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Basic nutritional investigation

High-fat, high-protein, and high-carbohydrate diets affect sphingolipid profile in pancreatic steatosis in Wistar rats

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ABSTRACT

Objectives: Imbalanced diets (e.g., excessive protein, fat, and carbohydrates) may contribute to numerous disorders, such as steatosis. However, previous studies in the pancreas are scarce and limited to the evaluation of sphingolipid metabolism in the islets of Langerhans that constitute only ~5% of the organ mass. The aim of this study was to assess the effects of high-fat, high-protein, and high-carbohydrate diets on the development of pancreatic steatosis in conjunction with sphingolipid profile in the organ.
Methods: The experiments were conducted on 40 male Wistar rats (initial age 8 wk) randomly allocated to experimental groups. After 8 wk, plasma and tissue sphingolipid levels were measured by means of high-performance liquid chromatography. Blood glucose levels were measured with a glucometer, whereas insulin concentration was determined using chemiluminescence.
Results: We demonstrated that a chronic feeding with three different types of improper diets exerts multifarious effects on sphingolipid metabolism in the pancreas. The most important finding of the present study was that all three diets predisposed toward the onset and development of pancreatic steatosis, as evidenced by an excessive ceramide accumulation.
Conclusions: As it has been established that pancreatic steatosis is a disease with growing prevalence and possible serious complications, further investigations of the topic are warranted. The complete and precise comprehension of pancreatic steatosis pathogenesis could contribute to the invention of novel therapies for the disease.

Introduction

Poor nutritional habits, including excess protein, fat, and carbohydrate consumption, contribute to numerous metabolic diseases [1]. According to data published by the World Health Organization (WHO) in 2016, ~650 million people (which is ~10% of the global population) were obese [1]. Moreover, about 3.7 million people die every year resulting from obesity-related diseases worldwide. In addition, it has been estimated that obesity prevalence has almost tripled since the mid-1970s [2]. Poor nutritional habits lead to the accumulation of lipids in various tissues and organs. This process, called steatosis, affects primarily the liver, heart, skeletal muscle, kidneys, and pancreas [3]. Interestingly, it seems that effects on the pancreas are gaining more interest among investigators [3].

Pancreatic steatosis was described for the first time, based on the results of autopsy, in 1933 [4]. Furthermore, the relationship between obesity and the occurrence of the disease was established in 1978 [5]. According to the current epidemiologic data, pancreatic steatosis affects 16% to 35% of the global population (including ~10% of pediatric and adolescent individuals) and its prevalence is slightly higher in Asian countries [6]. The risk factors for the onset and development of pancreatic steatosis include congenital diseases such as cystic fibrosis, alcohol abuse, viral infections such as Reoviridae infection, a history of acute or chronic pancreatitis, overuse of some medications (e.g., glucocorticoids, octreotide), and poor dietary habits [7]. These factors contribute to focal necrosis of

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the pancreatic parenchyma followed by its subsequent progressive infiltration with lipids (Fig. 1A). In the histologic assessment, a distortion of the lobular structure of the pancreas resulting from the accumulation of adipose tissue is observed. The most serious consequence of the described pathologic sequence is pancreatic fibrosis and the development of its exocrine and endocrine insufficiency [7].

Sphingolipids (Fig. 1B) are an extensively studied class of biologically active molecules found to be implicated in a diverse range of physiological and pathologic processes. Numerous studies conducted over the past 2 decades have revealed their significance for several important cellular processes, including differentiation, proliferation, or inflammation [8,9]. A central hub of sphingomyelin signaling pathway is ceramide (Fig. 1B), a molecule capable of stimulating many enzymes, that is, belonging to phosphatases and kinases families, and even transcription factors [10]. Ceramide originates mainly in the so-called de novo synthesis pathway or, to a lesser extent, from sphingomyelin hydrolysis [11,12]. The activation of ceramide signaling pathway has been described to take part in the regulation of cellular growth, differentiation, proliferation, necrosis, and apoptosis [13,14]. On the contrary, ceramide derivative sphingosine-1-phosphate (S1 P) is believed to be a tumor-promoting particle presenting the antagonizing effects to ceramide and involved in the inhibition of cellular proliferation, migration, apoptosis, and angiogenesis [15]. Although some studies concerning pathophysiological mechanisms in pancreatic steatosis have been published [16], the possible role of sphingolipids in its pathophysiology remains largely unexplained.

The aforementioned information regarding the high prevalence of pancreatic steatosis in combination with its serious complications and dependence on nutritional factors indicate it as a worthy candidate for a thorough scientific examination. Therefore, the main goal of this experiment was to assess the effects of high-fat, high-protein, and high-carbohydrate diets on the development of pancreatic steatosis in conjunction with the examination of sphingolipids profile in the organ in the course of the disease.

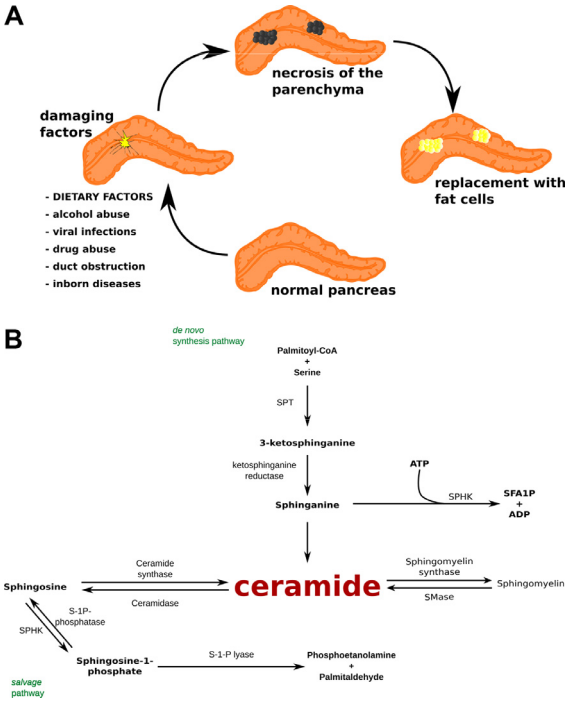

Fig. 1. (**A**) The sequence of pathologic features contributing to the onset and development of pancreatic steatosis. Modified from Cantazaro et al. [8]. (**B**) Sphingolipd signaling pathway. SFA1 P, sphinganine-1-phosphate; SPHK, sphingosine kinase; SPT, serine palmytoiltranspherase.

