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Original article

Dietary fatty acid content influences the expression of genes involved in the lipid turnover and inflammation in mouse colon and spleen

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ABSTRACT

Background: Dietary interventions can improve gastrointestinal (GI) symptoms. We determined the effects of fatty acids (FAs) supplementation with medium- and long-chain saturated FAs on mouse GI motility and correlated them with the expression of genes for free FA receptors (FFAR)1-4, FA binding protein 4 (FABP4) and inflammation.

Methods: Forty-eight BalbC were assigned to: standard diet (STD), diet rich in medium-chain saturated FAs (COCO) and long-chain saturated FAs (HF) (7% by weight). Body weight (BW) and food intake (FI) were monitored for 8-weeks. GI motility was determined by fecal pellet output (FPO) and colon bead expulsion tests. FABP4 inhibitor, BMS309403 (1 mg/kg, *ip*) was injected to half of each group 2 days/week. mRNA expression of *FABP4,* (FFAR)1-4, and pro-inflammatory cytokines were measured in colonic and splenic tissues using real-time PCR.

Results: COCO and HF decreased FI. COCO accelerated overall GI transit ($p < 0.05$). COCO increased the mRNA expression of *FFAR2* ($p < 0.001$) and *TNFα* ($p < 0.01$); HF increased the expression of *FABP4* and *FFAR4* ($p < 0.05$), and *FFAR2* ($p < 0.001$) in the colon, and decreased *FFAR1* and *FFAR4* ($p < 0.001$), *TNFα* ($p < 0.01$) and *IL-1β* ($p < 0.05$) in splenic tissues. BMS309403 decreased the FI and delayed colonic transit in STD+BMS and COCO+BMS *vs.* STD ($p < 0.05$). HF+BMS increased colonic expression of *FFAR3* ($p < 0.01$), *TNFα* ($p < 0.01$), *IL-6* ($p < 0.01$), and reduced *FFAR4* ($p < 0.05$); COCO + BMS decreased *TNFα* ($p < 0.01$).

Conclusion: Diversification in the dietary lipid content affected GI motility in mice and the expression of FFARs and pro-inflammatory cytokines *in vivo*.

Introduction

The implementation of appropriate dietary habits can substantially improve gastrointestinal (GI) ailments [1,2]. Several attempts have been made to establish the impact of dietary fat on the GI motor and secretory activity and their association with the course of disease, in both healthy individuals and in patients suffering from GI disorders [3–5]. Changes in the intake of fats are also believed to activate gut endocrine cells and modify the inflammatory process in the gut wall.

Dietary free fatty acids (FFAs) are important factors that modulate lipid turnover and immune response in the body. Various studies support the role of polyunsaturated fatty acids (FAs) intake in lowering the risk of inflammatory GI diseases, such as ulcerative colitis or colorectal cancer [1,2,6,7]. The effects of saturated FAs have already been evaluated in both animals and humans, yet without a clearly stated conclusion [4,8,9].

Several G protein-coupled receptors for FFAs have been identified: FFAR1, FFAR2, FFAR3 and FFAR4 [10]. FFARs belong to the family of luminal-facing receptors that are responsive to nutrients; FFAR1 and FFAR4 are activated by medium- to long-chain FFAs, whereas FFAR2 and FFAR3 are being activated mostly by short-chain FFAs [10,11]. FFAR4 can also be activated by long-chain saturated FAs [12]. FFARs are localized in the GI tract

Abbreviations: BW, body weight; COCO, coconut oil; Ct, threshold cycle; FAs, fatty acids; FABPs, fatty acid binding proteins; FABP4, fatty acid binding protein 4; FFAs, free fatty acids; FFAR1, free fatty acid receptor 1; FFAR2, free fatty acid receptor 2; FFAR3, free fatty acid receptor 3; FFAR4, free fatty acid receptor 4; FGIDs, functional gastrointestinal disorders; FI, food intake; FPO, fecal pellet output; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLP-1, glucagon-like peptide-1; HF, high fat diet; HPRT1, hypoxanthine-guanine phosphoribosyltransferase 1; *ip*, intraperitoneal; IBS, irritable bowel syndrome; LA, linoleic acid; MCFAs, medium-chain fatty acids; MCP-1, monocyte chemoattractant protein-1; PBS, phosphate-buffered saline; STD, standard diet.

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(i.e. predominantly in the intestinal epithelial cells and L cells) and are possibly involved in the maintenance of intestinal epithelial cells function. FFARs can also regulate the immunological response observed in several mouse models of colitis and inflammation [13,14].

Fatty acid binding proteins (FABPs) mediate the transport of FAs to their site of action. FABPs bind to hydrophobic ligands (saturated and unsaturated FAs) to coordinate their intracellular trafficking and control their metabolic pathways [15]. Within the family of FABPs, a fatty acid binding protein 4 (FABP4) has been detected in intestinal epithelial cells and in colonic mouse samples [16,17]. We have previously demonstrated that modulation of the expression of FABP4, by applying a selective FABP4 inhibitor, BMS309403, accelerated GI transit time and improved the defecation pattern in mice [17]. Although both FFARs and FABPs are sensors for FFAs, it is still a lack of research showing whether FFARs and FABPs cooperate with one another or function independently.

In this study, we determined the effects of saturated FA intake on GI motility, and examined whether the type of dietary saturated FAs can influence the expression of genes participating in the lipid turnover and inflammation in the mouse colonic and splenic tissues. We also evaluated whether the observed effects might be dependent on the function of FABP4, by administering the selective FABP4 inhibitor, BMS309403.

Materials and methods

Animals and study design

Experimentally naive male BalbC mice, weighing 22–24 g were obtained from the vivarium at the University of Lodz, Poland and housed under controlled laboratory conditions (22–23 °C, relative humidity: 45–55 %, 12:12–h light/dark cycle, lights on at 6:00 a.m.) in sawdust–lined plastic cages. To minimize circadian influence, all experiments were performed between 7:00 h and 16:00 h after at least 7 days of acclimatization. Tap water was available *ad libitum*. The weight-matched animals (n = 48) were randomly assigned to three groups fed with either standard laboratory diet (STD) containing 7% fat by weight, diet containing 7% coconut oil (COCO), composed of medium-chain saturated FAs (MCFAs) or diet containing 7% lard (HF), rich in long-chain saturated FAs. The content of FAs reflects the average amount of FAs ingested in humans. Total dietary calories provided by fat in each diet was equal 16%. Half of animals from each group received the intraperitoneal (*ip*) injection of the FABP4 inhibitor, BMS309403 (1 mg/kg) or vehicle twice a week [17]. The dosage of BMS309403 was selected based on previous experiments [17]. Animals were fed for 8 weeks. All diets were formulated to meet the nutritional requirements of growing mice and manufactured by the external company specialized in animal food supply (ZooLab, Poland). The composition of diets and the content of FAs are listed in Tables 1A and 1B , respectively [18].

