile ducts and mucinous peribiliary glands [6]. The role of Tff3 in intestinal inflammatory diseases [7] and regulation by multiple regulatory pathways [5] is well recognized. Potential role of *Tff3* gene in the metabolism relevant condition called diabesity (combination of diabetes and obesity) was revealed for the first time by quantitative trait locus (QTL) analysis of the mouse diabesity model, Tally Ho mouse strain [8]. When comparing mRNA transcriptome in relevant tissues (adipose tissue, pancreas, and liver), *Tff3* mRNA was found as being most affected in the liver, where it was reduced by almost 600 times in the early diabetic state of these mice [8]. In another study, serum level of Tff3 was increased by insulin, glucose and food intake in T1D patients as well as in healthy subjects [9]. In addition, Tff3 overexpression *in vivo* improved glucose tolerance in a dietary induced obesity mouse model without affecting the body weight, fasting insulin, triglyceride, cholesterol and leptin levels [10]. Recently, Xue et al. [11] reported that overexpression of Tff3 in primary mouse hepatocytes inhibited the expression of gluconeogenic genes and improved glucose tolerance and insulin sensitivity. They also showed that hepatic Tff3 expression levels were decreased in obese mice and in the high fat diet induced mouse model. Moreover, hepatic *Tff3* expression levels were decreased in obese and high fat diet induced mice compared to control mice [12].

Blood glucose homeostasis is regulated by the liver: in fasting time, the liver synthesizes glucose through both glycogenolysis and gluconeogenesis, or in the fed state, hepatocytes store excess glucose as glycogen [13]. In the fed state, hepatocytes use glycolytic products in the process of *de novo* lipogenesis to synthesize fatty acids. Long chain fatty acids in hepatocytes are incorporated into triacylglycerol, phospholipids, and/or cholesterol esters. These complex lipids are stored in lipid droplets and membrane structures, or secreted into the circulation as very low density lipoprotein particles [14]. Deregulated liver metabolism promotes insulin resistance, diabetes and nonalcoholic fatty liver diseases (NAFLD) [15]. Recently it was demonstrated that liver triglyceride accumulation does not cause cellular injury *per se* in the liver. Free fatty acids or their metabolites are primarily responsible for liver injury via increased oxidative stress (OS) [16]. Changes in lipid metabolism, in particular the increase of saturated fatty acids, is associated with increased endoplasmatic reticulum (ER) stress, oxidative stress and liver injury in the course of development of fatty liver disease [17]. Oxidative stress, as an overall state of substantial imbalance between reactions of free radicals, as oxidizing species, and antioxidants within the cells and tissues, leads to oxidative damage of proteins, lipids, and DNA. Accumulation of lipid (hydro)peroxides and their degradation products, due to lipid peroxidation processes and stimulating glutathione depletion, leads to oxidative damage of different organelles in liver tissue and participates in fatty liver disease development [18]. Increased Tff3 gene expression is noticed in the late phase of response of human tumor cells to oxidative stress [19].

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SIRT1 has attracted a lot of interest as it plays a key role in metabolic regulation, adaptation and oxidative stress. It acts as a nuclear metabolic sensor and deacetylates a wide range of targets, leading to epigenetic modifications of histones and modulation of transcription factors or metabolic enzymes [20]. It has been shown that downregulation of sirtuins has an important role in the pathophysiology of fatty liver disease in humans and in animal models [21]. Besides SIRT1, peroxisome proliferator-activated receptors (PPARs) also play an important role in cell metabolism. They are ligand inducible transcription factors (nuclear receptors) that modulate the expression of genes involved in adipogenesis, lipid and glucose metabolism, energy homeostasis and inflammation [22]. In mammals, there are three PPARs: PPARα, PPARβ/δ and PPARγ. Among them, PPARγ as a nuclear receptor central to glucose and lipid homeostasis appears to play a major role in promoting development of fatty liver [23].

The aim of this study was to investigate the effect of Tff3 deficiency on lipid and carbohydrate metabolism by using Tff3 knockout mouse model (accepted nomenclature for: genotype: *Tff3 -/-*; protein: Tff3 deficiency). We have determined specific markers of health status in the sera of Tff3 deficient mice, as well as the glucose level and response to glucose and insulin overload. Oxidative stress level was assessed by determination of lipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS), antioxidative capacity (FRAP) and antioxidative enzyme activity. Given that the liver is one of the major metabolic organs involved in lipid metabolism, we have determined the impact of Tff3 deficiency on the level of hepatic neutral lipid accumulation and FAs profile, which was correlated with FAs profile in blood serum. Furthermore, the expression of several genes related to metabolism of lipids, carbohydrates and oxidative stress (CAT, GPx1, SOD2, PPARα, PPARγ, PPARδ, HNF4α and SIRT1) was assessed. The protein level of relevant genes was determined to provide possible mechanistic explanation.

Materials and Methods

All used chemicals were of analytical reagent grade purity: KOH (Merck, Germany), KCl, EDTA, NADPH, sodium azide, H_2O_2, MDA, TBA, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), Trolox, $MgCl_2$ (Sigma-Aldrich, Germany). All solvents (Merck, Germany) were used without further purification.

Animals and sample collection
All animal housing conditions and research protocols were approved by the National Local Ethical Committee. Trefoil factor 3 protein-deficient mice were a gift from Prof. W. Hoffmann (previously described [2] (nomenclature: genotype: *Tff3 -/-*; protein: Tff3), as well as the appropriate wild type (WT) mice of mixed background (C57Bl6/ Sv/129 mice). They were kept at the Animal Facility of the Faculty of Medicine Osijek (Croatia). Care and use of the animals and the experimental protocol were reviewed and approved by the Institutional Ethical Committee. Mice were kept under standard conditions and fed (*ad libitum*) with standard chow diet until 12 weeks old, when they are deemed to represent physiologically matured mice. The mice were fasted for 6 hours before being sacrificed. The animals (males, min. 7 per group) were sacrificed, and blood was collected for further general blood chemistry analysis comprising the following routine blood biochemistry analysis: Fe, high-density lipoprotein (HDL), glucose, cholesterol, aspartate transaminase (AST), alanine transaminase (ALT), urea, creatinine, bilirubin, triglycerides (TG), alkaline phosphatase (AP), total proteins (TP), Na, K, amylase, lactate dehydrogenase (LDH), creatine kinase (CK). Liver tissue samples were snap-frozen in liquid nitrogen or fixed in 10% formalin and processed for regular histological analysis (HE, PAS) and lipid staining (Oil Red). Tissue sections were examined by experienced pathologist blinded to genetic origin of the mice.