Materials and methods

Protocol of the experiment

The protocol for this study was approved by the Local Ethical Committee for Animal Experiments of Medical University of Bialystok. The experiment was carried out on male Wistar rats (N = 40) obtained from a licensed breeder. The initial age of the rats was 8 wk and their starting weight was in the range of 200 to 250 g. The animals were held in an appropriate facility where they had ensured stable temperature (21°C–22°C), humidity, and reversed light/dark cycle (12/12 h). During the study period, the rats were provided unrestricted access to fodder and tap water. At the onset of the study, the animals were randomly assigned into one of the four dietary groups (based on a computer-generated sequence of numbers). There were 10 rats in each group. Rats in the control group were fed a standard rodent diet (Agropol, http://www.agropolmotycz.pl/gryzonie.html; Table 1). The high-fat diet group (HFD; Research Diets, Inc.; D12492) was fed a diet containing 60% fat, 20% carbohydrates, and 20% protein; https://www.researchdiets.com/formulas/d12492; Table 2). Rats in the high-protein diet group (HPD; Research Diets, Inc.; D03012801) were fed a diet containing 14% fat, 44% protein, and 33% carbohydrates (Table 2). The high-carbohydrate diet group (HCD; Research Diets, Inc. D12450 B) was fed a diet containing 10% fat, 20% proteins, and 70% carbohydrates; https://www.researchdiets.com/formulas/d12450 b; Table 2)

After 8 wk of the experiment, the animals were sacrificed. Before sacrifice, all the rats were anesthetized by intraperitoneal pentobarbital injection (80 mg/kg body weight) after an overnight (12 h) fast. Samples of pancreatic parenchyma were then excised, frozen at once in aluminum clamps (already precooled in liquid nitrogen), and stored until further analyses in −80°C. The blood samples (obtained from the abdominal aorta) were collected into heparinized syringe and stored in −80°C until further analyses.

Plasma glucose and insulin levels

Fasting plasma glucose levels (mg/dL) were evaluated with commercially available glucometer (Accu-Check, Bayer, Germany). The chemiluminescence method was used (Abbott, Princeton, New Jersey, USA) to measure plasma insulin levels (μU/mL).

Measurement of sphinganine, sphinganine-1-phosphate, sphingosine, and sphingosine-1-phosphate contents in pancreatic samples

The contents of sphinganine (SFA), sphinganine-1-phosphate (SFA1 P), sphingosine (SFO), and sphingosine-1-phosphate (S1 P) in pancreatic samples (total tissue homogenates) were measured in accordance with the method described previously [17]. In the first step of the procedure, before the homogenization and ultrasonication of the samples, internal standards (i.e., C17-sphingosine and C17-sphingosine-1-phosphate; Avanti Polar Lipids, UK) were added. Then, sphingoid bases were transformed to their *o*-phthalaldehyde derivatives and analyzed with high-performance liquid chromatography (HPLC) system (ProStar; Varian, Inc., Palo Alto, CA, USA) equipped with fluorescence detector and C18 reversed-phase column (OmniSpher 5, 4.6 × 150 mm; Varian, Inc.).

Table 1
Formulation of control diet from Agropol (http://www.agropolmotycz.pl/gryzonie.html, LSM diet)

Ingredient	Control
Protein, min (%)	23
Fat, min (%)	3
Ash, max (%)	7.5
Fiber, max (%)	5
Lysine (%)	1.5
Methionine + cysteine, min (%)	0.8
Calcium, min (%)	1.1
Phosphorus, min (%)	0.7
Sodium, max (%)	0.2
Vitamin A [mg/kg]	8000
Vitamin B_3 [mg/kg]	1000
Vitamin E [mg/kg]	50

Table 2
Formulation of HPD (#D03012801), HFD (# D12492), HCD (# D12450 B)*

Nutrient Class	Ingredient	HPD (gm%)	HFD (gm%)	HCD (gm%)
Protein	Casein	42.3	25.8	19
	Cystein L	0.6	0.4	0.3
Carbohydrate	Lodex10	0	16.2	3.3
	Sucrose	18.3	9.4	33.6
	Corn Starch	8.1	0	29.9
	Maltodextrin	5.3	0	0
Fiber	Solka Floc, FCC200	0	6.5	4.7
	Celulose	5.3	0	0
Fat	Lard	11.4	31.7	1.9
	Soybean oil, USP	2.6	3.2	2.4
Mineral	S10026	1.1	6.5	4.7
Vitamin	Choline bitartrate	0.2	0.3	0.2
	Vitamin mix	1.1	0.1	0.1

*From Research Diets Inc. (https://researchdiets.com/).
gm%, gram percent, i.e. [g] of ingredient per 100 [g] of diet; HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet.

Measurement of SFA, SFA1, SFO, and S1 P contents in blood samples

Plasma contents of SFA, S1 P, and SFO were measured according to the procedures described previously [17]. At the beginning of their method internal standards (C17-sphingosine and C17-sphingosine-1-phosphate; Avanti Polar Lipids) were added to the samples. Sphingoid bases were converted to their *o*-phthalaldehyde derivatives and subsequently examined with HPLC system (ProStar; Varian, Inc), which was equipped with fluorescence detector and C18 reversed-phase column (OmniSpher5, 4.6 × 150 mm; Varian, Inc.).

Measurement of ceramide content in pancreatic samples

A small portion of the chloroform phase was transferred to a fresh tube containing internal standard (i.e., C17-sphingosine; Avanti Polar-Lipids). In the next step, the organic phase, which contained ceramide, underwent hydrolysis at the temperature of 90°C for 60 min in the solution of 1 M KOH in 90% methanol. After that, the liberated sphingosine content was assessed via HPLC. *N*-palmitoylsphingosine (Avanti Polar-Lipids) was applied as a standard for the preparation of calibration curve. The evaluated ceramide amount was then standardized with respect to the level of free sphingosine obtained from the same sample (total tissue homogenate).

Measurement of ceramide content in blood samples

A small part of the chloroform phase was moved to a fresh tube containing C17-sphingosine (Avanti Polar Lipids, internal standard). The organic phase containing ceramide was then hydrolyzed at 90°C for 60 min in 1 M KOH in 90% methanol solution. Liberated in the aforementioned process, sphingosine was subsequently analyzed by means of HPLC. Calibration curve was prepared using *N*-palmitoylsphingosine as a standard (Avanti Polar Lipids). The measured amount of ceramide was corrected with respect to the level of free sphingosine in a given sample.

Statistical analysis

All of the presented results are expressed as the mean + standard deviation. Statistical differences were determined based on the results of one-way analysis of variance followed by an appropriate posthoc test (i.e., pairwise Student's *t* test). If assumptions were violated, nonparametric Kruskall–Wallis test followed by an appropriate post hoc test (i.e., pairwise Wilcoxon test) were applied. The obtained *P*-values were adjusted using Benjamini–Hochberg correction because of the multiple comparisons performed. Any difference with $P < 0.05$ was considered statistically significant.

Results

Effects of HPDs, HFDs, and HCDs on body weight, plasma glucose, and insulin levels

No differences were observed with respect to initial body weights between the groups. Although there was no significant difference in the final body weight between the control and HPD groups, a markedly increased final body weight was seen in the HFD and HCD groups compared with the control group (+18% and +15%, respectively, $P < 0.05$). Furthermore, when compared with the control group, rats from the HFD and HCD groups were characterized by a significant elevation in plasma glucose level (+61% and +87%, respectively, $P < 0.05$). Finally, a dramatic increment in plasma insulin level was observed in the HFD and HCD groups compared with the control group (+1188% and +995%, respectively, $P < 0.05$). On the other hand, there were no differences in plasma glucose and insulin contents between the HPD and control groups (Table 3).

