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ANALYSIS OF GENETIC POLYMORPHISMS OF OCT1, MATE1, MATE2 AND GLP1R IN RATS WITH EXPERIMENTALLY INDUCED OBESITY

M. Kalinkova[1], T. Kadiyska[2], K. Dabchev[2], G. Bogdanov[1], T. Handjieva-Darlenska[1]

[1]Department of Pharmacology and Toxicology, Faculty of Medicine, Medical University – Sofia, Bulgaria
[2]Department of Physiology and Pathophysiology, Faculty of Medicine, Medical University – Sofia, Bulgaria

Abstract. *The experimental model of obesity based on a cafeteria diet is a common model to investigate different aspects of obesity. The present study aimed to evaluate the link between genetic variants of OCT1, MATE1, MATE2 and GLP1R and the treatment effects in male obese rats. After 19-weeks of feeding with a standard chow food and Cafeteria-diet (CAF), the rats were divided into three groups: control group (only CAF), metformin group (CAF and metformin treatment) and liraglutide group (CAF and GLP1 agonist treatment). The genetic variations of the receptors for metformin in liver and kidney (OCT1, MATE1, MATE2) and for liraglutide (GLP1R) were examined. The results demonstrated a significant decrease in body mass index, blood glucose and a significant increase in plasma HDL-cholesterol levels in both groups treated with either metformin or liraglutide compared to the control group. No effect on plasma triglycerides and VLDL-cholesterol levels was shown between the three groups. According to the genetic analysis, all rats were "wild type" for the genetic variants tested in OCT, MATE1, MATE2 and GLP1R, not affecting the effects of treatment. This raises the possibility of other potential genes implicated in the underlying mechanism of obesity and metformin/liraglutide therapy.*

Key words: *obesity, metformin, liraglutide, polymorphisms, receptors*

Corresponding Author: *Mariya Kalinkova, MD, tel: +359885219702, e-mail: mariakkalinkova@gmail.com*

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INTRODUCTION

Obesity is a global public health problem observed in all age groups. It generates a socioeconomic impact since it affects people's health and quality of life. Moreover, junk food consumption is the major factor responsible for the weight gain, because of its high palatability and high-energy nutrients [1].

The latest guideline for obesity management demonstrates that the first step in its treatment is change in one's lifestyle [2]. When this does not lead to body weight reduction, medical pharmacotherapy is considered. The therapy could include liraglutide 3.0 mg/daily, a glucagon-like-peptide-1 receptor agonist and metformin off label [3]. A modern approach to enhance the therapy of obese patients is to use precision medicine. This method is based on using genetic and molecular analysis to find the most appropriate therapy for a patient, leading to better overall efficiency. An innovative approach for obesity treatment is the examination of the genes for the receptors for metformin and liraglutide [4].

In obese patients after lifestyle changes and ineffective results, the first line pharmacotherapy includes the group of glucagon-like-peptide-1 (GLP-1) agonists such as liraglutide and semaglutide [5]. There are six GLP-1 agonists: albiglutide, dulaglutide, exenatide, liraglutide, lixizenatide and semaglutide. They act as incretin mimetics and stop the degradation of the endogenous GLP-1 agonist and the gastric inhibitor peptide (GIP) [6]. Numerous clinical trials are aimed at the role of GLP-1 agonists, because of their insulinotropic action in patients with metabolic syndrome. The incretin signalization results in insulin secretion, blocks the glucagon secretion, and reduces the appetite [7].

Most of the studies concerning the treatment with GLP-1 agonist are focused on the gene encoding this protein as well as its variations. Several variations are estimated, which change the function of the receptor for GLP-1 and in this way change the insulin secretion [8]. According to a recent pilot survey, which compares exenatide and liraglutide, people with GLP1R rs6923761 recessive allele A (AA_AG) who take exenatide or liraglutide have a slower emptying of the stomach (GE T1/2) in comparison to the normal [117.9±27.5 (SEM) minutes and 128.9±38.32 minutes] compared to GG genotype (95.8±30.4 minutes and 61.4±21.4 minutes) [9]. The recessive allele A for GLP1R (rs6923761) is associated with even slower emptying of the stomach (GE T1/2) in response to the therapy with liraglutide and exenatide [10]. These studies give data on the effect of GLP1R (rs6923761) in weight loss therapy with GLP-1 agonists.