Glucose and insulin tolerance test
Tff3 -/- and WT mice were used to perform glucose or insulin tolerance test. Mice (n=7) were fasted for 6 hours before intraperitoneal injection of glucose (2 g/kg body mass; Merck, Germany) or insulin (0.75 IU/g body weight; Levemir, Novo Nordisk, UK). Blood glucose concentration measurements were taken

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from the tail vessels of the mice, at the following points in time: 0 (just before glucose injection), 15, 30, 60, 90 and 120 min after glucose or insulin injection.

Analysis of fatty acids

Liver tissue (0.2 g) was cut into small pieces and homogenized in PBS. Total lipids were extracted from serum (50 μl) and from tissue homogenates according to Bligh and Dyer [24]. The lipid extract was treated with 0.5 M KOH/MeOH (Merck, Germany) for 20 min at room temperature [25], and the corresponding fatty acid methyl esters (FAMEs) were formed, extracted with n-hexane (Merck, Germany), and analyzed by gas chromatography (GC). An internal standard (C19:0, Sigma Aldrich, Germany; 8.13 μg) was added to all samples. The FAME samples from liver and serum were diluted with 250 μl and 100 μl of n-hexane, respectively. 1 μl was injected to GC in a split mode (20:1). GC analyses of FAME were performed by using Varian 450-GC equipped with a flame ionization detector. A Stabilwax column (crossbond carbowax polyethylene glycol, 60m × 0.25 mm; Restek) was used as a stationary phase at a programmed temperature, with helium (Messer, Germany) as the carrier gas. The heating was carried out at a temperature of 150 °C for 1 min, followed by an increase of 5 °C/min up to 250 °C. The methyl esters were identified by comparison with retention times of standard commercially available mixtures (Marine oil FAME mix, Restek, USA). Individual fatty acids were determined in the liver (n=7) and serum (n=7) of wild type and Tff3 deficient mice and expressed as relative percentages of FAME obtained from total lipid extract. The relative amount of each fatty acid (percentage of FAME) was quantified by integrating the area under the peak and dividing the results by the total area for all fatty acids.

Quantitative real-time PCR analysis

Total RNA was extracted from individual mouse livers by RNeasy spin columns (Qiagen, MD, USA), followed by DNase treatment in accordance with the manufacturer's protocol. RNA was reverse transcribed by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA), according to the manufacturer's instructions, by using 1 μg of total RNA. qPCR analysis was carried out on an ABI 7300 sequence detection system by using predesigned TaqMan Gene Expression Assays, Thermo Fisher scientific (MA, USA): Beta-actin (Mm00607939_s1) as reference gene; Superoxide dismutase 2 (Mm01313000_m1), Catalase (Mm00437992_m1), PPARα (Mm00627559_m1), PPARβ/δ (Mm01305434_m1), PPARγ (Mm00440940_m1), HNF4α (Mm00433964_m1), GPx1 (Mn00656767_g1) and SIRT1 (Mm00490758_m1). The data were presented as whisker-box plots based on the permutated expression data. Expression variation is visualized for each gene in a whisker-box plot to provide additional information about the skew of the data distributions that would not be available simply by plotting the sample mean [26].

Western blot analysis

Liver samples were homogenized (Potter-Elvehjem homogenizer; 1300×g) with RIPA buffer (Thermo Fisher scientific MA, USA) supplemented with proteinase inhibitors (10% w/v) (Roche, Switzerland), sonicated (3 x 30 sec) and centrifuged at 16 000 g/20 min in a refrigerated centrifuge. Supernatant was collected and total cellular proteins (100 μg per lane) were resolved by denaturing SDS-PAGE and transferred on a PVDF or nitrocellulose membrane (Bio-Rad, CA, USA). Membranes were blocked in 5% nonfat dry milk (Bio Rad, CA, USA) in TN buffer (50 mM TRIS; Merck, Germany; 150 mM NaCl; Merck, Germany pH=7.4) and incubated overnight (4 °C) with appropriate primary polyclonal antibody SIRT1, iNOS and PPARγ (Santa Cruz Biotechnology, TE, USA; diluted 1:200), PMP70 (Thermo Fisher scientific, MA, USA; diluted 1:500). Antibodies against SOD2, CAT, GPx1 (Abcam, UK; diluted 1:200) were incubated 3 hours at room temperature. Donkey anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, UK) was incubated for 3 hours at room temperature. Equality of loading was confirmed by using AmidoBlack (Sigma Aldrich, USA), which was also used for normalization of the bands [27]. The chemiluminescence signals were detected and analyzed with the Alliance 4.7 Imaging System (UVITEC, Cambridge, UK).

Oxidative stress parameters
Antioxidant enzyme activities assay

Liver samples were weighed and crushed with liquid nitrogen and homogenized in an adequate solution with Ultra turrax T10 homogenizer while kept on ice. For SOD and GPx activity assay, liver tissue was homogenized (1:10, w/v) in 50 mM phosphate buffer (pH=7.8) and for CAT activity assay (1:10, w/v) in 100 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Total SOD activity was determined by inhibition

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of reduction of cytochrome C (0.05 mM) in PBS buffer saline with 0.1 mM EDTA in the system of the xanthine (1 mM)/xanthine oxidase (50 U). GPx activity was assessed indirectly at absorbance wavelength of 340 nm during 5 minutes of NADPH oxidation to NADP+. The assay mixture consisted of 50 mM phosphate buffer with 0.4 mM EDTA and 1 mM sodiumazide (pH= 7.0), 0.12 mM NADPH, 3.2 units of GR, 1 mM glutathione, and 0.0007% (w/w) H_2O_2. One unit of GPx activity catalyzes the oxidation by H_2O_2 of 1.0 µmol of reduced glutathione to oxidized glutathione per minute at pH=7.0 and 25 °C. GPx activity was calculated by using molar extinction coefficient for NADPH disappearance (ε = 6220 dm^3 mol^{-1} cm^{-1}) [28]. CAT activity was measured using H_2O_2 (0.036%) as a substrate in the reaction mixture with 50 mmol/L phosphate buffer pH (7.0). Absorbance was measured at 240 nm during 2 minutes, every 10 seconds after adding the sample. One unit of activity corresponds to the loss of 1 µmol of H_2O_2 per minute. CAT activity was calculated by using molar extinction coefficient (ε = 0.04 dm^3 mol^{-1} cm^{-1}) [28]. Activity of enzymes was expressed as U/mg protein (U/mg P) and protein concentration was determined by Bradford protein assay. All measurements were done at Lambda 25 UV-Vis spectrophotometer equipped with UV WinLab 6.0 software package (PerkinElmer For the Better, Waltham, Massachusetts, USA) [29].