Body weight (BW) was measured twice a week; the food intake (FI) was monitored every morning between 7 and 8 a.m. At the end of feeding, animals were used in GI motility studies, i.e. fecal pellet output (FPO) and colon bead expulsion tests. Animals were euthanized and colonic and splenic tissues were resected and further used for the measurement of the expression of selected genes, using real-time PCR method.

The experimental protocols followed the European Communities Council Directive of 22 September 2010 (2010/63/EU), were in accordance with Polish legislation on animal experimentations, and were approved by the Local Ethics Committee at the Medical University of Lodz (#63/LB77/2017). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Table 1A
Composition of diets included in the study.

Ingredients (g/kg diet)	STD [a, b]	COCO [b]	HF [b]
Corn starch	397.486	397.486	397.486
Casein	200.00	200.00	200.00
Maltodextrin	132.00	132.00	132.00
Sucrose	100.00	100.00	100.00
Soybean oil	70.00	0.00	0.00
Coconut oil	0.00	70.00	0.00
Lard	0.00	0.00	70.00
Fibre	50.00	50.00	50.00
[a]AIN93 G Mineral mix	35.00	35.00	35.00
[a]AIN93 G Vitamin mix	10.00	10.00	10.00
L-Cystine	3.00	3.00	3.00
Choline bitartrate	2.50	2.50	2.50
Tert-butylohydrochinon	0.014	0.014	0.014
	1000.0	1000.0	1000.0

Full ingredient list for the diets in this study, formulated by ZooLab, Poland. [a]STD refers to rodent diet 93 of the American Institute of Nutrition (Reeves et al. [18]). [b]All diets: total energy: 4029,94 kcal; protein (% energy): 20; fat (% energy):16; carbohydrated (% energy): 64.

Table 1B
Fatty acid composition of experimental diets.

Type of FA	Content of FA in a diet (%)		
	STD	COCO	HF
Saturated MCFAs	9,7	69,4	0,0
Saturated LCFAs	5,1	21,0	39,0
Monounsaturated LCFAs	23,0	6,7	45,0
Polyunsaturated LCFAs	54,4	1,8	11,0
Others	7,8	1,1	5,0
TOTAL	100	100	100

COCO: diet containing 7% of virgin coconut oil; FA: fatty acid. HF: diet containing 7% of lard; LCFAs: long-chain Fas. MCFAs: medium-chain FAs; STD: standard diet.

BMS309403 was dissolved in 5% dimethyl sulfoxide and diluted in 0.9% saline to desired concentrations as selected in preliminary studies. BMS309403 was injected (*ip*) at the dose of 1 mg/kg twice a week for 60 days. 0.9% Saline was used as vehicle (100 u L/mouse, *ip*). The vehicles had no effects on the observed parameters in mice. The anaesthesia was induced by the *ip* injection of ketamine/xylazine solution 1 mL and 0.5 mL, respectively, diluted in 8.5 mL of 0.9% sodium chloride.

All drugs and reagents, were purchased from Sigma-Aldrich (Poland), unless stated otherwise. BMS309403 was purchased from Tocris (UK); Isoflurane from Baxter Healthcare Corp. (IL, USA); Phosphate-buffered saline (PBS) from Polgen (Poland) and TRIsure from Bioline (UK). The PCR TaqMan Gene Expression Assay probes used for the quantification of the mRNA expression of genes were purchased from Life Technologies (CA, USA).

GI motility studies

GI transit

The FPO test was performed to measure the whole GI transit in non–fasted animals after 8 weeks of feeding, as described previously [19].

Colonic motility

Distal colonic bead expulsion test was performed after 8 weeks of feeding following an overnight fasting (for 16 h) with water *ad libitum*, as described previously [19]. In animals, allocated to the group receiving the FABP4 inhibitor, BMS309403, or vehicle, the colon bead expulsion test was conducted 15 min after *ip* administration of the respective solution, under light anesthesia with isoflurane [20].

RNA extraction and quantitative real-time PCR

Immediately after cervical dislocation segments from the distal colon and splenic tissues were isolated and rinsed with PBS. Fecal contents from the colonic samples and the connective tissue residues were gently removed. All tissues were transferred into new tubes and stored at −80 °C until analysis.

For the quantification of mRNA expression, we applied the real-time fluorescence detection PCR method with FAM dye-labeled TaqMan probes (Applied, Biosystems, USA). Mouse colonic and splenic RNA were isolated according to manufacturer's protocol using Total RNA Mini kit (A&A Biotechnology, Poland). Briefly, tissue samples were homogenized in TRIsure reagent (Bioline, UK) using an ultrasound homogenizer (Bandelin Sonoplus HD3100, Germany). The purity and quantity of the isolated RNA were measured using a Colibri Microvolume Spectrometer (Titertek Berthold, Colibri, Germany). The sample was characterized with A260 nm/A280 nm ratio, which was in the range of 1.79-2.01. Total RNA was eluted using diethyl pyrocarbonate treated water. A total of 4 μg of RNA was converted into cDNA with Maxima First Strand cDNA synthesis kit (Fermentas, Canada) with the three step incubation: 25 °C for 10 min, 50 °C for 15 min and 85 °C for 5 min. The quantification was performed in a 20 μL volume containing 1 μL of RNA and 19 μL of the reaction mixture: 1 μL of FAM dye-labeled TaqMan probe, 8 μL of RNA-free water and 10 μL of TaqMan Gene Expression Master Mix (Life Technologies, CA, USA) following manufacturer's instructions. Quantitative analysis was performed using fluorescently labeled TaqMan probes Mm00445878_m1 for mouse *FABP4*, Mm00809442_s1 for mouse *FFAR1*, Mm02620654_s1 for mouse *FFAR2*, Mm02621638_s1 for mouse *FFAR3*, Mm00725193_m1 for mouse *FFAR4*, Mm00443258_m1 for mouse *TNFα*, Mm00434228_m1 for mouse *IL-1β*, Mm00446190_m1 for mouse *IL-6*, Mm01545399_m1 for mouse hypoxanthine-guanine phosphoribosylatransferase 1 (*HPRT1*) and Mm99999915_g1 for mouse glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Life Technologies, CA, USA). *HPRT1* was used as endogenous control for the quantification of genes in colonic samples, whereas the *GAPDH* served as a housekeeping gene for splenic samples. The analysis was performed on Lightcycler (Roche, Switzerland). Each sample was run in triplicates. The threshold cycle (Ct) values for studied genes were normalized to Ct values obtained for a housekeeping genes, *HPRT1* and *GAPDH*. Relative amount of mRNA copies was calculated using following equation: $2^{-\Delta Ct}$ x 1000 [21].