Metformin is a well-known oral anti-hyperglycemic drug. Moreover, it is also prescribed for obesity treatment off label [11, 12]. Metformin specifically reduces hepatic gluconeogenesis without increasing insulin secretion, inducing weight gain or risk of hypoglycemia. This preferential action of metformin in hepatocytes is due to the predominant expression of organic cation transporters 1 (OCT1) that are responsible of hepatic uptake of metformin [13]. OCT1 belongs to the solute carrier family (SLC22A) and is localized in the sinusoidal membrane of rat and human hepatocytes. Other reported locations of human OCT1 include the lateral membrane of intestinal epithelial cells, the luminal (apical) membrane of ciliated cells in the lung, and of tubule epithelial cells in the kidney [14]. The human SLC22A1 gene encoding OCT1 consists of 11 exons, has been mapped to chromosome 6q26 and spans about 37kb. OCT1 is highly polymorphic in ethnically diverse populations and mediate differences in transporter function. This helps to provide a possible mechanism to account for individual variations in the metformin responses. Furthermore, metformin is also transported by the multidrug and toxin extrusion proteins (MATEs), namely MATE1 and MATE2 [11, 12]. MATE1 is highly expressed in the kidney and liver with lower expression in skeletal muscle and adipose tissue [15, 16]. According to some studies, MATE1 and OCT1 have been shown to mediate transcellular transport of metformin in vitro and to affect metformin response in diabetic patients. Additionally, MATE1 and OCT1 polymorphisms affect metformin response in diabetic patients [17].

Regarding pharmacodynamics, the transporters play a major role in metformin renal elimination. MATE1 and MATE2 contribute to the efflux of metformin into the urine. There have been studies showing that renal clearance and tissue distribution of metformin is altered in MATE1 (−/−), but not in Mate1 (+/−) mice [18, 19, 20]. However, the effects of genetic variation in MATE1 and MATE2 on the pharmacokinetics of metformin in humans remain unclear. Clinical studies that are focused on the effects of promoter variants of drug disposition and response have been less well studied than coding and intronic region polymorphisms [21].

Animal models of obesity are used in both basic and clinical research. It is crucial to have valid animal models to approach and mimic human's disorders [22]. Cafeteria diet (CAF) has been used to induce obesity in experimental animals since 1970s and now it is one of the most frequently used diets for obesity imitation [1]. On this diet, the laboratory animals eat the same ultra-processed and unhealthy products that humans consume, e.g., cookies and chips. They are allowed to standard chow and water while concurrently offered highly palatable, energy dense, unhealthy human foods ad libitum. It provokes voluntary hyperphagia that results in rapid weight gain and increase in fat mass [23, 24]. All these result in prediabetic parameters such as glucose and insulin intolerance. The CAF diet model resembles a certain pattern of problematic human consumption, resulting in weight gain and obesity. Inducing obesity in laboratory rodents is a good model to investigate the effect of the different anti-obesity drugs [25].

In the present study, we hypothesized that the promoter variations for the receptors for metformin (MATE1, MATE2 and OCT1) and for liraglutide (GLP1RA) determine the effect of the therapy with metformin and liraglutide. They also have an impact on the liver metabolism, renal clearance and glucose-lowering response in rats with experimentally induced obesity and the consequent metabolic disorders.

MATERIALS AND METHODS

Study design and animals

The study was conducted in accordance with the Code of Ethics of Medical University – Sofia and was carried in accordance with the European Communities Council Directive (86/609/EEC of 24 November 1986 on the approximation of laws).

A total of thirty-nine male two-months Wistar rats with an average weight of around 200 g were used. The animals were sourced from The Institute of Neurobiology at BAS (Bulgarian Academy of Science). They were housed three per cage in a humidity-controlled room at a temperature between 20-22°C. Prior to the experiment, they were brought to a standard 12:12 light: dark cycle (lights on at 07:00 h), for one week acclimation period.

The first part of the experiment consisted of inducing obesity for 19 weeks. During this period the rats were exposed to unlimited access to standard laboratory chow diet, water and CAF diet consisting of high-caloric, palatable foods (Table 1). The monitoring and progress of the model was recorded weekly.