TBARS and FRAP

Liver samples were weighed and crushed with liquid nitrogen and homogenized in ice-cold 1.15% KCl solution (1:10, w/v) by using Ultra turrax T10 homogenizer while kept on ice. Malondialdehyde (MDA), the final degradative product of lipid hydroperoxides formed in lipid peroxidation process, reacts with thiobarbituric acid and the colored products were used as indicators of oxidative stress [30]. The absorbance of the sample was measured at 572 and 532 nm against the reagent and results were expressed as µmol MDA by using a calibration curve obtained with MDA.

Antioxidant capacity was assessed by using FRAP. Presence of antioxidants caused blue discoloration of solution due to reduction of the (Fe^{3+} TPTZ) complex to (Fe^{2+} TPTZ) complex [31]. Trolox was used as standard (Sigma). The absorbance of the sample was measured at 593 nm and results were expressed as mM Trolox (mM TE). Both spectrophotometric methods were carried out on Nanophotometer P300 UV/VIS, IMPLEN.

Lipid hydroperoxide analysis in isolated lipid extracts

Liver samples were snap-frozen and stored at -80 °C until analysis. Lipids were extracted according to the modified method of Kramer et al. [32]. Briefly, 0.2 g of liver tissue was cut and homogenized in PBS. Lipid extraction started by adding 5 ml of $CHCl_3$ to the homogenate, followed by vigorous shaking. The solution was washed with 1.5 ml of 0.034% $MgCl_2$, centrifuged (2 min, at 3000×g) and the aqueous layer was drawn off by aspiration using a Pasteur pipette. The washing procedure was repeated with 2.5 ml of 2M KCl/MeOH (4:1, v/v) to eliminate all proteins and non-lipid contaminants. $CHCl_3$ layer was finally washed with $CHCl_3$/MeOH (2:1, v/v) and centrifuged for 5 min at 3000×g. The organic layer containing lipids was carefully transferred to a glass tube and solvent was removed on a rotary evaporator. After weighing, the lipids were stored at -20 °C until analysis of lipid hydroperoxides (LOOH). Spectrophotometric ferric thiocyanate assay was used for determination of LOOH concentration. Samples were prepared according to the following procedure [33]. The analyzed samples were prepared by diluting with a deaerated mixture of CH_2Cl_2/MeOH (2:1, v/v). The concentrations of LOOH were calculated by using the molar absorptivity of the complex $[FeNCS]^{2+}$ formed per mol of LOOH, 58 440 dm^3 $mol^{-1}cm^{-1}$, at 500 nm [33].

Statistical analysis

The differences between WT and *Tff3-/-* mice were compared with Student t test or with Two-way ANOVA and Bonferonni post-Hock test (GTT and ITT). For all tests, the significance level was set at p≤0.05. Gene expression data were analyzed by REST 2009 expression analysis software [26].

Results

Biochemistry

Generally, Tff3 deficiency did not affect body weight so there was no significant difference between WT (24, 43±1.05) and *Tff3 -/-* mice (23.28±0.54) on a standard diet (mean value ±

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SEM). Biochemical analysis of WT and Tff3 deficient mice sera (Table 1) showed no difference in kidney function (urea and creatinine), lipid metabolism (HDL, TG) or liver function (ALT, AST). Tff3 deficient mice had a significantly lower total protein level (reduced by 20%) and serum amylase concentration (22% reduction) compared with WT mice (Table 1).

Glucose and insulin tolerance test

Blood glucose concentration was not changed in Tff3 deficient mice compared with WT mice (Table 1). Glucose tolerance test (Fig. 1A) showed that Tff deficient mice had a significantly lower level of glucose at 15 and 30 min upon its administration, while at other time intervals the glucose concentration was similar to glucose concentration in the blood of WT mice. Insulin tolerance test (Fig. 1B) showed that Tff3 deficient mice utilize glucose from the blood stream more effectively at 15 and 30 min upon insulin injection, compared with WT mice. Glucose concentrations were similar at other time points.

Histomorphology of liver

Histomorphology of the liver was not affected by Tff3 deficiency and the PAS staining showed no change in liver glycogen amount (data not presented). Oil Red staining of the liver (Fig. 2A, B) demonstrated that Tff3 deficient mice had an increased number of smaller lipid containing vesicles in the hepatocytes compared with WT mice on a standard diet.

Liver and serum fatty acids profile

Composition of fatty acids (FAs) was determined in liver and serum of WT and Tff3 deficient mice

(Table 2 and 3). The major fatty acids detectable were: palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1, ω-9), linoleic acid (LNA, 18:2, ω-6), arachidonic acid (AA, C20:4, ω-6) and docosahexaenoic acid (DHA, 22:6, ω-3). The monitored FAs were expressed as a relative percentage of FAME obtained from total lipid extract of the liver (Fig. 3A) and serum (Fig. 3B). Total lipid content showed no statistically relevant change, although it was slightly higher in Tff3 deficient mice (9.2±1.8 [mg/mg liver]) compared with WT mice (7±0.8 [mg/mg liver]).

Tff3 deficient mice had a significantly changed ratio of numerous FA in liver (Fig. 3A) and sera (Fig. 3B) compared with WT mice. The ratio of myristic acid (C14:0) was reduced (1.6-fold) in the liver of *Tff3 -/-* compared with WT mice, while stearic (C18:0) and arachidic (C20:0) FA ratio was increased (both 1.5-fold) in the liver of *Tff3-/-* mice. Serum ratio of arachidic acid (C20:0) increased 1.8-fold, as the only statistically affected