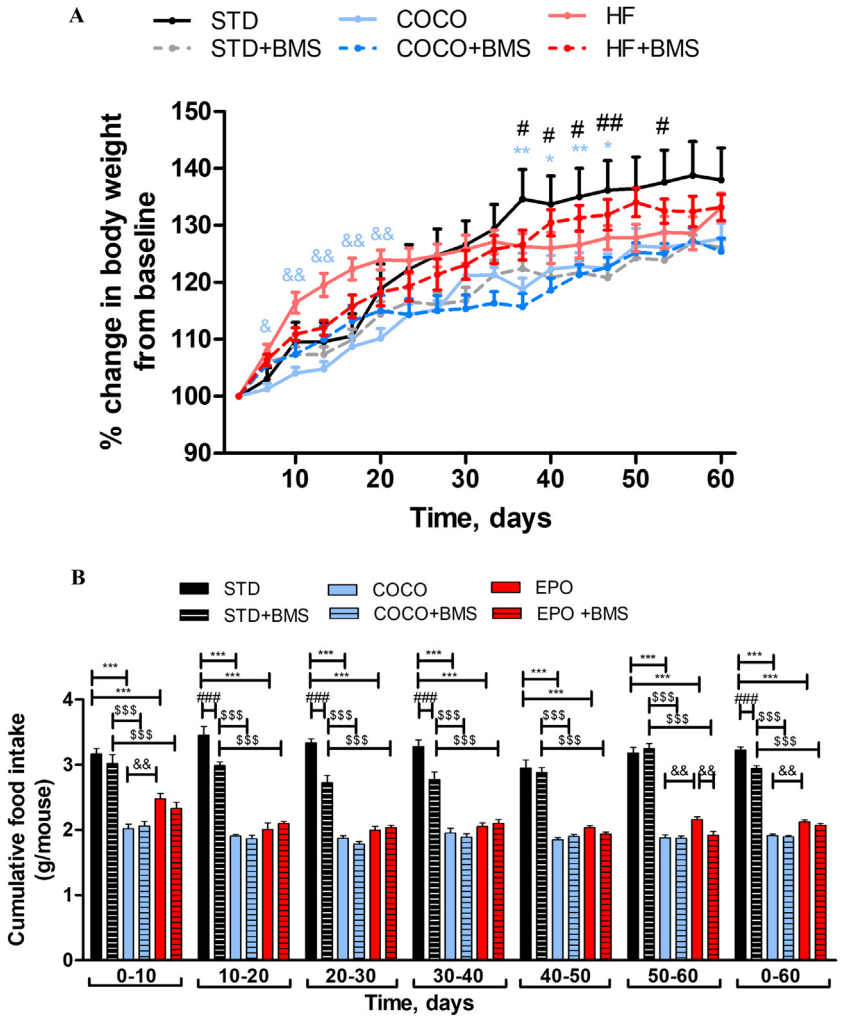

Fig. 1. Representative graphs for body weight changes (A) and average food intake (B) in mice fed with standard diet (STD), medium-chain saturated FAs (COCO) and long-chain saturated FAs (HF) with and without treatment with BMS309403 (1 mg/kg; *ip*). Data represent mean ± SEM of n = 8 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* STD; $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$, *vs.* HF diet; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, *vs.* respective dietary intervention without BMS309403; $^{\$\$\$}p < 0.001$, *vs.* STD with BMS309403.

Statistical analysis

Statistical analysis and curve–fitting were performed using Prism 5.0 (GraphPad Software Inc., CA, USA). The data are expressed as means ± SEM. Two–way ANOVA followed by the Newman–Keuls *post–hoc* test was used for analysis of multiple treatment means. p Values < 0.05 were considered statistically significant. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [22].

Results

Body weight and food intake

A noticeable decrease in BW, from the 36th day of feeding till the end of the treatment, was noted among animals fed with COCO and HF diet *vs.* STD (Fig. 1A). However, the statistically significant differences were observed only in COCO between the 36th and 46th day of feeding ($p < 0.01$ for 36th and 43rd, and $p < 0.05$ for 40th and 46th day of treatment). COCO presented with the lowest BW gain after 60 days of interventional period. Between the 4th and 20th day of feeding a significant increase in BW was reported in HF *vs.* COCO diet ($p < 0.05$ for 4th day of feeding, and $p < 0.01$ between the 10th and 20th day of treatment).

The administration of the FABP4 inhibitor, BMS309403, reduced the BW in STD + BMS *vs.* STD group, in particular between the 36th and 46th ($p < 0.01$ for 36th, 40th, 43rd and 46th day) as well as in 53rd day of treatment ($p < 0.05$) (Fig. 1A). BMS309403 neither affected the BW of animals from COCO + BMS or HF + BMS *vs.* STD + BMS groups, nor COCO + BMS and HF + BMS when compared to the respective dietary modifications without the BMS309403 intervention (animals belonging to COCO and HF, respectively).

Mice fed with COCO and HF diets consumed significantly less than those from STD group, throughout the course of the study ($p < 0.001$) (Fig. 1B). Their FI was similar during the feeding period.

BMS309403 led to a significant decrease in FI in STD + BMS *vs.* STD between the 10th and 40th day ($p < 0.001$), and in HF+BMS *vs.* HF between 50th and 60th day of feeding ($p < 0.01$). No difference between the FI of COCO+BMS *vs.* COCO were noted (Fig. 1B). Animals in both COCO + BMS and HF + BMS groups ingested significantly less of their chow than animals from STD + BMS group ($p < 0.001$). The FI for COCO+BMS as well as HF+BMS groups were similar throughout the interventional period ($p < 0.001$) (Fig. 1B).

GI transit

Animals fed with COCO diet had significantly accelerated GI transit, which was observed as an increase in the number of pellets excreted over 60 min ($p < 0.05$ *vs.* STD). No significant difference in the GI transit was observed in HF group *vs.* STD (Fig. 2A).

Chronic administration of the BMS309403 did not affect overall GI motor activity, regardless of the interventional treatment (Fig. 2A).

Mouse colonic transit

None of the diets produced significant changes in the colonic motility; however, a slight tendency towards an accelerated colonic transit was noted in the COCO group (Fig. 2B).

The *ip* injection of BMS309403 considerably prolonged the time to bead expulsion in STD + BMS and COCO + BMS groups *vs.* animals belonging to STD and COCO, respectively ($p < 0.05$) (Fig. 2B). The intestinal motor activity in the HF + STD group was similar to the STD + BMS or HF groups (Fig. 2B).