Effects of HPDs, HFDs, and HCDs on SFA contents in pancreatic and plasma samples

The comparison of pancreatic SFA concentrations revealed no significant differences between the control and all other groups. However, plasma SFA contents in the HPD and HFD groups were markedly elevated when compared with the control group (+294% and +679%, respectively, $P < 0.05$). There were no differences in plasma SFA level between HCD and control animals (Fig. 2A, B and Tables 4 and 5).

Effects of HPDs, HFDs, and HCDs on SFA1 P contents in pancreatic and plasma samples

The comparison of pancreatic SFA1 P contents showed that there were no significant differences between the control and other groups. However, plasma SFA1 P contents in the HPD, HFD, and HCD were significantly increased compared with the control group (+44%, +198%, and +100%, respectively, $P < 0.05$; Fig. 3A, B and Tables 4 and 5).

Effects of HPDs, HFDs, and HCDs on ceramide contents in pancreatic and plasma samples

Compared with the control group, the rats originating from the diet groups were characterized by a significant elevation in pancreatic ceramide concentration (+33%, +26%, and +31%, respectively, $P < 0.05$). Interestingly, only the rats from the HFD group were characterized by a markedly decreased ceramide content in blood plasma compared with the control group (−42%; $P < 0.05$). No changes in the plasma ceramide level were seen between the

Table 3
Effects of HPD, HFD, and HCD feeding for 8 wk on body weight, glucose level, and insulin level

Variable	Control	HPD	HFD	HCD
Initial body weight (g)	230.5 ± 12.7	219.4 ± 12.4	228.3 ± 20.4	226.2 ± 14.2
Final body weight (g)	325.6 ± 16	317.7 ± 29.5	385.4 ± 18.1*	374.7 ± 25.3*
Glucose level (mg/dL)	104.3 ± 6.7	108.7 ± 5.6	167.5 ± 14.6*	194.6 ± 30.5*
Insulin level (μU/mL)	4.4 ± 0.6	4.8 ± 0.8	56.7 ± 5.7*	48.2 ± 8.4*

HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet.
*$P < 0.05$ compared with control.

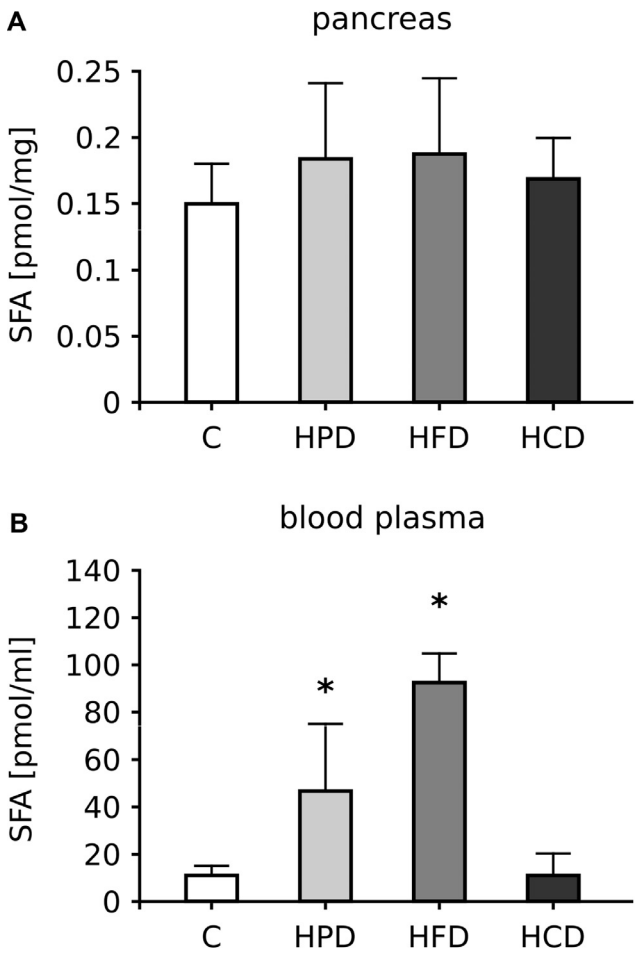

Fig. 2. (**A**) Alterations in pancreatic SFA concentrations (pmol/mg) resulting from HPD, HFD, and HCD. (**B**) Alterations in plasma SFA concentrations (pmol/mg) resulting from HPD, HFD, and HCD. *Difference versus control; $P < 0.05$. HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet; SFA, sphinganine.

remaining groups and the control group (Fig. 4A, B and Tables 4 and 5).

Effects of HPDs, HFDs, and HCDs on SFO contents in pancreatic and plasma samples

No differences were observed in the pancreatic SFO contents between the HPD and control groups. However, the rats from the HFD group were characterized by a significant decrease in pancreatic SFO content compared with the C group (−50%; $P < 0.05$). On the other hand, the comparison between the animals from the HCD and control group revealed a significant elevation in pancreatic SFO level (+19%; $P < 0.05$). Furthermore, plasma SFO contents were markedly elevated in both the HPD and HFD groups compared with the control group (+156% and +330%, respectively; $P < 0.05$). There were no differences in plasma SFO levels between the HCD and control groups (Fig. 5A, B and Tables 4 and 5).

Effects of HPDs, HFDs, and HCDs on S1 P contents in pancreatic and plasma samples

HPD and HFD animals were characterized by a significant elevation in pancreatic S1 P levels compared with the control group (+58% and 31%, respectively; $P < 0.05$). However, no differences in pancreatic S1 P contents were observed between the HCD and control groups. Finally, the rats from both the HFD and HCD groups were characterized by a significant increment in plasma S1 P concentrations compared with the control group (+41% and +86%, respectively; $P < 0.05$). No differences in the plasma S1 P content were found after comparison with the control and HPD groups (Fig. 6A, B and Tables 4 and 5).

Discussion

Pancreatic steatosis, a disease characterized by growing prevalence, remains a challenging issue in both diagnosis and management. Based on previously published studies, it has been

Table 4
Effects of HPD, HFD, and HCD feeding for 8 wk on plasma SFA, SFA1 P, CER, SFO, and S1 P levels

Variable	Control	HPD	HFD	HCD
SFA	12.07 ± 3.272	47.29 ± 28.471*	93.54 ± 11.669*	12.19 ± 8.445
SFA1 P	82.55 ± 17.264	177.43 ± 36.071*	246.33 ± 64.015*	165.6 ± 37.367*
CER	5741.65 ± 855.363	5481.86 ± 1296.933	3304.72 ± 484.181*	5063.7 ± 1908.936
SFO	39.43 ± 5.763	100.92 ± 32.742*	169.85 ± 40.819*	66.38 ± 35.047
S1 P	421.12 ± 26.377	512.79 ± 189.008	595.72 ± 175.827*	781.85 ± 145.291*

CER, ceramide; S1 P, sphingosine-1-phosphate; SFA, sphinganine; SFA1 P, sphinganine-1-phosphate; SFO, sphingosine.
Data presented as mean ± SD (pmol/mL).
*$P < 0.05$ compared with control.