Table 1. Biochemical analysis of the blood sera. Data are presented as mean ± SEM and *Tff3 -/-* mice compared with wild type mice by using Student t-test. Statistically significant changes are marked (**p≤0.01 and ***p≤0.001). a-aspartate transaminase; b-alanine transaminase; c-triglycerides; d-alkaline phosphatase; e- total proteins; f-lactate dehydrogenase; g-creatine kinase

|	Unit	Wild type	Tff3-/- mice
Fe-PI	μmol/l	32.4±2.13	29.2±1.67
HDL	mmol/l	1.58±0.05	1.18±0.13
Glucose	mmol/l	11.5±0.99	10.7±0.52
Cholesterol	mmol/l	2.09±0.08	1.84±0.13
AST[a]	U/l	323.2±89.20	349.7±70.20
ALT[b]	U/l	18.7±8.83	25.8±6.88
Urea	mmol/l	11.7±0.23	10.8±0.52
Creatinine	μmol/l	40.1±1.13	37.4±1.05
Bilirubin	μmol/l	2.03±0.13	2.18±0.26
TG[c]	mmol/l	1.40±0.07	1.28±0.16
AP[d]	U/l	93,0±16.72	82.8±6.95
TP[e]	g/l	53.5±2.16	47.8±0.93**
Na-C	mmol/l	153.0±1.13	149.2±1.66
K-C	mmol/l	6.37±0.65	9.65±0.33
Amylase	U/l	1738,7±198.40	1344.4±159.60
LDH[f]	mmol/l	655.0±193.85	643.0±109.69
CK[g]	mmol/l	771.2±194.16	794.1±228.62
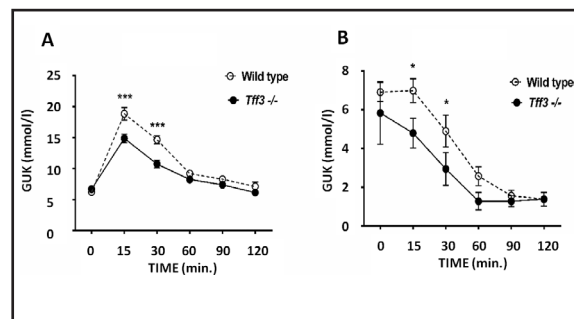
Fig. 1. Intraperitoneal glucose tolerance (GTT) and insulin tolerance (ITT) test in wild type and *Tff3 -/-* mice. (A) Glucose tolerance test (2 g/kg body mass) and (B) insulin tolerance test (0.75 IU/g body weight) at time points 0, 15, 30, 60, 90 and 120 min after glucose or insulin injection. Data are presented as mean values ± SEM and compared with WT mice using Two-way ANOVA and Bonferonni post-Hock test. Statistically significant time points are marked as ** p≤0.01 and *** p≤0.001.

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saturated FA in *Tff3 -/-* mice. The level of saturated FA was slightly but statistically significantly increased (1.1-fold) in the liver of *Tff3 -/-* mice, while the sera content was unaffected.

Monounsaturated fatty acids (MUFAs) were significantly reduced in the liver of *Tff3 -/-* mice compared with their WT controls. Palmitoleic acid (C16:1) was reduced 2.6-fold, oleic acid (C18:1) was reduced 1.8-fold, vaccenic acid (C18.1) was reduced 1.7-fold, and eicosenoic acid (C20:1) was reduced 1.3-fold. Significantly changed MUFAs in sera of Tff3 deficient mice compared with WT were: vaccenic acid (C18:1), with 1.5-fold reduction, and eicosenoic acid (C20:1), with an almost 2-fold increased level. Altogether, levels of MUFAs were statistically decreased 1.7-fold in the liver of *Tff3 -/-* mice, while the proportion in sera was not affected by Tff3 deficiency.

At the same time, polyunsaturated fatty acid (PUFAs) had a statistically changed ratio in the liver of Tff3 deficient mice: eicosadienoic acid (C20:2) was increased 1.45-fold,

Fig. 2. Oil red staining of neutral lipids in the liver of male WT (A) and *Tff3*-/- (B) mice

Table 2. Relative percentage of fatty acids as methyl esters (FAME) of FAME in liver homogenate of wild type and *Tff3 -/-* mice expressed as % in total lipid content. RATIO ω-3/ω -6=(ALA+DHA+EPA)/(AA+LNA). Data are presented as mean value ± SEM (n=7 per genotype; Student t test). Statistically significant changes are marked as * p<0.05, **p>0.01 and ***p<0.001

Fatty acids	Wild type [rel % FAME]	Tff3 -/- [rel % FAME]	Fold change
SATURATED			
C14:0 myristic acid	0.34±0.05	0.21±0.03***	1.6↓
C16:0 palmitic acid	27.92±2.57	28.06±0.88	
C18:0 stearic acid	8.80±0.81	13.16±0.65***	1.5↑
C 20:0 arachidic acid	0.28±0.06	0.42±0.07**	1.5↑
C 22:0 behenic acid	0.11±0.04	0.09±0.01	
C 24:0 lignoceric acid	0.61±0.13	0.65±0.09	
MONOUNSATURATED			
C16:1 palmitoleic acid (ω-9)	3.30±0.62	1.28±0.27***	2.6↓
C18:1 oleic acid (ω-9)	20.10±2.36	11.22±1.55***	1.8↓
C18:1 vaccenic acid (ω-7)	2.80±0.81	1.7±0.28**	1.7↓
C20:1 eicosenoic (gondoic acid) (ω-9)	0.56±0.09	0.43±0.04**	1.3↓
C22:1 erucic acid (ω-9)	0.06±0.02	0.06±0.01	
POLYUNSATURATED			
C18:2 (LNA) linoleic acid (ω-6)	20.32±2.78	20.49±0.98	
C20:2 eicosadienoic acid (ω-6)	0.25±0.01	0.34±0.01***	1.4↑
C20:4 (AA) arachidonic acid (ω-6)	6.36±0.90	9.69±0.64***	1.5↑
C18:3 (ALA) alpha linolenic (ω-3)	0.63±0.16	0.42±0,08**	1.5↓
C20:5 (EPA) eicosapentaenoic (ω-3)	0.88±0.21	0.95±0.14	
C22:6 (DHA) docosahexaenoic (ω-3)	6.68±0.48	10.83±1.77***	1.6↑
SATURATED FAs	38.06±3.33	42.59±1.67***	1.1↑
MONOUNSATURATED FAs(MUFAs)	26.82±2.60	14.69±2.00***	1.7↓
POLYUNSATURATED FAs (PUFAs)	35.12±2.60	42.72±1.30***	1.2↑
RATIO ω-3/ω-6	0.30±0.049	0.40±0.06*	1.3↑
Total	100%	100%	

arachidonic acid (AA, C20:4, ω-6) increased 1.4-fold, alpha linolenic acid (ALA, C18:3, ω-3) decreased 1.5-fold and docosahexaenoic acid (DHA, C22:6, ω-3) increased 1.6-fold. Statistically changed PUFAs in sera of Tff3 deficient mice were: linoleic acid (C18:2, ω-6), which increased 1.3-fold, and alpha linolenic acid (ALA, C18:3, ω-3), the proportion of which was reduced 3-fold.