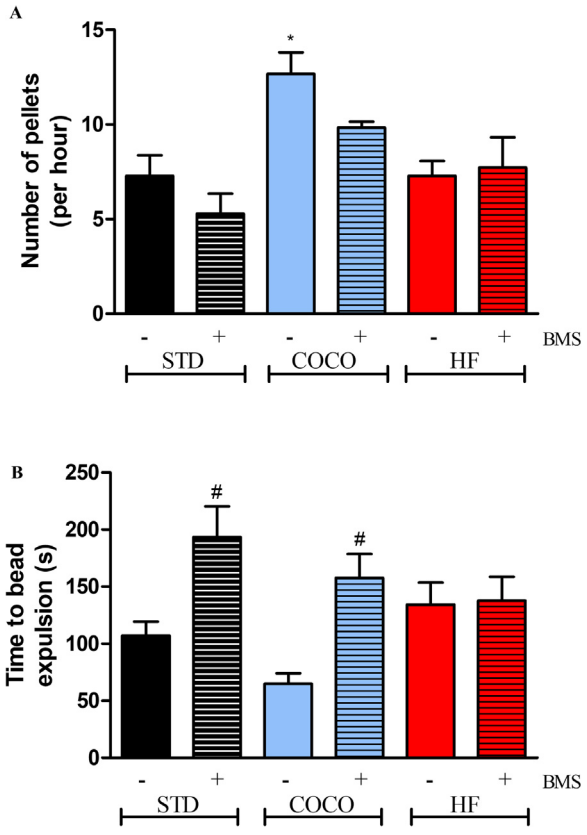

Fig. 2. The effects of different dietary interventions (STD, COCO and HF) on (A) fecal pellet output and (B) colonic transit in mice with or without the administration of BMS309403 (1 mg/kg, *ip*) Data represent mean ± SEM of n = 6–8 mice per group. $^{\#}p < 0.05$, *vs.* respective dietary intervention without the intervention of BMS309403.

Regulation of gene expression in the mouse colon

Under dietary intervention with COCO diet, we did not observe any significant changes in the expression level of *FABP4*, *FFAR3* or *FFAR4* (Fig. 3). HF diet significantly increased the level of *FABP4* and *FFAR4* vs. STD ($p < 0.05$); however, similarly to COCO diet, it did not affect the gene expression of *FFAR3*. COCO and HF diets slightly reduced the expression of *FFAR1* ($p < 0.05$) and significantly elevated the level of mRNA expression of *FFAR2* ($p < 0.001$) *vs.* STD (Fig. 3B,C).

An elevated expression levels of *FFAR2* and *FFAR3* were noted in STD + BMS *vs.* STD group ($p < 0.01$) and in HF+BMS *vs.* STD+BMS ($p < 0.01$), respectively (Fig. 3C,D). Moreover, the treatment with HF + BMS significantly decreased the expression level of *FFAR4* *vs.* STD + BMS group ($p < 0.05$). Regardless of dietary intervention, BMS309403 did not alter the expression of *FABP4* or *FFAR1* in mouse colonic samples (Fig. 3A and B, respectively).

COCO diet significantly increased the expression level of *TNFα* ($p < 0.01$) *vs.* STD (Fig. 4A), but not of *IL-1β* or *IL-6*. HF diet did not change the expression of *TNFα*, *IL-1β* or *IL-6*.

BMS309403 exerted different effects on the expression level of *TNFα* depending on the type of FAs presented in the diet (Fig. 4A). BMS309403 significantly diminished the expression of *TNFα* in COCO + BMS *vs.* COCO group ($p < 0.01$), and increased its expression level in HF+BMS *vs.* HF or STD+BMS groups ($p < 0.01$ and $p < 0.01$, respectively). The expression level of *IL-1β* did not change upon the administration of BMS309403 and displayed similar levels in all groups (Fig. 4B). BMS309403 caused increasing tendency in the expression level of *IL-6* in STD + BMS, COCO + BMS and HF + BMS, *vs.* their respective dietary treatment without