To define obesity, body mass index (BMI) was calculated. The formula for BMI is weight in kilograms divided by height in meters squared. For rats the normal BMI is 0.45-0.68 g/cm^2, above this, they are considered obese [26]. BMI was calculated from week 9 and every second week onwards. Due to not enough weight gain on week 12, a soft drink (banana juice) was added ad libitum to the diet with high consistence of sugar (12g/100 ml), in accordance with similar experiments [27, 28]. Upon measurement on week 19, the rats reached obesity levels calculated by BMI levels.

The second part of the experiment consisted of dividing the rats into three groups and starting the drug treatment (metformin or liraglutide) over period of six weeks to study their metabolic effects. Rats were randomly assigned into three groups: control group (n = 11, CAF only), second group treated with metformin (n = 17, CAF, and metformin) and third group treated with liraglutide (n = 11, CAF and GLP1 agonist). Along with the therapy, all three groups had unlimited access to standard chow food and water. Metformin hydrochloride (Metfodiab, Teva) was applied in a dose of 250mg/kg/day intragastrical. Liraglutide (Victoza, NovoNordisk) was applied in a dose of 75 µg /kg/day subcutaneously. Body weight and naso-anal length were measured weekly.

Biochemical analysis

At the end of the experimental period (week 25), rats were anesthetized and decapitated. Blood was collected in 4 ml vacuum tubes with Gel/Clot Activator (Biomed Global). After centrifugation (3900 g for 10 min. at 4°C) the serum was collected in a separate tube. Serum levels of glucose, triglycerides, HDL-cholesterol, and VLDL-cholesterol were also analyzed using colorimetric kits in accordance with the manufacturer's instructions. BMI was used as an anthropometric parameter for obesity evaluation.

DNA purification from rats' preparations

For genetic analysis of the rats, a part of the neck muscles (~1 cm2) was taken from each model and stored in distilled water.

The DNA isolation and purification was performed using Qiagen (QIA amp DNA Mini Kit)

following the exact protocol.

PCR assays and Sanger sequencing

Amplification reactions were performed for the corresponding genes using the following primers: OCT1 ex7 forward (5'-CTCTTCAGGCCATCACGG-3') and reverse (5'- GTACCGCCCTGCCTTTG-3'); MATE1 ex8-10 forward (5'-CTGCCAGGGTGGAGAGG -3') and reverse (5'-CCAGTCCCAGCTCCAGG-3'); MATE2 ex4 (5'GGGGAGAGGAGGGACATG-3') and reverse (5'- ACCCCAAGCGCTCACAT -3'); GLP1R ex 5 (5'-GGTCACCTCATGTCCTTGGA-3') and re-

Table 1. Cafeteria diet and chow food composition per 100 g

Food	Energy (kcal)	Protein (g)	Total fat (g)	Total carbohydrate (g)	Sugar (g)	Trans-unsaturated fatty acids (g)
Standard chow food	285	20.7	2	45	4	0
Cacao balls	386	7.3	2.6	81	26	0.4
Cheese corns	528	4.3	32.	52	3.4	14
Mini- Waffles	549	3.56	32.86	59.73	28.75	14.8
Biscuits	500.4	7.3	23.2	65.6	27.3	9.8
Banana juice	50	0	0	12	12	0

verse (5'-TCCAGAGAGCAGGACTCTCTG -3'). The cycling conditions were as follows: 5 min denaturation at 95ºC, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 40s, and a final extension step of 5 min at 72°C to obtain a 417bp product for OCT1, 591bp for MATE1, 323bp for MATE2 and 293bp for GLP1R. PCR products were verified by 3% agarose gel electrophoresis, visualized with Ethidium Bromide. Sanger sequencing of the OCT1, MATE1, MATE2 and GLP1R genes was performed by BigDye® Terminator cycle sequencing kit v.3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI 3130 sequencer.

Statistical analyses

Data was analyzed using the IBM SPSS 21.0 statistical software. Continuous variables were expressed as mean value ± SD, and categorical variables were expressed as absolute numbers. One-way ANOVA was used for the statistical analysis of the variables. Results were considered statistically significant when $p < 0.05$.