The amount of total PUFAs was significantly increased (1.2-fold) in the liver of *Tff3 -/-* mice, while the proportion in the sera was not affected by Tff3 deficiency. *Tff3 -/-* mice exhibited significant modification of the ω-3/ω-6 ratio. Liver ω-3/ω-6 ratio was increased (1.3-fold), while the serum ratio was decreased (1.9-fold).

The complex synthesis relationship of monitored FAs is presented (Fig. 4) with marked changes in *Tff3 -/-* mice compared with wild type (elevated ↑, decreased ↓ and no change θ). Activity of SCD-1 enzyme was calculated based on the ratio of specific FAs content (C18:1/C18:0 and C16:1/C16:0), which was reduced 2.7-fold in Tff3 deficient mice compared with WT ones.
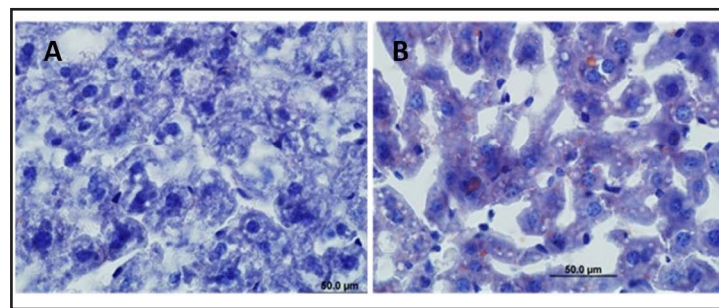
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Expression of SIRT1, PPARα, PPARγ, PPARδ, SOD2, CAT, GPx1 and HNF4α genes in liver

Expression of several oxidative stress and FA metabolism regulation relevant genes (SIRT1, PPARα, PPARγ, PPARδ, SOD2, CAT, GPx1 and HNF4α) was monitored in the liver of WT and Tff3 deficient mice (Fig. 5). Tff3 deficient mice exhibit a rather scattered but strong reduction of PPARγ mRNA (it was reduced 7.7-fold compared with WT). Expression of mRNA for SIRT1, PPARα, PPARδ, SOD2, CAT, GPx1, HNF4α was not significantly changed by Tff3 deficiency.

SOD2, CAT, GPx1, PPARγ, SIRT1 and PMP70 protein expression in liver

Relevant protein expression in liver homogenate of *Tff3-/-* mice was presented relative to the content in WT mice, which was set as 100% (Fig. 6). Level of SOD2, CAT, GPx1, PMP70 was not affected by Tff3 deficiency. No activation of inducible NOS (iNOS) was found in any of the genotypes. *Tff3-/-* mice had a strong reduction of nuclear receptor SIRT1 (reduction to a level equivalent to 25% of WT level; p<0.001) and PPARγ protein level (reduction to 54% of WT level; p=0.003).

Activity of antioxidative enzymes, oxidative stress (TBARS), antioxidative capacity (FRAP) and lipid peroxidation in the liver of WT and Tff3 -/- mice

Activity of liver SOD was not significantly affected in *Tff3 -/-* (Fig. 7). Tff3 deficient mice had a significantly reduced activity of liver CAT (68 % of WT activity), while the activity of GPx was significantly increased (158 % of WT activity). Tff3 deficiency had not affected the liver oxidative stress [TBARS (mmol MDA/mg tissue): wt ($3.60 \times 10^{-5} \pm 1.38 \times 10^{-5}$) vs *Tff3-/-* ($6.38 \times 10^{-5} \pm 1.77 \times 10^{-5}$)], antioxidative capacity [FRAP (mmol Trolox/mg tissue): wt (0.40 ± 0.01) vs. *Tff3-/-* (0.39 ± 0.02)] and lipid peroxidation [LOOH(mmol LOOH /mg total lipids): wt ($1.90 \times 10^{-6} \pm 2.79 \times 10^{-7}$) vs. *Tff3-/-* ($2.25 \times 10^{-6} \pm 3.76 \times 10^{-7}$)].

Table 3. Relative percentage of fatty acids as methyl esters (FAME) in blood serum of wild type and *Tff3 -/-* mice expressed as % of FAME in total lipid content. Nd. Means not detected due to low concentration. RATIO ω-3/ω-6 = (ALA+DHA+EPA) / (AA+LNA). Data are presented as mean value ± SEM (n=6 per genotype, Student t test). Statistically significant changes are marked as * p≤0.05, **p≤0.01 and ***p≤0.001

| Fatty acids	Wild type [rel % FAME]	Tff3 -/- [rel % FAME]	Fold change
SATURATED			
C14:0 myristic acid	1.17±0.44	1.13±0.34	
C16:0 palmitic acid	24.42±1.42	24.89±1.69	
C18:0 stearic acid	10.70±1.51	10.61±1.01	
C20:0 arachidic acid	0.26±0.07	0.46±0.10***	1.8↑
C22:0 behenic acid	Nd	Nd	
C24:0 lignoceric acid	0.48±0.11	0.41±0.07	
MONOUNSATURATED			
C16:1 palmitoleic acid ω-7	2.40±0.74	1.65±0.49	
C18:1 oleic acid ω-9	14.89±2.04	13.71±0.84	
C18:1 vaccenic acid ω-7	2.82±0.42	1.44±0.28**	1.5↓
C20:1 eicosenoic (gondoic acid) ω-9	0.46±0.29	0.89±0.18*	1.9↑
C22:1 erucic acid ω-9	Nd	Nd	
C24:1 nervonic acid (ω-9)	4.73±1.10	4.43±1.01	
POLYUNSATURATED			
C18:2 (LNA) linoleic acid (ω-6)	25.98±3.20	32,01±1.93**	1.3↑
C20:2 eicosadienoic acid (ω-6)	0.24±0.10	0.61±0.22	
C20:4 (AA) arachidonic acid (ω-6)	6.23±1.35	4.64±1.08	
C18:3 (ALA) alpha linolenic (ω-3)	3.13±1.53	1.02±0.27*	3.0↓
C20:5 (EPA) eicosapentaenoic (ω-3)	1.30±0.48	1.40±0.38	
C22:6 (DHA) docosahexaenoic (ω-3)	0.46±0.36	0.55±0.25	
C20:3 eicosatrienoic acid (ω-3)	0.33±0.40	0.15±0.16	
SATURATED FAs	37.03±1.24	37.5±2.13	
MONOUNSATURATED FAs(MUFAs)	25.3±2.59	22.12±3.69	
POLYUNSATURATED FAs (PUFAs)	37.67±3.81	40.38±4.18	
RATIO ω-3/ω-6	0.15±0.04	0.08±0.02**	1.9↓
Total	100%	100%	
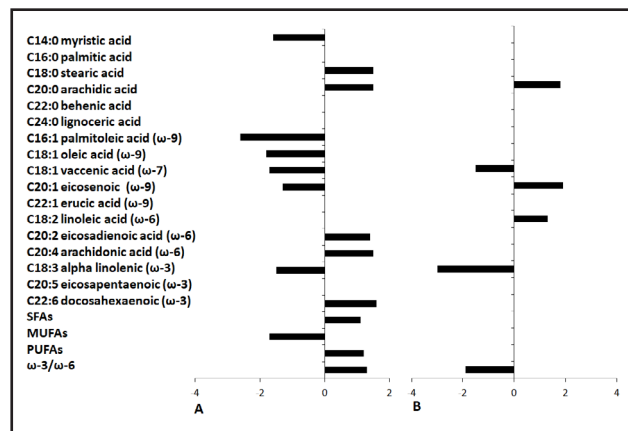
Fig. 3. Fold change of individual fatty acids in liver homogenate (A) and serum (B) between wild type and *Tff3 -/-* mice.