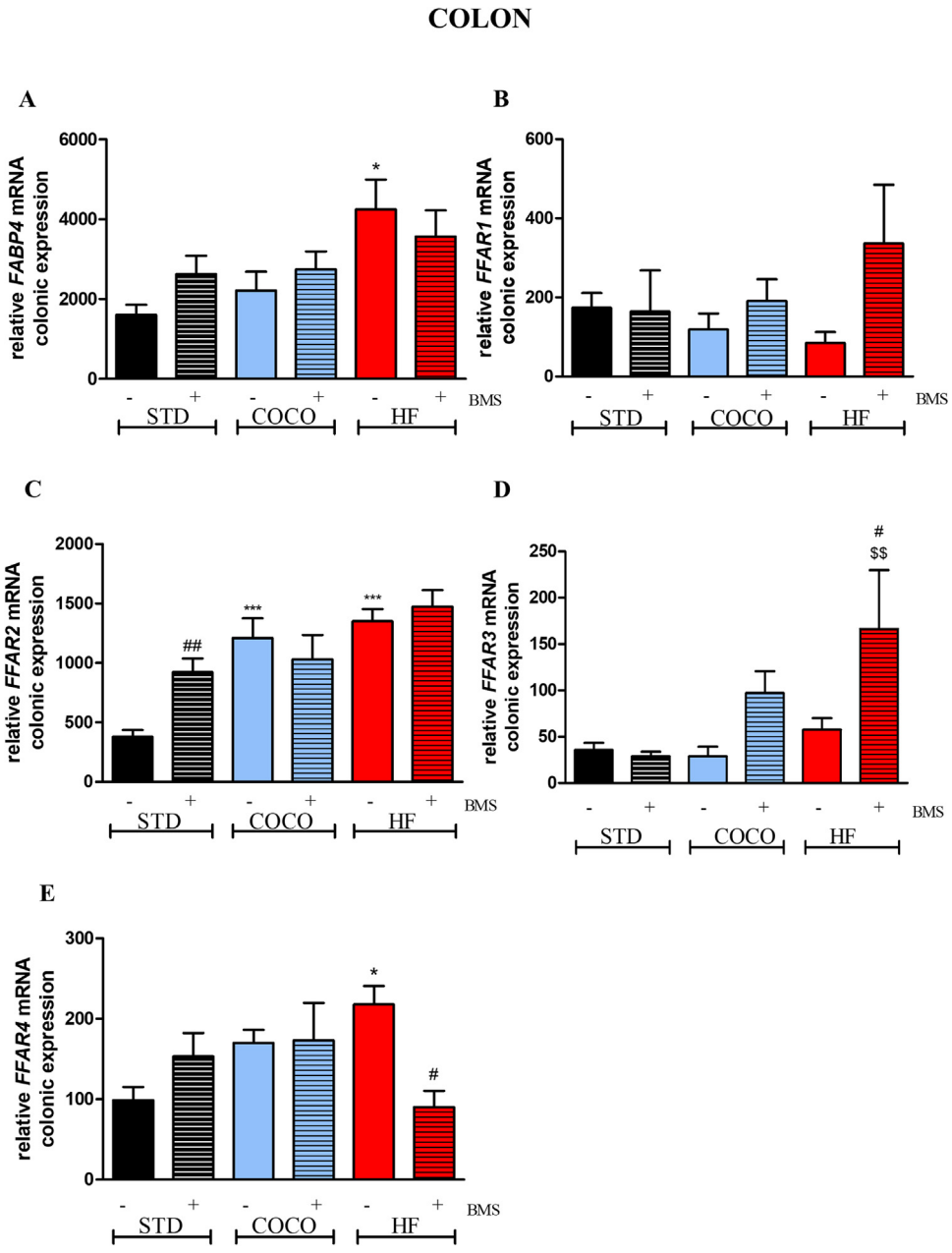

Fig. 3. Relative mRNA expression of FABP4 (A), FFAR1 (B), FFAR2 (C), FFAR3 (D) and FFAR4 (E) in mouse colon. Tissues were isolated from animals exposed to different dietary interventions (STD, COCO and HF) with or without the injection of BMS309403 (1 mg/kg, *ip*). Studied genes were normalized to the expression of the housekeeping gene *GAPDH*. Data represent mean ± SEM of n = 6–8 mice per group. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs.* STD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *vs.* respective dietary intervention without the intervention of BMS309403; $^{\$\$}p < 0.01$, *vs.* STD with BMS309403.

BMS309403 administration; the statistical significance was observed only in the HF + BMS group ($p < 0.05$) (Fig. 4C).

Regulation of gene expression in the mouse splenic tissues

Both COCO and HF diets increased the expression level of *FABP4* but the result did not reach statistical significance (Fig. 5A). COCO-fed mice had similar expression of *FFAR1, FFAR2, FFAR3* and *FFAR-4* as STD-fed mice. Regarding the HF diet, we observed a significant decrease in the expression level of *FFAR1* and *FFAR4 vs.* STD ($p < 0.001$) (Fig. 5B and E, respectively). The expression level of *FFAR2* and *FFAR3* in the HF group showed comparable values with the STD group (Fig. 5B and D, respectively).

BMS309403 significantly decreased the expression level of *FFAR1* in the STD + BMS ($p < 0.05$) *vs.* STD group (Fig. 5B). BMS309403 significantly decreased the expression of *FFAR4* in the STD + BMS group *vs.* STD group ($p < 0.01$). There were no significant differences in *FABP4, FFAR1, FFAR2, FFAR3* and *FFAR4* mRNA expression in COCO + BMS or HF + BMS *vs.* STD + BMS or its respective dietary intervention without BMS309403.

COCO diet had no effect on the mRNA expression of *TNFα*, *IL-1β* or *IL-6* (Fig. 6A–C) in mouse splenic tissue. In contrast, HF group had significantly decreased expression level of both *TNFα* and *IL-1β* (Fig. 6A and B), but did not change the level of *IL-6*.

Tested diets with simultaneous administration of BMS309403 had no influence on the mRNA expression of *TNFα*, *IL-1β* and *IL-6*.

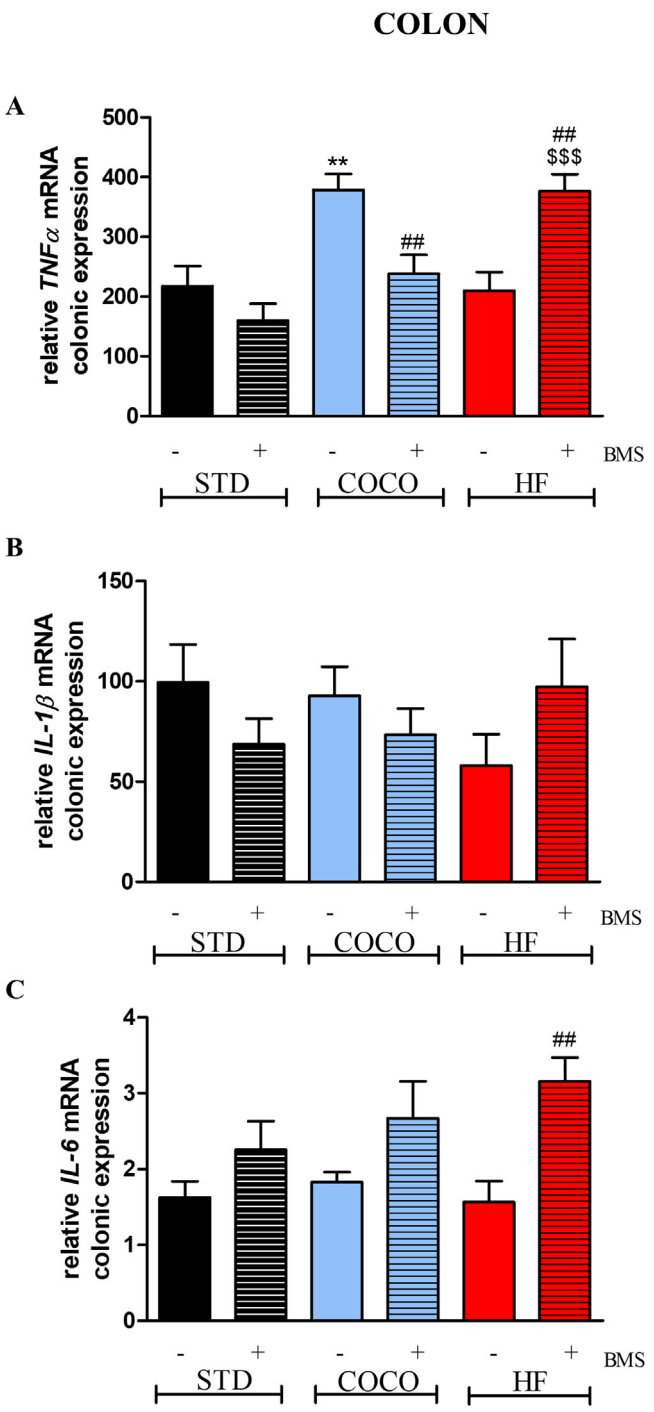

Fig. 4. Relative mRNA expression of TNFα (A) IL-1β (B) and IL-6 (C) in mouse colon. Tissues were isolated from animals exposed to different dietary interventions (STD, COCO and HF) with or without the injection of BMS309403 (1 mg/kg, *ip*). Studied genes were normalized to the expression of the housekeeping gene *GAPDH*. Data represent mean ± SEM of n = 6–8 mice per group. $^{**}p < 0.01$, *vs.* STD; $^{\#\#}p < 0.01$, *vs.* respective dietary intervention without the intervention of BMS309403; $^{\$\$\$}p < 0.001$, *vs.* STD with BMS309403.