RESULTS

Changes in anthropometric parameters

The results demonstrated a significant increase in body weight (g) after 19 weeks on a modified CAF diet, p< 0.0001 (Fig. 1). The mean body weight at baseline was 240 g and the mean value at the end of the nutritional period was 456 g. In addition, all rats reached the value of BMI for obesity which was 0.81 ± 0.12 (lower 95% CI of mean 0.75 and upper 95 % CI of mean 0.83). Based on the anthropometric changes a successful model of obesity was induced in the studied rats.

After the induction of obesity, rats were divided into three groups and the second phase of the study started with treatment with metformin or liraglutide. The results demonstrated a significant decrease in BMI in both groups treated with either metformin or liraglutide compared to the control group, p< 0.0001 (Fig. 2).

Blood glucose changes in the three groups are presented in figure 3. The results demonstrated a significant decrease in serum glucose levels in the groups treated with either metformin or liraglutide compared to the control group, $p < 0.0001$. The mean serum glucose levels in the control, metformin and liraglutide groups were 6.6 ± 0.7 mmol/l (lower 95% CI of mean 6.1 – upper 95% CI of mean 7), 5.2 ± 0.9 mmol/l (lower 95% CI of mean 4.7 – upper 95% CI of mean 5.6) and 5.7 ± 0.6 mmol/l (lower 95% CI of mean 5.7 – upper 95% CI of mean 6.5), respectively.

The data demonstrated a significant increase in the serum levels of HLD-cholesterol following treatment with metformin and liraglutide, p< 0.05 (Fig. 4). The mean serum HDL-cholesterol levels in the control, metformin and liraglutide groups were 0.8 ± 0.3 mmol/l (lower 95% CI of mean 0.6 – upper 95% CI of mean 1.1), 1 ± 0.3 mmol/l (lower 95% CI of mean 0.8 – upper 95% CI of mean 1.2) and 1.2 ± 0.4 mmol/l (lower 95% CI of mean 0.1 – upper 95% CI of mean 1.5), respectively.

No effect of metformin or liraglutide was detected on serum triglyceride and VLDL-cholesterol levels (Figs 5 and 6).

For the genetic analysis the base calling reading software novo SNP was used to visualize the data from

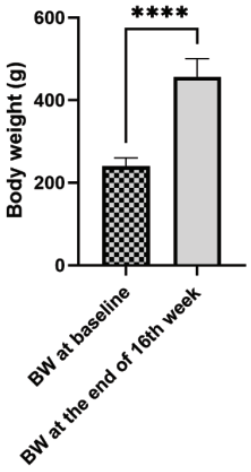

Fig. 1. Comparison of body weight (g) at baseline and at the end of week 16. Data was presented as mean ± SD, **** p< 0.0001

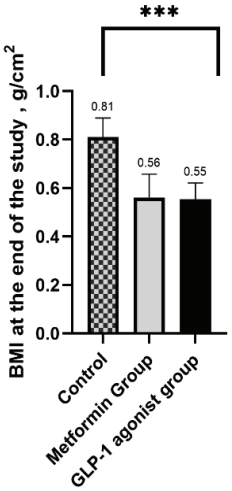

Fig. 2. BMI levels at the end of the experiment in the three groups. Data are presented as mean ± SD, *** p < 0.0001. Abbr. BMI, body mass index.

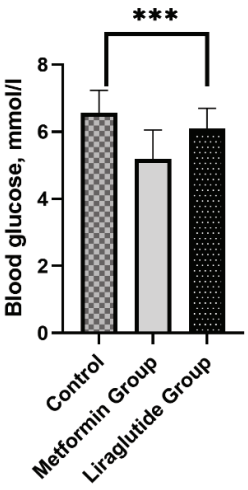

Fig. 3. Changes in plasma glucose levels in the three groups. Data are presented as mean ± SD, *** p< 0.0001

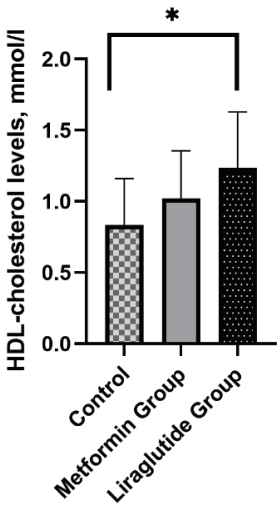

Fig. 4. Changes in plasma HDL-cholesterol levels in the three group. Data are presented as mean ± SD, *p< 0.05