Discussion

The first function of Tff3 protein was connected with protection of gastrointestinal tract injuries but recent data connect Tff3 protein with metabolically relevant conditions

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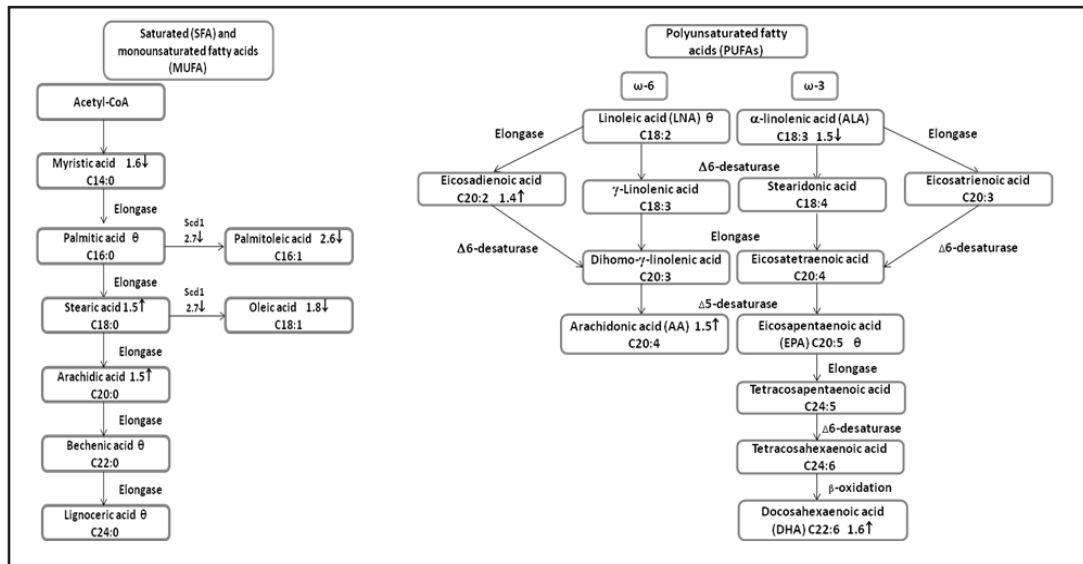www.karger.com/cpb

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Fig. 4. Fatty acids synthesis in the liver with marked changes (elevated ↑, decreased ↓ and no change θ) of individual fatty acids in *Tff3 -/-* mice compared with wild type.

[8, 12]. The results of the present study showed that Tff3 protein deficiency affects the metabolism of fatty acids in the liver (Fig. 3A) and that Tff3 deficient mice have an increased pool of small lipid vesicles in their hepatocytes (Fig. 2B), with no effect on liver oxidative stress markers (i.e. TBARS, FRAP and LOOH) that are often connected with changed liver lipid homeostasis. Due to its wide distribution in the body and presence in blood circulation, we have monitored the overall impact of Tff3 deficiency on health status and metabolic response on glucose/insulin overload.

Tff3 deficiency had no effect on general blood parameters since they were similar to values found in WT mice (Table 1). A statistically relevant change was noticed in the level of total protein concentration, which was reduced by 11% compared with the level

Fig. 5. Relative expression of CAT, GPx1, SOD2, PPARα, PPARγ, PPARδ, HNF4α and SIRT1 gene in liver of *Tff3 -/-* mice (n=6) compared with wild type mice. Ct values obtained by quantitative real time PCR were analyzed with REST 2009. Data are presented relative to WT mice (expression set as 1) as Whisker-box plots in order to see expression median and distribution of permutated expression values with 95 % confidence level.
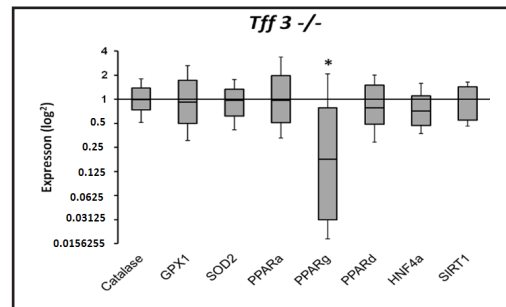

Fig. 6. Relative protein level of SOD2, CAT, GPx1, PMP70, SIRT1 and PPARγ in liver homogenate of wild type and *Tff3 -/- mice*. Protein level is presented relative to WT mice as mean ± SEM of specific protein band density normalized with amido black. The differences between groups were compared with by using Student t-test. Statistically significant changes are marked appropriately **p≤0.01 and ***p≤0.001.
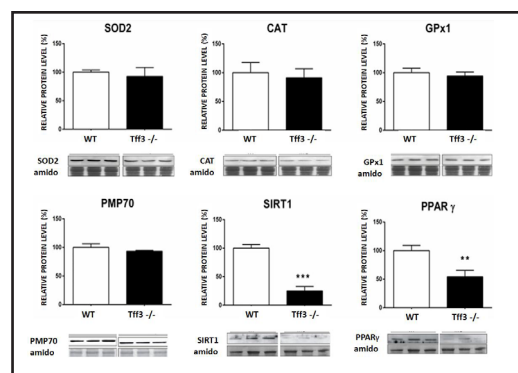
found in WT mice. The measurement of total protein level showed changes in two of the most representative proteins, albumin and globulin, which could be caused by reduced liver production or increased loss by urine.