Discussion

There is a strong foundation of epidemiological and experimental studies to support the role of environmental modifiers, particularly high dietary fat intake and the development of GI diseases. Consequently, increasing evidence suggests that the type and source of dietary FAs are important and should be considered while establishing dietary recommendations for patients with GI disorders.

FAs in a diet are considered as potential factors affecting appetite and modulating motility of the GI tract. Despite the fact that increased dietary fat intake apparently leads to overconsumption in both animals and humans [23,24], fat can also induce changes in the GI tract that favor less energy intake and therefore inhibit the appetite [4,25]. In our study, two sources of saturated FAs were used: coconut oil, which is considered as a non-obesogenic oil in clinical and rodent studies [26,27], and lard, which can cause obesity depending on the amount consumed and length of feeding. Even though both oils were used in relatively low concentrations, they were able to induce changes in the BW and FI. We observed that although the BW of animals corresponded to the amount of food ingested, the dynamics of BW and FI varied depending on the intervention treatment, i.e. medium- (COCO) or long-chain (HF) saturated FAs caused significant differences in the BW of animals at the beginning of the dietary intervention. Animals from HF diet gained weight faster than animals from the COCO group; accordingly, their FI was also elevated. Taking into account the fact that the metabolism of long- and medium- chain FAs differs, it is reasonable to consider the observed changes as an adaptation to the diet abundant with long-chain saturated FAs [28]. HF-fed animals required more time to get enough energy from the diet to cover their daily energy demand than animals fed with medium-chain FAs, from which the energy is obtained almost immediately after ingestion. Consequently, even when HF-fed animals obtained enough energy to cover their daily requirements, the amount of long-chain saturated FAs they had consumed accumulated in the body causing the increase in the BW. Differences in BW and FI dynamic observed in the study may also stem from the fact that higher intake of long-chain saturated FAs can alter the satiety response in the postprandial state [29], unlike medium-chain saturated FAs.

Regardless of the source of FAs in a diet, COCO- and HF-fed animals consumed significantly less of their chow than the STD group; still, their BW was positively correlated with their FI. Since the composition of each diet was balanced in terms of the energy content, it seems that the changes observed in the FI could be affected solely by the origin and the chain length of FAs presented in each diet [9] and not by other factors. It is important to highlight that in our study, the composition of each diet was equivalent to the average amount of FAs ingested in humans.

The main function of cytoplasmic FABPs is to facilitate FFA solubility and transport to specific enzymes and cellular compartments e.g. mitochondria for oxidation, endoplasmic reticulum for re-esterification, or to the nucleus for regulation of gene expression [30]. FABP4 has high affinity towards saturated as well as unsaturated FAs. BMS309403 is a potent and selective inhibitor of FABP4 that competes with endogenous FFAs for binding to the FA binding pocket. We showed that BMS309403 administered to COCO and HF groups had minimal effect on the BW and FI of mice; which is in accordance with other findings [31,32]. Although few studies showed that BMS309403 reduces the weight of animals fed with saturated fat, most of them were performed in FABP4-deficient animals where they used a diet composed of 60% fat [33,34]. Moreover, recent analysis identified elevated levels for palmitoleate in the plasma of mice lacking FABP4, which may suggest that monounsaturated FAs are the main drivers for the metabolic phenotype correspondent to the deficiency of the FABP4 [35,36]. This in turn may explain the highest drop in the BW and FI of animals from STD + BMS group *vs*. STD; more than one fourth of FAs in STD belonged to monounsaturated FAs.

Studies in animals, and to a limited extent in humans, show that the consumption of a fatty diet modulates the GI responses and contributes to impairments in gut function, including motility and