Table 5
Effects of HPD, HFD, and HCD feeding for 8 wk on the pancreas SFA, SFA1 P, CER, SFO, and S1 P levels

Variable	Control	HPD	HFD	HCD
SFA	0.15 ± 0.029	0.18 ± 0.056	0.19 ± 0.055	0.17 ± 0.031
SFA1 P	0.04 ± 0.019	0.04 ± 0.03	0.07 ± 0.031	0.04 ± 0.005
CER	307.48 ± 17.468	409.53 ± 53.25*	387.03 ± 36.075*	401.16 ± 62.72*
SFO	3.21 ± 0.149	3.84 ± 0.844	1.64 ± 0.69*	3.82 ± 0.465*
S1 P	0.14 ± 0.022	0.23 ± 0.059*	0.19 ± 0.046*	0.13 ± 0.047

CER, ceramide; S1 P, sphingosine-1-phosphate; SFA, sphinganine; SFA1 P, sphinganine-1-phosphate; SFO, sphingosine.
Data presented as mean ± SD (pmol/mL).
*$P < 0.05$ compared with control.

demonstrated that long-term, untreated pancreatic steatosis contributes to the development of systemic diseases such as metabolic syndrome, atherosclerosis (as a result of plasma lipid alterations) or diabetes (both type 1, as a result of the destruction of the islets of Langerhans, and type 2, because of the development of insulin resistance). In addition, it has been demonstrated that pancreatic steatosis predisposes individuals to many other pancreatic diseases, including acute and chronic inflammation, fibrosis, and even pancreatic cancer [18]. In clinical practice, the diagnostic management includes radiologic examinations: transabdominal and endoscopic ultrasonography, computed tomography, and magnetic resonance imaging. The methods are characterized by high sensitivity and specificity and allow for a visualization of the adipose tissue presence within the pancreatic parenchyma [19–21]. However, it is extremely difficult or even impossible to evaluate the content of lipid fractions and specific sphingolipids moieties because of the anatomic location of the pancreas in the retroperitoneal space. The localization often forces an investigator to obtain the tissue samples by means of invasive procedures (fine-needle aspiration, open biopsy). For this reason, data concerning sphingolipid profile in the course of pancreatic steatosis are missing. Thus, the present study presents, presumably for the first time, the effect of three kinds of improper diets on sphingolipid metabolism in the pancreas.

In the first stage of the present study, we assessed whether chronic HPD feeding could exert an influence on sphingolipid metabolism in the rat pancreas. Recently, the so-called commercially available "high-protein diets" are gaining in popularity as a remedy for weight loss. Among them, the two most commonly used are the Atkins and Dukan diets [22]. In addition to their unquestionable beneficial effect on body weight reduction (these diets do not contain any carbohydrates), their additional potential applications also were demonstrated. First of all, it was found that an HPD could be useful in treating febrile convulsions in children [23]. Moreover, it improves cognitive functions of elderly patients with Parkinson's or Alzheimer's diseases [23]. Despite the aforementioned undoubted advantages resulting from the use of HPDs, their effect on different organs such as the kidneys, liver, small bowel, and bones seems to be unfavorable [24–26]. In detail, in the kidneys, the HPD contributes to increased glomerular filtration pressure (secondary to elevated plasma protein concentration) and hyperfiltration. As a result, damage to the glomeruli and the development of subsequent renal failure may arise [27]. Moreover, the effects of an HPD on liver function seem to be adverse. In a study performed on rats by Monteiro et al. [28] it was demonstrated that 8-wk HPD feeding contributes to the development of liver steatosis, induction of hepatocytes apoptosis (programmed cell death), and a significant increment in the amount of liver enzymes in the peripheral blood [28]. Because it has been established that fatty liver disease predisposes to the development of liver cirrhosis and

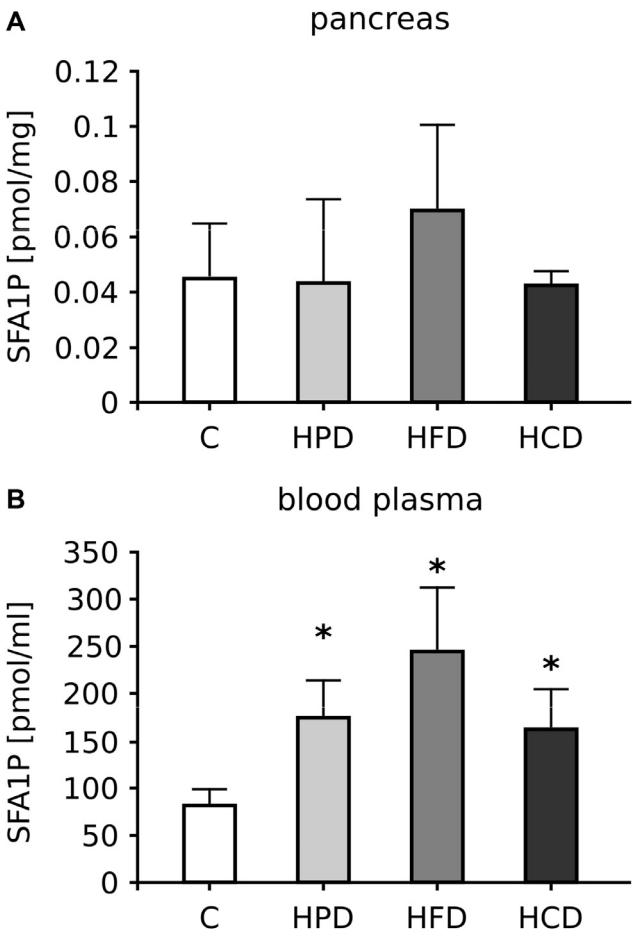

Fig. 3. (**A**) Alterations in pancreatic SFA1 P concentrations (pmol/mg) resulting from HPD, HFD. and HCD. (**B**) Alterations in plasma, *Difference ersus control; $P < 0.05$. HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet; SFA1 P, sphinganine-1-phosphate.

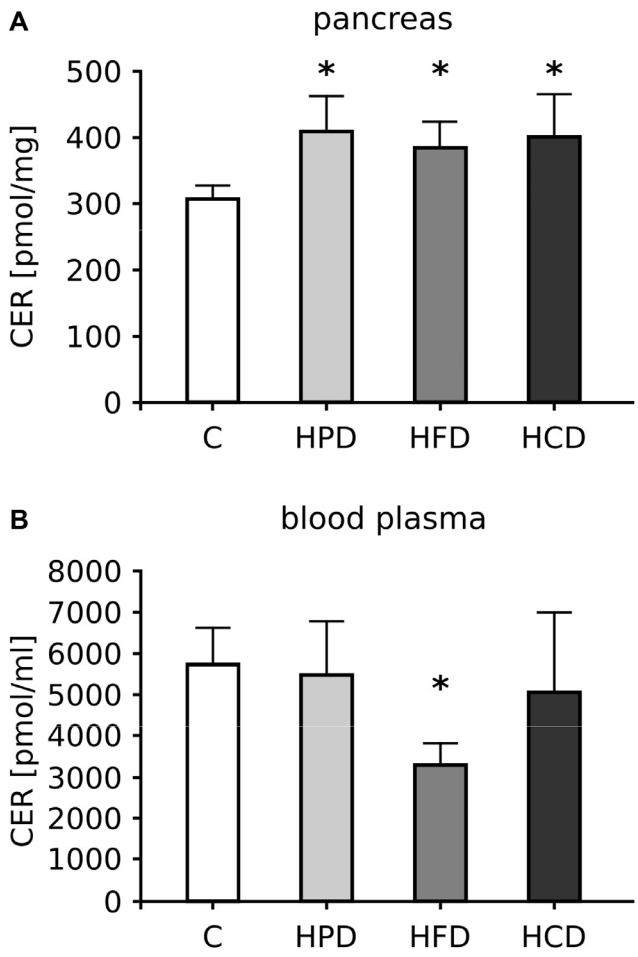

Fig. 4. (**A**) Alterations in pancreatic CER concentrations (pmol/mg) resulting from HPD, HFD. and HCD. (**B**) Alterations in plasma. (**B**) Alterations in plasma CER concentrations (pmol/mg) resulting from HPD, HFD. and HCD. *Difference versu control; $P < 0.05$. CER, ceramide; HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet.