However, GTT and ITT show faster utilization of glucose in Tff3 deficient mice upon glucose or insulin administration (Fig. 1). Direct effect of Tff3 on hepatocytes was demonstrated by overexpression of Tff3 in primary mouse hepatocytes (mice fed by standard diet), which inhibited the expression of gluconeogenic genes and decreased cellular glucose output [11]. This is in line with our observation of miRNA species being used to identify glycolysis/gluoconeogenesis pathways as a possible target of Tff3 action [34]. Another study by Ge at al., also in the diet-induced obesity mouse model, showed that overexpression of Tff3 *in vivo* improved glucose tolerance without affecting body weight, fasting insulin, triglyceride, cholesterol and leptin levels [10].

Fig. 7. Activity of antioxidative enzymes in liver homogenate of wild type and *Tff3* -/- mice. Enzyme activity (U/mg of protein) is presented as means ± SEM. The differences between groups were compared by using Student t test and statistically significant changes are marked as *($p \leq 0.05$).

The fact that our *Tff3 -/-* mice exhibit improved glucose tolerance as well as Tff3 protein overexpressing obese mice models [9] is contradictory at the first moment. This can be explained by different mouse strains (different genetic background) and different diets, contributing to different metabolic and expressional conditions. All of the above contributes to the complexity of this issue and additionally underlines the complexity of metabolic regulation. Nevertheless, reduced expression of liver Tff3 was noticed in different obese mice models of various genetic background (High fat induced obesity, Lepr^{db}/Lepr^{db} and Lepr^{ob}/Lepr^{ob}) [10], early diabetic mice [8] and steatotic liver [12], connecting Tff3 deficiency with conditions caused by specific diseases.

Additionally, liver Tff3 expression was most dramatic (reduction by 600x) in the early diabetes phase of multigene diabesity model called Tally Ho mice [8]. Therefore, we have focused our investigations on the liver. Blood analysis showed that activity of liver enzymes ALT/AST was not affected by Tff3 deficiency, indicating no effect on liver function. Staining of liver sections with H&E and PAS did not show any differences between Tff3 deficient and WT mice (data not shown). Conversely, Tff3 deficiency caused an increased number of small lipid containing vesicles (Fig. 2B). Besides energy storage, lipid droplets provide reservoirs of structural lipids (such as sterols, fatty acids and phospholipids) used for membrane formation. Given their diverse functions, lipid droplets are situated at the crossroads of membrane biology and energy metabolism as important organelles in maintaining cell homeostasis [35]. Tff3 deficiency did not significantly change the lipid content in the liver, but the content of small lipid vesicles was higher, raising the possibility that Tff3 protein deficiency affects formation of lipid droplets *per se*. Tff3 is a small protein able to interact with different partners. Lubka et al. identified BAG6 protein as a possible Tff3 interacting partner by using the yeast two-hybrid system [36]. BAG6 complex is a binding factor of tail-anchored (TA) transmembrane proteins and some of them are involved in membrane biogenesis, apoptosis, vesicular trafficking and signal transduction [37]. It is possible that Tff3 protein deficiency affects interaction of BAG6 with TA proteins involved in formation and trafficking of lipid vesicles, resulting in an increased number of small lipid vesicles. Correlating with histological findings (Fig. 2B), total lipids were slightly although not significantly increased in the liver of Tff3 knockout mice. Taking also into account that there was no change in TG level in the sera, we could only confirm increased small lipid vesicle formation with no signs of liver steatosis.
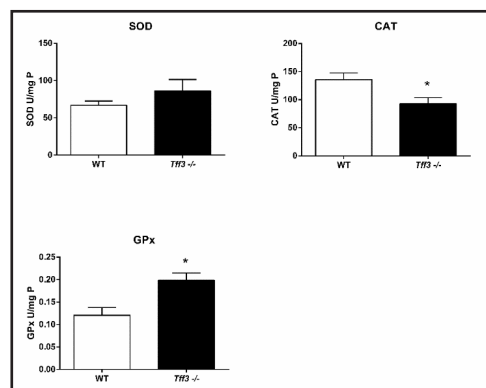
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Fatty acids profile analysis showed that Tff3 deficient mice had a statistically significant different level of numerous fatty acids in the liver and serum compared with WT mice (Fig 3.; Table 2, 3). Thus, the level of SFA (1.1 x) and PUFAs (1.2 x) were increased while the level of MUFAs was decreased (1.7 x) in the liver of Tff3 deficient mice compared with WT ones. In addition, the ω-3/ω-6 ratio was elevated in liver (1.3x) and decreased in serum (1.9x) of Tff3 deficient mice compared with WT ones. However, total serum levels of cholesterol, TG or HDL did not differ among the groups. Interestingly, content of essential fatty acids that needs to be taken by food is also affected. ALA (C18:3, ω-3) is reduced in the liver (1.5-fold) and sera (3-fold) while LNA (C18:2, ω-6) is increased 1.3-fold in sera and not affected in the liver. Although the level of specific FAs in the serum was changed, total content of the monitored SFAs, MUFAs and PUFAs was not changed due to Tff3 deficiency, demonstrating no effect on FA utilization and transport. In addition, we also did not observe changes in fatty acid composition in adipose tissue of Tff3 deficient mice (data not shown). All these data indicate that liver is the main site of impaired fatty acid metabolism. Hepatic FAs may derive from endogenous lipogenesis or from the free FA (FFA) plasma pool via active uptake into the hepatocyte. Hepatic lipogenesis includes *de novo* synthesis of FAs from acetyl-CoA (Fig. 4) or malonyl-CoA that are produced from a number of different pathways within the cell, most commonly carbohydrate catabolism [38]. In mammals, FA synthesis is catalyzed by acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) an enzyme that is complexly regulated by various nuclear receptors (PPARs and the bile acid receptor/farnesoid X receptor [FXR]) [39]. In *de novo* lipid synthesis pathway (Fig. 4) we have found a decreased amount of myristic (C14:0) fatty acid (1.6 x), suggesting the inhibition of *de novo* lipidogenesis in the liver of Tff3 deficient mice. Further steps in the synthesis scheme show no differences in palmitic, bechenic and lignoceric acid level. In contrast, the levels of stearic and arachidonic acids were elevated (1.5-fold). Additionally, the ratios of FA, C16:1/C16:0 and C18:1/C18:0, were different in Tff3 deficient mice compared with WT ones. Stearoyl CoA desaturase (SCD1) is a rate-limiting enzyme that catalyzes the biosynthesis of MUFAs from SFAs, mainly palmitoleic (C16:1) and oleic acid (C18:1) from palmitic (C16:0) and stearic acids (C18:0), respectively [40]. Desaturation index (determined by ratios of C18:1/C18:0 or C16:1/C16:0) has been widely used as a marker of SCD1 activity [41], and it was reduced 2.7-fold in Tff3 deficient mice compared with WT ones (Fig. 4). This could be due to lower protein expression or SCD1 activity. There are two possible explanations for this. First, SCD1 gene transcription is regulated by PPARγ and SIRT1 which were found to be downregulated in Tff3 deficient mice (Fig. 6). Second, free PUFAs could bind directly to the transcription factors that are involved in SCD1 expression and inhibit their activity and nuclear abundance [42]. It is important to mention that besides conversion to palmitoleic acid (9*cis*-16:1) by SCD1, palmitic acid (C16:0) can be converted to another isomer called sapienic acid (6*cis*-16:1) by fatty acid desaturase 2 (FADS2) enzyme, which is also involved in ω-3 and ω-6 pathway. Sapienic acid detection in human blood and in cancer breast cells demonstrates that it may be synthesized in different tissues and that it plays a significant role in lipid metabolism [43, 44]. Considering the results of this study, which showed impaired metabolism of MUFA and ω-3/ω-6 fatty acids in *Tff3-/-* mice, it is possible that FADS2 enzyme activity is also changed. However, more detailed and extensive studies will be needed to clarify this assumption.