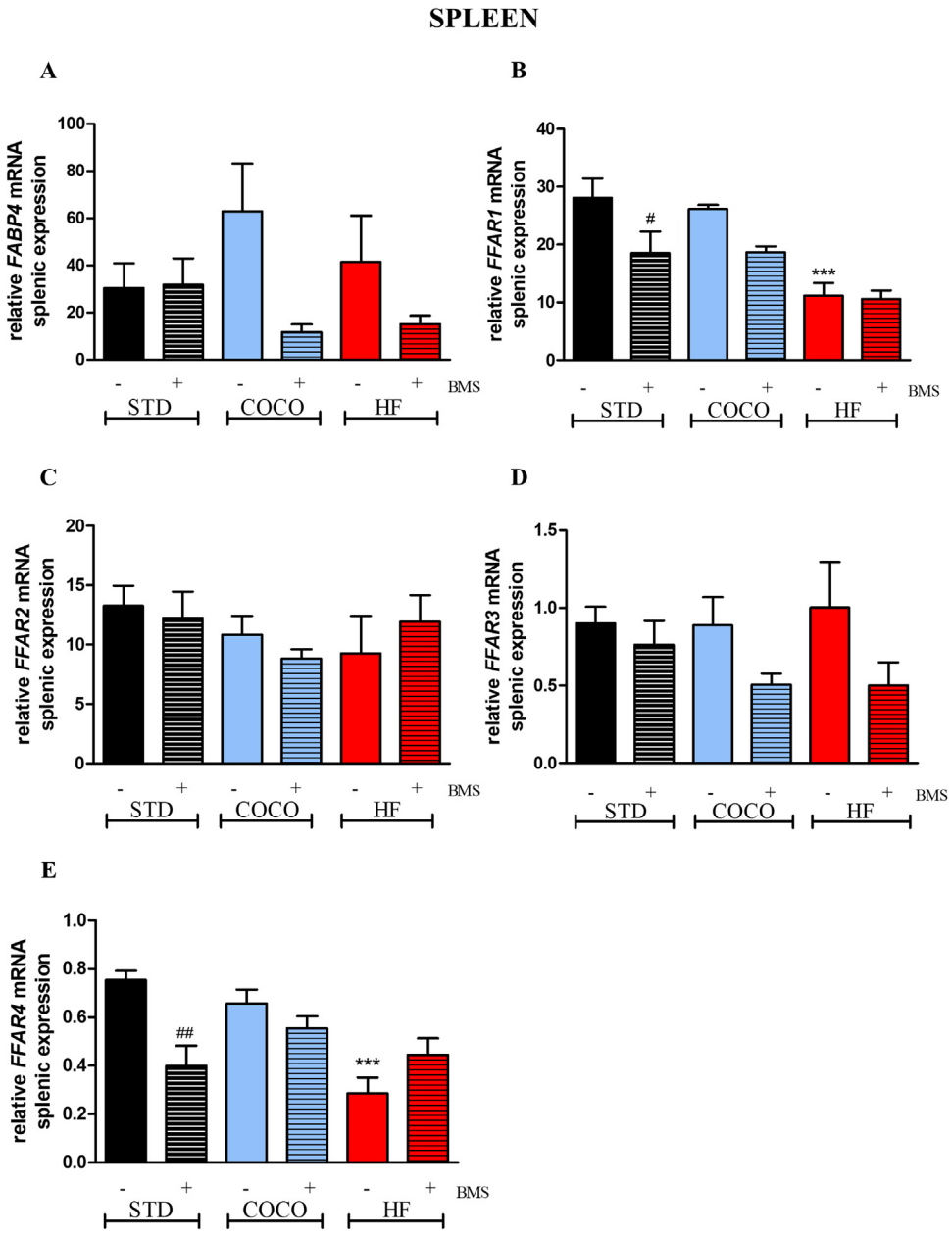

Fig. 5. Relative mRNA expression of FABP4 (A), FFAR1 (B), FFAR2 (C), FFAR3 (D) and FFAR4 (E) in mouse spleen. Tissues were isolated from animals exposed to different dietary interventions (STD, COCO and HF) with or without the injection of BMS309403 (1 mg/kg, *ip*). Studied genes were normalized to the expression of the housekeeping gene *HPRT1*. Data represent mean ± SEM of n = 6–8 mice per group. $^{***}p < 0.001$ *vs.* STD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *vs.* respective dietary intervention without the intervention of BMS309403.

secretion [4,5,37,38]. A high intake of fatty diets is associated with impairments of fat-induced activation of vagal afferent pathways [28,38] and can delay the gastric emptying in rats [39]. We showed that 8 weeks of dietary intervention accelerated the overall GI motility in animals fed with medium-chain saturated FAs (COCO), but not in those fed with long-chain saturated FAs (HF). Neither diet rich with medium- nor with long-chain saturated FAs influenced the colonic motility; albeit a slight increase was reported in the COCO *vs.* STD group. Such observations indicate that the effect of FAs on GI function depends on site of action of FAs in the GI tract and the length of the FA chain (meaning that different parts of the GI tract can respond differently, when exposed to either medium- or long-chain saturated fat).

We have previously evaluated the effects of acute and chronic administration of FABP4 inhibitor, BMS309403, on overall GI and colonic motility [17]. Only acute administration accelerated overall GI and colonic transit, whereas chronic inhibition for 6 and 13 consecutive days, did not induce any changes [17]. In the current study, in turn, we observed that chronic (8 weeks) FABP4 inhibition significantly delayed mouse colonic motility in STD + BMS and in COCO + BMS groups. The observed changes, associated to the effects of dietary interventions as well as BMS309403 injection, were not related to the mRNA expression of *FABP4* in the colon, but rather on the content of FAs in a diet.

BMS309403 has been found to improve lipid profiles and modulate the inflammatory response *in vivo* [31]. Therefore, we verified whether changes in the dietary FA content alone, or together with the implementation of FABP4 inhibitor can disturb immune response in the colon. COCO-fed animals had higher mRNA expression level of *TNFα*, which suggests that the diet could

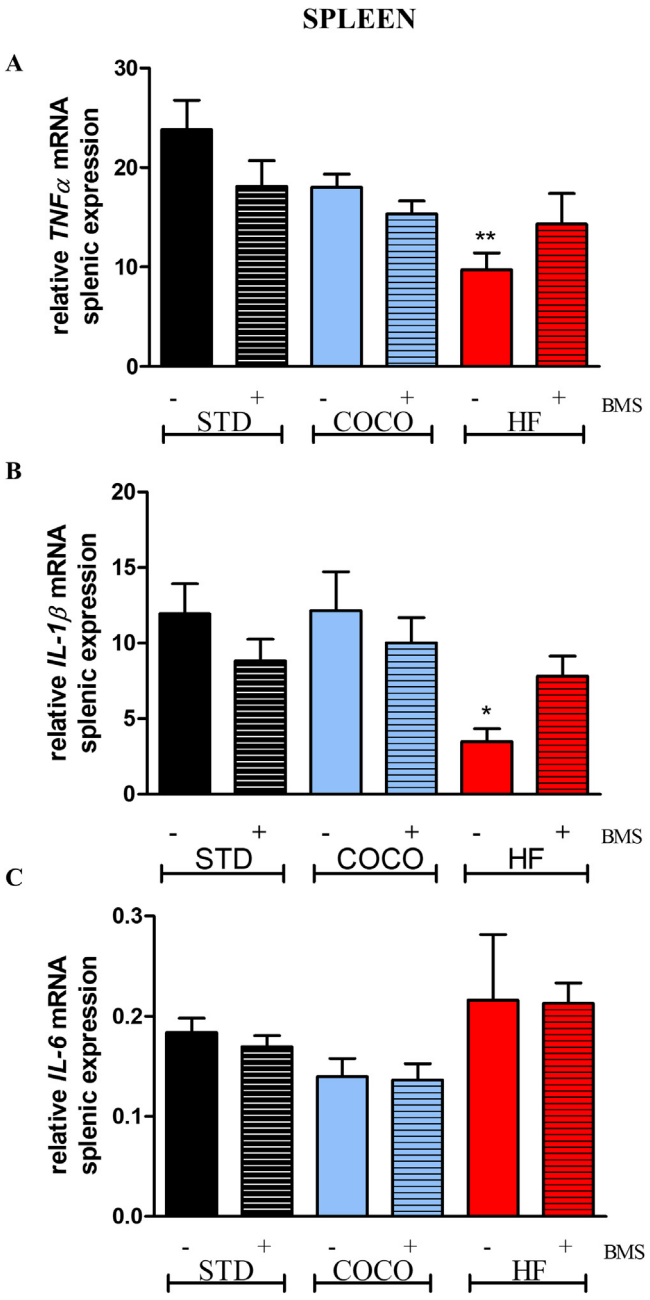

Fig. 6. Relative mRNA expression of TNFα (A), IL-1β (B) and IL-6 (C) in mouse spleen. Tissues were isolated from animals exposed to different dietary interventions (STD, COCO and HF) with or without the injection of BMS309403 (1 mg/kg, *ip*). Studied genes were normalized to the expression of the housekeeping gene *HPRT1*. Data represent mean ± SEM of n = 6–8 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, *vs.* STD.

initiate the inflammatory response in the colon; chronic administration of BMS309403 could diminish its high level. Our results also indicate that the anti-inflammatory properties of BMS309403 are specifically linked to the type of FAs in a diet. The administration of BMS309403 to COCO group caused an opposite effect when compared to HF group, in which an increased mRNA expression levels of *TNFα* and *IL-6* were noted. The anti-inflammatory properties of BMS309403 have already been described *in vivo* [40]. Of note, it has been reported that the health benefits from the reduction or absence of FABP4 are linked to inhibited production of inflammatory mediators produced by macrophages [40]. In a macrophage cell line, chemical inhibition or absence of FABP4 markedly diminished expression of pro-inflammatory cytokines and chemokines e.g. IL-1β, IL-6 and monocyte chemoattractant protein-1 (MCP-1) [41].