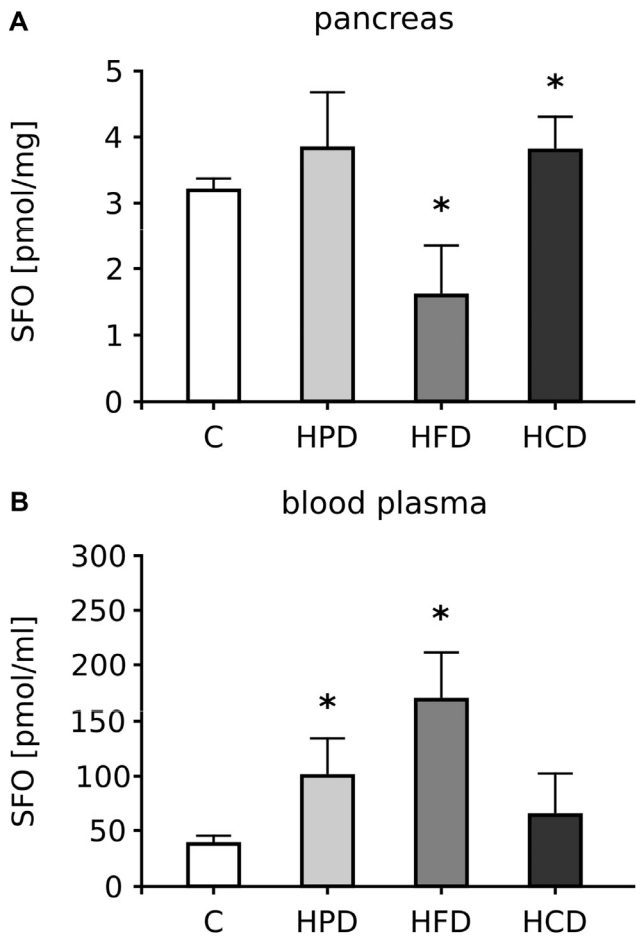

Fig. 5. (**A**) Alterations in pancreatic SFO concentrations (pmol/mg) resulting from HPD, HFD. and HCD. (**B**) Alterations in plasma SFO concentrations (pmol/mg) resulting from HPD, HFD. and HCD. *Difference versus control; $P < 0.05$. HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet; SFO, sphingosine.

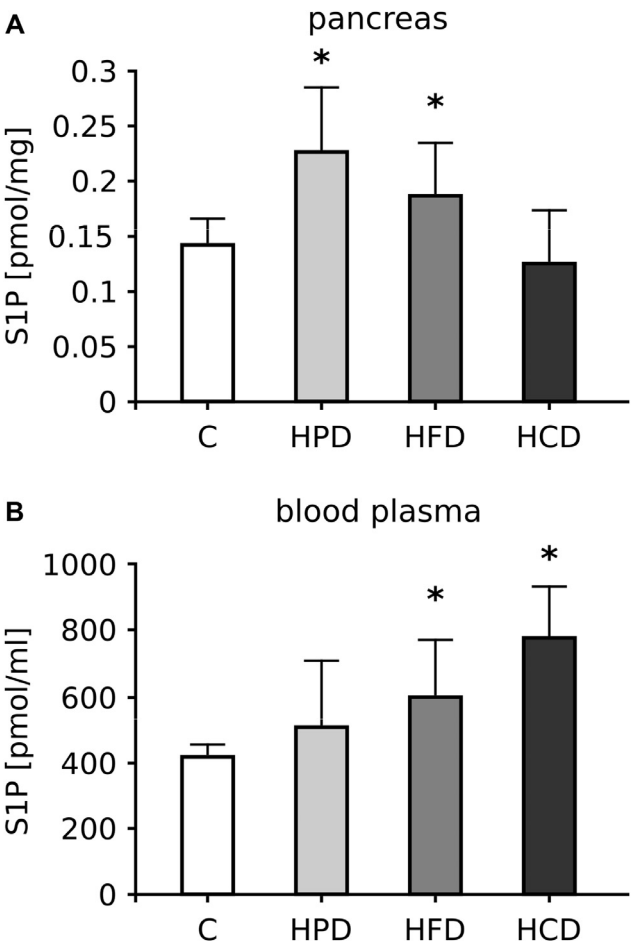

Fig. 6. (**A**) Alterations in pancreatic S1 P concentrations (pmol/mg) resulting from HPD, HFD, and HCD. (**B**) Alterations in plasma S1 P concentrations (pmol/mg) resulting from HPD, HFD, and HCD. *Difference versus control; $P < 0.05$. HCD, high-carbohydrate diet; HFD, high-fat diet; HPD, high-protein diet; S1 P, sphingosine-1-phosphate.

subsequent hepatic failure, the effect of an HPD on the liver should not be underestimated.

Interestingly, alterations in lipids metabolism similar to the one observed in hepatic steatosis are also present in the pancreas. In the present study, we demonstrated that chronic HPD feeding caused some alterations in sphingolipid metabolism. First of all, the concentrations of ceramide precursors SFA and SFA1 P were markedly elevated in the rats fed an HPD (+294% and +44%, respectively; $P < 0.05$; Figs. 2B and 3B), which strongly suggests activation of ceramide de novo synthesis in the course of pancreatic steatosis. Surprisingly, these changes were observed only in the samples obtained from peripheral blood, but not from the pancreas itself. These findings could have possible applications in clinical practice because we have proven that the evaluation of ceramide precursors levels in peripheral blood samples is an easy and cost-effective method of diagnosis of early pancreatic steatosis [29]. Furthermore, we found a significant and excessive accumulation of ceramide and its metabolite, S1 P, in the pancreas of the rats fed an HPD (+33% and +31%, respectively; $P < 0.05$; Figs. 4A and 6A), which confirms that this diet contributes to the onset and development of pancreatic steatosis. However, because previously published data regarding the possible effects of an HPD on pancreatic steatosis are limited, any comparisons are virtually impossible to make. In a sole paper on the topic published by Hara et al. [30], it was revealed that an 8-wk HPD regime resulted in pancreatic lobules overgrowth. This led to the exacerbation of the pancreatic exocrine function manifested in the increased activity of its digestive enzymes. Furthermore, the disturbances in pancreatic exocrine functions most likely result from the increased synthesis of the protein enzymes (i.e., amylases and lipases) resulting from excessive protein supplementation in the diet [30]. The aforementioned manifestations of pancreatic exocrine and endocrine insufficiency are typically observed in the course of chronic pancreatitis, a disease entity that could be a result of pancreatic steatosis. Moreover, a recent paper by Drummond et al. [31] might shed more light on the discussed topic. In the research, the authors tested the influence of casein hydrolystaes (casein is a main distinctive feature of our HPD, Table 2) on pancreas glycemic control properties. The study showed that casein tends to moderately increase insulin secretion and reduce glucose levels, but only in the case of obese ob/ob mice and not in the case of their wild-type C57 BL/6 counterparts [31]. This seems to be in line with the present findings because we did not observe changes in plasma glucose or insulin concentration (Table 3). Regrettably, the aforementioned research did not investigate pancreas lipid metabolism. However, the authors observed a significant reduction in the overall liver steatosis (−43% in overall liver fat, −28% in the average lipid droplet volume) [31]. Unfortunately, the authors only reported the changes for ob/ob animals, not for their C57 BL/6 littermates, therefore