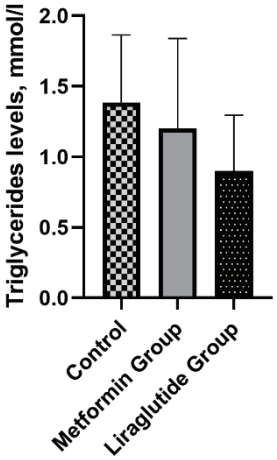

Fig. 5. Changes in serum triglyceride levels in the three groups. Data are presented as mean ± SD

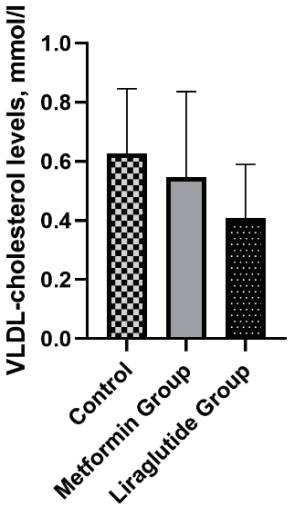

Fig. 6. Changes in serum VLDL levels in the three groups. Data are presented as mean ± SD

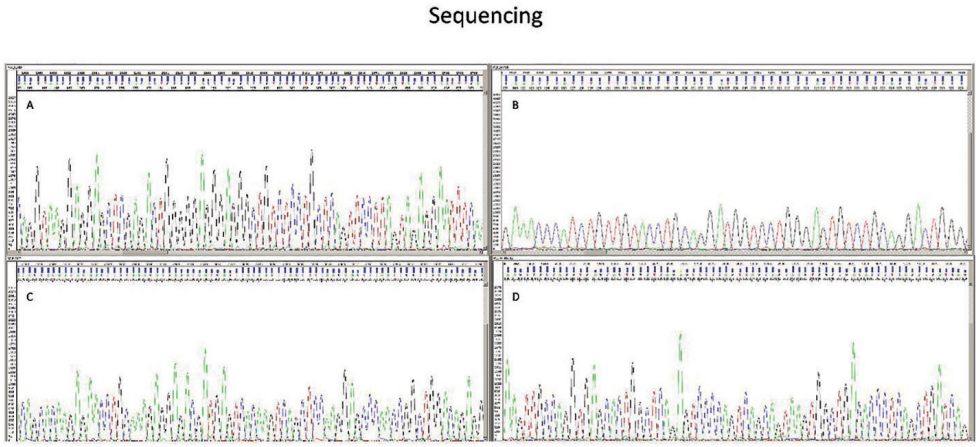

Fig. 7. Graphic representation of data from genetic sequencing using novo SNP software for the following genes: A) GLP1R ex5; B) MATE1 ex 8-10; C) OCT1 ex7 and D) MATE2 ex4

ABI 3130 sequencer. The data was aligned and compared to reference sequences from the same "wild type" genetic models. Based on the analysis, during sequencing no polymorphisms were observed for the tested regions: OCT1 ex7, MATE1 ex 8-10, MATE2 ex4 and GLP1R ex5. This ranks the results as negative, classifying the genetic models as "wild type" for the above-mentioned genes.

DISCUSSION

Obesity is a major health problem as it is considered as a risk factor for insulin resistance, type 2 diabetes mellitus, cardiovascular diseases, and endocrine disorders [29].

CAF diet is calorically dense and extremely palatable. It is easily over consumed. Therefore, the CAF diet provides a relevant model of obesity in terms of examining the human diet with junk and palatable food. In the current study we confirm that the modified CAF diet with added soft drink successfully induces weight gain and obesity in male Wistar rats. A significant increase in the measured weight and BMI was estimated on the 19th week. Moreover, other reports in which obesity is induced with CAF diet, have similar results. According to a study, for 15 weeks CAF-diet fed rats exhibited voluntary hyperphagia and grossly elevated fat intake which resulted in significant weight gain [28, 30]. Another study applying a modified CAF diet with a soft drink added, results in a confirmation that 20 weeks of CAF feeding leads to a rapid and significant weight gain [27].