At least some of the activities attributed to FAs are mediated through their interaction with a number of G protein–coupled receptors. By interacting with short-, medium- or long-chain FAs they are able to transduce signals across the cell membrane. Whether they act as nutrient sensing receptors independent or dependent on FABP4 is unconfirmed. In addition to these observed effects, it is still unknown whether the presence of FFARs in the intestines has an impact on GI motility.

Up to now, the role of FFAR1 was mainly examined in beta cells, taste buds [42], the brain and in the GI tract [43]. Adachi et al. [44] reported that FFAR1 is expressed in scattered cells distributed throughout the mouse GI tract i.e. from the gastric pylorus to the colon. Of note, the expression in the stomach and intestine was mostly related to the appearance of differentiated endocrine cells of the GI tact. This may indicate that FFARs located on these cells can be involved in the mechanism by which dietary FAs change the GI motility. Our study showed that FFAR1 is expressed in the mouse colon, and was detected in all dietary groups, regardless of dietary intervention. However, we did not observe any changes in the level of mRNA expression of *FFAR1* between groups. Therefore, we presume that the specificity of FFAR1 is independent of the degree of saturation of FAs and their chain length in the colon. This observation is in line with other studies [45].

The short-chain FAs are the most potent agonists of FFAR2 and FFAR3 [46]. The expression of FFAR2 is high in adipose tissue, spleen, pancreas and peripheral blood mononuclear cells [47]. Unlike FFAR2, high expression of FFAR3 is also found in cells of the distal ileum and colon [47]. In this study, albeit both *FFAR2* and *FFAR3* were detected in mouse colonic samples, only the expression level of *FFAR2* varied according to changes in dietary content of FAs. In line, mice fed with COCO and HF diets displayed a significant augmentation in the mRNA expression of *FFAR2 vs.* STD. Consistent with this, the outcomes described herein indicate that the expression of FFAR2 and FFAR3 can be changed upon the consumption of medium- and long-chain FAs; not only by short-chain FAs as described elsewhere [48]. After treatment with BMS309403 we observed a significant increase in the mRNA expression of *FFAR3*. It may suggest that by inhibiting FABP4 more long-chain saturated FAs remain in the intestinal lumen and stimulate the production of FFAR3.

Many studies point at beneficial, anti-inflammatory properties of FFARs. For example, FFAR2 is expressed on a variety of immune cells e.g. neutrophils, macrophages, mast cells, dendritic cells, lymphocytes and epithelial cells of colonic tissue. Studies show that FFAR2 is an important regulator of inflammation in mouse models of colitis [49]. Taking into account the above-mentioned information, our result may also indicate that the activation of pro-inflammatory cytokines in the colon could increase the expression level of *FFAR2* in the COCO group. Nonetheless, characterization of the role of FFAR2 in this concept is challenging since this receptor is widespread in the body and can be involved in many physiological processes.

FFAR4 has been implicated in the secretion of incretin hormones, such as glucagon-like peptide-1 (GLP-1) [44], from L-cells in the mouse gut. Incretin hormones and GLP-1 in particular, reduce appetite and FI, leading to weight loss in long term [50]. It is thus plausible that changes seen in the expression levels of *FFAR4* in our study were responsible for changes in BW and FI of animals. A statistically higher mRNA expression of *FFAR4* in mice fed with HF, *vs.* STD diets, could contribute to the increase in the secretion of incretin hormones and decrease in the blood glucose level, and therefore affecting the BW [51]. HF + BMS group exhibited a significant decrease in the mRNA expression of *FFAR4*, *vs.* HF group, and reduced FI at the end of the study (50–60 days of feeding). The

mRNA expression of *FFAR4* in the COCO and COCO + BMS groups were similar and did not significantly differ from the STD or STD + BMS, respectively. It has been proven that colonic administration of medium-chain FAs has no impact on GLP-1 in L-cells and thereby does not change the plasma glucose level [44]. It is worth mentioning that the BW and FI of COCO and COCO + BMS were similar throughout the study. The concept of incretin hormones is of particular interest and may explain, at least in part, the results presented herein. However, since we did not measure the hormone levels, our presumptions should be taken with caution.

The mRNA expression of the same set of genes (i.e. *FFAR(1–4), Fabp4, TNFα, IL-6* and *IL-1β*, as evaluated in colonic samples, were also measured in splenic tissues. The secretory function of spleen, the central organ regulating the inflammation-related immune response, can be modulated by dietary modifications [52]. In line, increased BW and the intake of HF affect the spleen function [53] and induce lower production of pro-inflammatory cytokines e.g. IL-6 or TNFα [54]. In our study, only HF diet triggered marked decrease in the mRNA expression of *FFAR1, FFAR4, TNFα* and *IL-1β*. Owing to the function of FFAR1 and FFAR4 in splenic macrophages [12], both receptors can regulate the immune response by exerting anti-inflammatory effects. However, the mRNA expression level of both genes were decreased which excludes the possible initiation of the inflammatory process after dietary treatment with long chain FAs. Philippe et al. [55] demonstrated that mice fed with HF diet had increased MCP-1 level but the effect occurred independently of the presence or absence of FFAR1. Our findings show that diet containing long-chain saturated FAs decrease the expression of pro-inflammatory cytokines and simultaneously decrease the expression of the anti-inflammatory FFA receptors, FFAR1 and FFAR4, in mouse spleen. Although we did not observe any changes in the expression of the *IL-6*, Since the administration of BMS309403 did not significantly affect the expression of (FFAR) 1-4 and pro-inflammatory cytokines, it seems that the FABP4 does not regulate their expression in the spleen.

Conclusion and future directions

Chronic dietary intervention with a relatively low concentration of saturated FAs in a diet can affect GI motor function, induce changes in the expression of several FFA receptors, and promote migration of pro-inflammatory cytokines. Depending on the organ, the same dietary intervention can bring about opposite effects. This is presumably related to the origin of saturated FAs, their chain length as well as the distribution of FFAs receptors in the body and their specificity towards FA ligands.

The first-line therapy for patients suffering from GI diseases involves dietary regimen and when implemented aids in improving major GI symptoms, including GI motility or abdominal pain. Our results can serve as a step toward establishing novel dietary recommendations for patients suffering from GI disorders.

Author contributions

PM and JF provided the overall concept and designed the research study; PM, AT and MS conducted experiments; PM analyzed the data and wrote the manuscript. PM, AT, AF, JK, KN, AB, AB, MS, JF regularly discussed the experiments and data, suggested adjustments of the experimental protocols, read and approved the final version of the manuscript.

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Conflict of interest

Authors declare no conflict of interest.

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