referring to the changes observed by us in the pancreas seems to be unjustified.

In the next step of the present experiment, we assessed whether chronic HFD feeding affects sphingolipid metabolism in the blood plasma and pancreas tissue. As expected, the dietary regime caused increases in the basic rats' characteristics (Table 3), namely, final body weight (+18%), glucose (+61%), and insulin concentration (+1188%). The above is in agreement with previous studies that we conducted [32,33] and those by others [34]. Moreover, it seems of vital importance because many studies [19,35] indicate the existence of a positive correlation between plasma glucose and insulin level and fatty pancreas. The measurements of SFA and SFA1 P levels in the tissue samples obtained directly from the pancreas revealed no significant differences between the control and other studied groups of animals (Figs. 2A and 3A). On the contrary, plasma levels of the aforementioned ceramide precursors were markedly increased in the HFD group compared with the control rats (+679% and +198%, respectively; $P < 0.05$; Figs. 2B and 3B). Furthermore, we found that HFD feeding led to an accumulation of ceramide and its derivative S1 P in pancreatic tissue (+26% and +31%, respectively; $P < 0.05$; Figs. 4A and 6A). This accumulation often is an indicator of a tissue steatosis. These observations remain in accordance with the alterations in sphingolipid metabolism found in other tissues, such as the liver and skeletal muscle, where they are a commonly considered to be a result of steatosis because of chronic HFD feeding [14,36]. Moreover, we must bear in mind our diet composition, namely, its significantly greater animal fat (i.e., lard) content (Table 2). This could well translate into increased sphingolipid saturation status and greater burden for the pancreas functioning. This could be the case as previous studies in insulin-sensitive tissues showed a relation between the increased "animal" (saturated) fatty acids in ceramides (especially the one with >16 carbons) and their deleterious consequences for the accumulating tissue functioning [32,37,38]. Surprisingly, we revealed that in the course of pancreatic steatosis, ceramide precursor levels are elevated in plasma samples, whereas ceramide and S1 P levels are increased in pancreatic samples. These changes indicate that the alterations in sphingolipid metabolism may start in peripheral blood and lead to pancreatic steatosis. Similar relationships were demonstrated for other lipid fractions [39]. A study by Novotny et al. reported that circulating triacylglycerides and free-fatty acids are more responsible for decreased insulin secretion than fatty tissue stored in pancreatic parenchyma [39]. Among other biomarkers of excessive ectopic fat accumulation in the pancreas were high-density lipoprotein (HDL) and glycated hemoglobin. Circulating levels of biomarkers, including ceramide precursors, appear to be the best currently available non-invasive markers of pancreatic steatosis. The approach, blood analysis screening toward pancreatic steatosis, should be a subject of further investigation because of its high accuracy, non-invasive nature, and relatively low cost [35]. Nevertheless, some opposite results have been published. In a study by Garbowska et al. [40], a significant reduction in ceramide concentration after HFD feeding was shown in the rat salivary glands (i.e., the organs with histologic architecture similar to the pancreas) [35]. Despite the indisputable clinical significance of steatosis and its deleterious effects on different tissues and organs, studies concerning the pathogenesis of pancreatic steatosis are scarce and limited to the evaluation of sphingolipid metabolism in the islets of Langerhans. Previously published studies demonstrated that ceramide impairs insulin secretion in primary β-cell cultures. Moreover, it has been revealed that ceramide inhibits proliferation and induces apoptosis of the islets of Langerhans [41]. However, it should be highlighted that the pancreatic islets constitute only ~5% of the organ mass, whereas the process of steatosis affects the entire pancreatic parenchyma. For this reason, the disease predisposes not only to the development of the endocrine organ insufficiency, but also, and mainly, to the disturbances in its exocrine function.

Finally, we evaluated the effects of an HCD on sphingolipid metabolism in the pancreas. First of all, like in the case of HFD feeding, we observed increases in the basic rats' characteristics (Table 3), namely final body weight (+15%), glucose (+87%), and insulin concentration (+995%). This seems of vital importance because a meta-analysis by Singh et al. [35] revealed that plasma glucose and insulin levels correlate positively with pancreas steatosis (glucose: $R = 0.36$, 95% confidence interval [CI], 0.15–0.57; insulin: $R = 0.38$, 95% CI, 0.33–0.43). The aforementioned data already indicate a possible steatosis of the examined organ. This is in line with a study by Polakof et al. [42], in which the authors revealed that an HCD led to glucose intolerance and alterations in lipid metabolism in the pancreas of rainbow trout [42]. Interestingly, the present study demonstrated that the HCD resulted in excessive accumulation of ceramide and its metabolite SFO in the pancreas (+31% and +19%, respectively; $P < 0.05$; Figs. 4A and 5A). Surprisingly, no differences in the S1 P content between the control and the HCD groups were found (Fig. 6A). However, the levels of S1 P and SFA1 P in the blood samples were markedly elevated in the HCD group (+86% and +100%; $P < 0.05$; Figs. 3B and 6B). On the other hand, based on the results of previously published studies, it can be postulated that the HCD predisposes to the development of pancreatic steatosis. In a study published by Hazakira et al. [43], it was been demonstrated that a chronic (12-wk) high-carbohydrate feeding leads to significant alterations in pancreatic parenchyma. In comparison with the rodents from the control group, rats fed the HCD developed necrosis and subsequent fibrosis of the pancreas [43]. Furthermore, the aforementioned changes also were accompanied by an increased deposition of lipid droplets in the pancreas [43]. The differences observed between the present study results and the one published previously most likely arise from two different time frames for high-carbohydrate feeding (8 versus 12 wk). That notion seems to be confirmed by a study by Hazakira et al. [43], in which restoration of a standard chow diet led to a partial regression of the aforementioned disturbances after only 4 wk of application [43]. Interestingly, it can be assumed that a chronic HCD leads to pancreatic endocrine dysfunction. Moreover, other studies have indicated the deleterious effects of carbohydrate excess on internal organ/tissue functioning [44]. Ruteledge et al. [44] demonstrated that sustained fructose oversupply results in perturbations of cell signaling. Moreover, it may stimulate certain inflammatory processes in insulin-sensitive tissues. This is achieved by the activation of tumor necrosis factor-α, c-Jun amino terminal kinase, (protein tyrosine phosphatase 1 B, or a combination of all three. Importantly, the molecules just cited are well-known pathologic factors mutually interrelated with sphingolipids signaling pathway in the liver and skeletal muscles [45,46]. Interestingly, some studies point to sphingolipids as a causative factor triggering inflammation and tissue (including the pancreas) degeneration/apoptosis [47,48]. Whether this is the case in HCD-induced steatosis remains to be determined. One possible way through which the HCD exerted its pathophysiological effects is via its high content of sucrose and starch (Table 2), which quite probably contributed to its increased glycemic index. In line with that notion, Sun et al. [49] tested the influence of so-called "resistant starch" (i.e., the one with a low glycemic index) on pancreas dysfunction in type 2 diabetic Sprague-Dawley rats fed a high-glucose fat diet. The authors found the treatment to be effective with respect to better management of lipids in blood plasma and the liver [49]. Furthermore, the rats also presented an improvement in glucose and insulin metabolism and probably decreased pancreatic damage [49]. Nevertheless, for now the theory of starch and sucrose as