An intriguing observation in the present study is that Tff3 deficient mice have elevated levels of PUFAs in the liver, especially AA (C20:4, ω-6) and DHA (C22:6, ω-3). Namely, in mammals ALA (C18:3, ω-3) is converted to eicosapentaenoic acid (EPA, C20:5, ω-3) in low amounts (8%) and -in even lower amounts- to DHA (C22:6, ω-3) (< 0.1%). PUFAs are derived from either LNA (C18:2, ω-6) or ALA (C18:3, ω-3) by an alternating series of desaturation and elongation reactions depending on the desaturase and elongase activities [45]. The major end products of ω-6 and ω-3 pathways are AA (C20:4, ω-6) and DHA (C22:6, ω-3). DHA is synthesized from ALA in the liver by a series of desaturations, elongations and β-oxidation (Fig. 4). The desaturations and elongations occur in the ER and the β-oxidation occurs in the peroxisome, where 24-carbon PUFAs are transferred. When β-oxidation is affected in the mitochondria, fatty acids are channeled for β-oxidation in peroxisomes and ω-oxidation

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in microsomes. In relation to this known mechanism, we did not observe any difference in the level of PMP70 protein, taken as a marker for peroxisomal proliferation (Fig. 6). *Tff3 -/-* mice had decreased levels of MUFA and elevated levels of PUFAs in the liver. Oleic acid is the major MUFA found in the membrane and the ratio of SFA to MUFA has been implicated in alteration of membrane fluidity [46]. There is a possibility that decrease of the MUFA content in the liver of *Tff3 -/-* mice is compensated by elevated levels of PUFAs. This ratio, due to the presence of more double bonds in the fatty acyl chain, could increase in membrane fluidity. Degree of insulin sensitivity and glucose transport is correlated to the amount of PUFAs in the membrane [47]. This might explain why *Tff3 -/-* mice had increased glucose tolerance and insulin sensitivity compared with WT ones. In addition, *Tff3 -/-* have decreased ω-3/ω-6 ratio in the liver and it is known that a decreased ω-3 deficiency ω-6 ratio is associated with an improvement in whole body glucose tolerance [48].

Tff3 deficiency influenced the synthesis and accumulation of FAs which are not only the source of energy, but also fine modulators of cellular signaling and metabolism [49]. The majority of genes that play key roles in lipid metabolism and their expression are modulated by SIRT1 and nuclear receptors PPARs [50]. SIRT1 is the epigenetic regulator acting as a metabolic sensor affecting expression and activity of various metabolic enzymes and transcription factors such as PPARγ, a nuclear receptor central to glucose and lipid homeostasis [20, 23]. To understand the underlying molecular mechanism(s) responsible for disturbances in fatty acid composition, we studied their genes and protein expression (Fig. 5 and 6). Tff3 deficient mice had a significantly reduced level of SIRT1 (4 fold) compared with WT ones (Fig. 6). Although the level of SIRT1 protein is reduced but not completely abolished in Tff3 deficient mice, they both show increased systemic glucose and insulin sensitivity. In addition, SIRT1 deficient mice also exhibit increased content of hepatic free fatty acid and deregulated fatty acid oxidation genes [51].

Furthermore, the expression of PPARγ was decreased 2-fold in Tff3 deficient mice. Interestingly, the liver specific knockout of PPARγ protected the mice against high fat diet induced obesity and improved their glucose tolerance. In addition, deletion of PPARγ downregulated the genes involved in lipogenesis and β-oxidation [52]. The reason for such discordant observation in Tff3 deficient mice could be in the existence of small lipid vesicles in contrary to macrovesicular steatosis that was found in the above mentioned work.

Considering that Tff3 knockout mice had an increased level of PUFAs which are susceptible to oxidation, and increased OS is noticed in lipid metabolism in the course of fatty liver development, the markers of OS and the endogenous antioxidant system were examined. The level of TBARS and FRAP in the liver of Tff3 deficient mice showed no difference compared with that found in WT ones, although the activity of some enzymes was changed (Fig. 7). We have detected reduced CAT and increased GPx activity while the protein level of SOD, CAT and GPx1 were not changed in Tff3 deficient mice compared with the levels found in WT ones (Fig. 6). Together, these findings indicate that changes in liver lipid metabolism of Tff3 deficient mice are not connected with oxidative stress and lipid peroxidation.

Conclusion

In summary, our results demonstrated that systemic Tff3 deficiency affects liver lipid metabolism and accumulation. Increased lipid droplets formation is not accompanied by liver oxidative stress, although expression/activity of monitored enzymes is deregulated compared with that of WT mice. We have revealed altered FAs profile and expression of SIRT1 and PPARγ. It is known that Tff3 expression is downregulated by TNFα and IL6, which were originally considered as classical inflammatory cytokines and are now considered to be major links between steatosis, insulin resistance and related inflammatory disorders [53]. Metabolism and maintenance of metabolic homeostasis are extremely complicated processes involving interactions of various organs. Loss of Tff3 gene expression (600-

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fold downregulation) in the liver as one of the most prominent events in the early phase of diabetes in diabetic/obese mice its presence in circulation and its regulation by insulin and food intake, suggest Tff3 as; an interesting candidate for further research in metabolic relevant conditions.

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Disclosure Statement

The authors declare that they have no conflicts of interests.

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