Moreover, in the current study we estimated that the treatment with both metformin and liraglutide for six weeks led to a significant decrease in body weight and BMI compared to the control group [31]. These

findings are expected and supported by several study results. Firstly, regarding the effect of metformin, it is well known that it has an anorectic effect by enhancing satiation signals secreted by the gut [32]. This leads to decrease in meal size during initial metformin treatment and reductions in meal number. The treatment with metformin has also shown a significant weight loss in diabetic and non-diabetic rats according to previous reports [33]. Liraglutide decreases the food intake and has an anorectic and gastric inhibitory effect that led to decreased consumed amount of food [34]. Moreover, it decreases the appetite and influences the appetite control. In a similar study, examining the effect of liraglutide on glucose metabolism and insulin resistance in Wistar rats treated only for 7 days with liraglutide, a significant body weight and BMI reduction was seen with liraglutide [35].

In our study, the treatment with both drugs led to significantly lower level of glucose and increase in HDL-cholesterol compared to the control group. Metformin is a well-known antidiabetic drug that lowers the hepatic glucose production. Moreover, multiple studies in mouse hepatocytes and transgenic mice prove the role of metformin in reducing hepatic gluconeogenesis and/or insulin sensitivity. According to the literature data, liraglutide has also a hypoglycemic effect by enhancing non–insulin-mediated glucose disposal and increasing glucose-dependent insulin secretion in mice and humans, thereby mimicking enhanced insulin effectiveness [36, 37]. In the current study, the treatment with metformin and liraglutide significantly increased the level of HDL-cholesterol. In other studies, it has been shown that metformin increases HDL-cholesterol levels in overweight rats. The decreased level of HDL-cholesterol is reported to be associated with insulin resistance [38]. One possible explanation is the positive effect of metformin on the insulin resistance in obese individuals. According to our data, the treatment with liraglutide has similar effect on the levels of HDL. Moreover, other studies also prove that liraglutide applied for a short period of time has an impact on the lipids, increasing HDL- cholesterol [39].

However, according to our data, neither metformin, nor liraglutide therapy led to significant reduction in the level of triglycerides and VLDL cholesterol. This contrasts with other studies, in which the therapy with metformin had a significant effect on the triglycerides. One possible explanation for our result might be due to the smaller cohort number. In comparison, a study examining homozygous human CETP transgenic female obese mice fed a Western-type diet and treated for 4 weeks with metformin (200mg/kg/daily) demonstrated a significant reduction in the level of triglycerides and VLDL cholesterol. However, in this study the dose and the type of animals that were examined are different. In contrast to our results, the therapy with liraglutide was also associated with a significant reduction in the level of triglycerides and VLDL cholesterol [40].

Considering the genetic analyses, the OCT1, MATE1, MATE2 and GLP1R genes were specifically selected because of the high homology between the human and the rat genes.

In human genes, these regions contain variants determining individual response to metformin and liraglutide therapy. Based on this, the relevant regions were sequenced in a rat sample consisting of control and metformin-treated rats. No variants were found in the studied exons of the genes listed above in the entire sample; neither at the positions in the coding sequence equivalent to pharmacogenetic variants in man, nor in the rest of the protein-coding DNA sequence. The conclusion of the conducted analysis is that the rats were “wild type”, which means that no changes in the relevant genes are observed. This means that if different outcomes are observed during and after therapy between the rats, this is not due to changes in the studied genes. Since the rats received the same amount of metformin, the dose can also be excluded as a reason for the different outcome of the therapy. One possible explanation might be that there are other genes responsible for the difference, and it is also possible that there are other factors (e.g., environment, diet, and stress).

CONCLUSION

The present study demonstrated the effects of metformin against obesity, dyslipidemia, and hyperglycemia in a cafeteria diet rat model of obesity. The successful induction of obesity in the rats was demonstrated by the body weight, Lee index, hyperglycemia, and dyslipidemia. Metformin and liraglutide treatment significantly attenuated the pathological characteristics of obesity, by reducing blood glucose and blood lipids. The mechanism of this effect may be associated with improved glycemic control, lipid metabolism, and anti-oxidative and anti-inflammatory functions. The results demonstrate that the cafeteria-diet is an efficient model to develop metabolic changes in rats, and to easily evaluate the effect of anti-obesity drugs. The genetic analyses lead to some more questions whether the genetic variations in people are similar in rats. The results did not meet the initial hypothesis. However, further investigation is required to fully elucidate the underlying mechanism.

Disclosure Summary: *The authors have nothing to disclose.*

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