main pathogenic agents in the HCD, although tempting, would need to be directly tested.

Moreover, it is worth noting that the disturbances in sphingolipid metabolism are observed in some other pancreatic diseases. More specifically, our own recent study demonstrated that ceramide downstream metabolite S1 P was markedly decreased in the blood plasma of patients with severe acute pancreatitis [29], as well as in the pancreas of rats with cerulein-induced acute pancreatitis [50]. Interestingly, the application of S1 P analog fingolimod (FTY720) prevents acute pancreatitis complications. On the other hand, it has been revealed that the inhibition of ceramide de novo synthesis with an SPT inhibitor—myriocin—restores fat accumulation in hepatocytes [36,51]. Thus, myriocin is a valid chemical agent in the treatment of non-alcoholic fatty liver disease, an entity that has similar pathogenesis to the one observed in pancreatic steatosis. Based on the data just cited, one may speculate that regulation of sphingolipd metabolism may act as a novel therapeutic strategy for the prevention and treatment of pancreatic steatosis. However, further studies in this area are mandatory to provide conclusive evidence.

Conclusion

The present study demonstrated, presumably for the first time, that chronic feeding with three different types of improper diets exerts multifarious effects on sphingolipid metabolism in the pancreas. The most important finding of the present study was that HPDs, HFDs, and HCDs predispose toward the onset and development of pancreatic steatosis, as evidenced by an excessive ceramide accumulation in pancreatic parenchyma. Because it has been established that pancreatic steatosis is a disease entity with growing prevalence and possible serious complications, further investigations of the topic are warranted. The complete and precise comprehension of pancreatic steatosis pathogenesis could contribute to the invention of novel therapies to address the disease.

References

[1] World Health Organization. Obesity and overweight. Factsheet no. 310. Available at: http://www.who.int/mediacentre/factsheets/fs310/en/. Accessed January, 2015

[2] World Obesity Federation. Obesity prevalence worldwide—adults. Available at: https://www.worldobesity.org/data/map/overview-adults. Accessed January, 2015

[3] Feldman M, Friedman LS, Brandt LJ. Sleisenger and Fordtran's gastrointestinal and liver disease e-book: pathophysiology, diagnosis, management. vol. 1. 10th ed. Philadelphia, PA, USA: Elsevier Health Sciences; 2015.

[4] Ogilvie RF. The islands of langerhans in 19 cases of obesity. J Pathol 1933;37:473–81.

[5] Olsen TS. Lipomatosis of the pancreas in autopsy material and its relation to age and overweight. Acta Pathol Microbiol Scand A 1978;86 A:367–73.

[6] Pham YH, Bingham BA, Bell CS, Greenfield SA, John SD, Robinson LH, et al. Prevalence of pancreatic steatosis at a pediatric tertiary care center. South Med J 2016;109:196–8.

[7] Catanzaro R, Cuffari B, Italia A, Marotta F. Exploring the metabolic syndrome: Nonalcoholic fatty pancreas disease. World J Gastroenterol 2016;22:7660–75.

[8] Coant N, Sakamoto W, Mao C, Hannun YA. Ceramidases, roles in sphingolipid metabolism and in health and disease. Adv Biol Regul 2017;63:122–31.

[9] Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. Nat Rev Cancer 2004;4:604–16.

[10] Hannun YA, Obeid LM. Principles of bioactive lipid signaling: lessons from sphingolipids. Nat Rev Mol Cell Biol 2008;9:139–50.

[11] Hanada K. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. Biochim Biophys Acta 2003;1632:16–30.

[12] Mikłosz A, Łukaszuk B, Chabowski A, Rogowski F, Kurek K, Żendzian-Piotrowska M. Hyperthyroidism evokes myocardial ceramide accumulation. Cell Physiol Biochem 2015;35:755–66.

[13] Kurek K, Łukaszuk B, Piotrowska DM, Wiesiołek P, Chabowska AM, Zendzian-Piotrowska M. Metabolism, physiological role, and clinical implications of sphingolipids in gastrointestinal tract. Biomed Res Int 2013:908907.

[14] Kurek K, Mikłosz A, Łukaszuk B, Chabowski A, Górski J, Żendzian-Piotrowska M. Inhibition of ceramide de novo synthesis ameliorates diet induced skeletal muscles insulin resistance. J Diabetes Res 2015:154762.

[15] Bartke N, Hannun YA. Bioactive sphingolipids: metabolism and function. J Lipid Res 2009;50(suppl):S91–6.

[16] Sadr-Azodi O, Orsini N, Andrén-Sandberg Å, Wolk A. Abdominal and total adiposity and the risk of acute pancreatitis: a population-based prospective cohort study. Am J Gastroenterol 2013;108:133–9.

[17] Kurek K, Garbowska M, Ziembicka DM, Łukaszuk B, Rogowski J, Chabowski A, et al. Myriocin treatment affects lipid metabolism in skeletal muscles of rats with streptozotocin-induced type 1 diabetes. Adv Med Sci 2017;62:65–73.

[18] Chang H-H, Moro A, Takakura K, Su H-Y, Mo A, Nakanishi M, et al. Incidence of pancreatic cancer is dramatically increased by a high fat, high calorie diet in krasg12 d mice. PLoS One 2017;12:e0184455.

[19] Lee JS, Kim SH, Jun DW, Han JH, Jang EC, Park JY, et al. Clinical implications of fatty pancreas: Correlations between fatty pancreas and metabolic syndrome. World J Gastroenterol 2009;15:1869–75.

[20] Kim HJ, Byun JH, Park SH, Shin YM, Kim PN, Ha HK, et al. Focal fatty replacement of the pancreas: usefulness of chemical shift mri. AJR Am J Roentgenol 2007;188:429–32.

[21] Kim MK, Chun HJ, Park JH, Yeo DM, Baek K-H, Song K-H, et al. The association between ectopic fat in the pancreas and subclinical atherosclerosis in type 2 diabetes. Diabetes Res Clin Pract 2014;106:590–6.

[22] Atkins Dr. RD. Atkins' new diet revolution. New York, NY, USA: HarpPeren; 2002.

[23] Bielohuby M, Menhofer D, Kirchner H, Stoehr BJM, Müller TD, Stock P, et al. Induction of ketosis in rats fed low-carbohydrate, high-fat diets depends on the relative abundance of dietary fat and protein. Am J Physiol Endocrinol Metab 2011;300:E65–76.

[24] Garbow JR, Doherty JM, Schugar RC, Travers S, Weber ML, Wentz AE, et al. Hepatic steatosis, inflammation, and ER stress in mice maintained long term on a very low-carbohydrate ketogenic diet. Am J Physiol Gastrointest Liver Physiol 2011;300:G956–67.

[25] Hammond KA, Janes DN. The effects of increased protein intake on kidney size and function. J Exp Biol 1998;201:2081–90.

[26] Cuenca-Sánchez M, Navas-Carrillo D, Orenes-Piñero E. Controversies surrounding high-protein diet intake: satiating effect and kidney and bone health. Adv Nutr 2015;6:260–6.

[27] Ko GJ, Obi Y, Tortorici AR, Kalantar-Zadeh K. Dietary protein intake and chronic kidney disease. Curr Opin Clin Nutr Metab Care 2017;20:77–85.

[28] Monteiro MEL, Xavier AR, Oliveira FL, Filho PJ, Azeredo VB. Apoptosis induced by a low-carbohydrate and high-protein diet in rat livers. World J Gastroenterol 2016;22:5165–72.

[29] Konończuk T, Łukaszuk B, Żendzian-Piotrowska M, Dąbrowski A, Krzyżak M, Ostrowska L, et al. Plasma sphingolipids in acute pancreatitis. Int J Mol Sci 2017;18.

[30] Hara H, Narakino H, Kiriyama S, Kasai T. Induction of pancreatic growth and proteases by feeding a high amino acid diet does not depend on cholecystokinin in rats. J Nutr 1995;125:1143–9.

[31] Drummond E, Flynn S, Whelan H, Nongonierma AB, Holton TA, Robinson A, et al. Casein hydrolysate with glycemic control properties: evidence from cells, animal models, and humans. J Agric Food Chem 2018;66:4352–63.

[32] Lukaszuk B, Miklosz A, Zendzian-Piotrowska M, Wojcik B, Gorski J, Chabowski A. Changes in the diaphragm lipid content after administration of streptozotocin and high-fat diet regime. J Diabetes Res 2017;2017:3437169.

[33] Matczuk J, Zalewska A, Łukaszuk B, Knaś M, Maciejczyk M, Garbowska M, et al. Insulin resistance and obesity affect lipid profile in the salivary glands. J Diabetes Res 2016;2016:8163474.

[34] Oza MJ, Kulkarni YA. Formononetin treatment in type 2 diabetic rats reduces insulin resistance and hyperglycemia. Front Pharmacol 2018;9:739.

[35] Singh RG, Yoon HD, Poppitt SD, Plank LD, Petrov MS. Ectopic fat accumulation in the pancreas and its biomarkers: a systematic review and meta-analysis. Diabetes Metab Res Rev 2017;33.

[36] Kurek K, Piotrowska DM, Wiesiołek-Kurek P, Łukaszuk B, Chabowski A, Górski J, et al. Inhibition of ceramide de novo synthesis reduces liver lipid accumulation in rats with nonalcoholic fatty liver disease. Liver Int 2014;34:1074–83.

[37] Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3 T3-l1 adipocytes and c2 c12 myotubes. Arch Biochem Biophys 2003;419:101–9.

[38] Blachnio-Zabielska AU, Chacinska M, Vendelbo MH, Zabielski P. The crucial role of c18-cer in fat-induced skeletal muscle insulin resistance. Cell Physiol Biochem 2016;40:1207–20.

[39] Nowotny B, Kahl S, Klüppelholz B, Hoffmann B, Giani G, Livingstone R, et al. Circulating triacylglycerols but not pancreatic fat associate with insulin secretion in healthy humans. Metab Clin Exp 2018;81:113–25.

[40] Garbowska M, Łukaszuk B, Mikłosz A, Wróblewski I, Kurek K, Ostrowska L, et al. Sphingolipids metabolism in the salivary glands of rats with obesity and streptozotocin induced diabetes. J Cell Physiol 2017;232:2766–75.

[41] Véret J, Bellini L, Giussani P, Ng C, Magnan C, Le Stunff H. Roles of sphingolipid metabolism in pancreatic β cell dysfunction induced by lipotoxicity. J Clin Med 2014;3:646–62.

[42] Polakof S, Skiba-Cassy S, Kaushik S, Seiliez I, Soengas JL, Panserat S. Glucose and lipid metabolism in the pancreas of rainbow trout is regulated at the molecular level by nutritional status and carbohydrate intake. J Comp Physiol B, Biochem Syst Environ Physiol 2012;182:507–16.

[43] Hazarika A, Kalita H, Chandra Boruah D, Chandra Kalita M, Devi R. Pathophysiology of metabolic syndrome: the onset of natural recovery on withdrawal of a high-carbohydrate, high-fat diet. Nutrition 2016;32:1081–91.
[44] Rutledge AC, Adeli K. Fructose and the metabolic syndrome: pathophysiology and molecular mechanisms. Nutr Rev 2007;65:S13–23.
[45] Straczkowski M, Kowalska I. The role of skeletal muscle sphingolipids in the development of insulin resistance. Rev Diabet Stud 2008;5:13–24.
[46] Kim MH, Ahn HK, Lee E-J, Kim S-J, Kim Y-R, Park J-W, et al. Hepatic inflammatory cytokine production can be regulated by modulating sphingomyelinase and ceramide synthase 6. Int J Mol Med 2017;39:453–62.
[47] Sergi D, Morris AC, Kahn DE, McLean FH, Hay EA, Kubitz P, et al. Palmitic acid triggers inflammatory responses in n42 cultured hypothalamic cells partially via ceramide synthesis but not via tlr4. Nutr Neurosci 2018: 1–14.
[48] Kowluru A, Kowluru RA. RACking up ceramide-induced islet β-cell dysfunction. Biochem Pharmacol 2018;154:161–9.
[49] Sun H, Ma X, Zhang S, Zhao D, Liu X. Resistant starch produces antidiabetic effects by enhancing glucose metabolism and ameliorating pancreatic dysfunction in type 2 diabetic rats. Int J Biol Macromol 2018;110:276–84.
[50] Konończuk T, Łukaszuk B, Mikłosz A, Chabowski A, Żendzian-Piotrowska M, Kurek K. Cerulein-induced acute pancreatitis affects sphingomyelin signaling pathway in rats. Pancreas 2018;47:898–903.
[51] Kurek K, Wiesiołek-Kurek P, Piotrowska DM, Łukaszuk B, Chabowski A, Żendzianendzian-Piotrowska M. Inhibition of ceramide de novo synthesis with myriocin affects lipid metabolism in the liver of rats with streptozotocin-induced type 1 diabetes. Biomed Res Int 2014;980815.