Characterization of Plasma Carnitine Level in Obese Adolescent Korean Women

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Abstract － Carnitine is known to be involved in lipid metabolism and affects body composition as well as energy metabolism of the whole body. Improvement of obesity by L-carnitine supplement suggests that obesity can be related with the abnormality of carnitine metabolism and therefore, plasma carnitine level in normal and obesity groups was investigated. For the characterization of plasma carnitine level in obese people, 60 plasma samples collected from Korean women subjects were analyzed using LC/MS and plasma fatty acid level was also determined using GC/MS. Additionally, several clinical chemical parameters including fasting glucose, cholesterol, AST, and ALT level were measured. All the data obtained were combined and pattern recognition analysis was carried out with the dataset. Obese group showed a different metabolic pattern compared with normal group. Plasma acylcarnitine level of the obese group was found to be 11.7 μg/ml, which was higher than that of normal group (8.0 μg/ml). Statistically significant differences in plasma fatty acid level were not observed between the two groups. Other clinical parameters for the obese group were within normal ranges but AST and ALT levels were slightly elevated compared to normal group. The obese group showed elevated plasma acylcarnitine level.

Keywords: Carnitine, Obesity, LC/MS, GC/MS, Metabonomics

INTRODUCTION

More than 50 individual dietary supplements and more than 125 commercial combination products are available for weight loss. These supplements are purported to increase fat oxidation (carnitine, conjugated linoleic acid), increase metabolic rate (ephedrine, pyruvate), or inhibit hepatic lipogenesis (hydroxycitrate). L-Carnitine is considered as one of the most popularly used products for weight loss (Saper *et al.,* 2004).

L-Carnitine is a naturallyoccurring substance required for energy metabolism in mammals. L-Carnitine is essential for the transport of long chain fatty acids across the mitochondrial membrane for subsequent fat degradation and energy production. Another important function of L-carnitine is the ability to shuttle short chain fatty acids from inside the mitochondria to the cytosol. Therefore, L-carnitine can influence lipid metabolism and body composition as well as energy metabolism of the whole body (Foster, 2004).

Various reports regarding the effects of carnitine supplementation on lipid parameters and obesity were published for experimental animal models such as mouse (Yang *et al.,* 2006), rat (Hong *et al.,* 2002; Yoon *et al.,* 2003), cat (Ibrahim *et al.,* 2003), and dog (Broekhuysen *et al.,* 1965). L-carnitine supplementation was reported to lead to a significant increase in fat oxidation in overweight subjects (Wutzke and Lorenz, 2004). These observations strongly supported effects of L-carnitine on weight loss and improvement of obesity.

Improvement of obesity by L-carnitine supplement conversely suggests obesity might be related with the abnormality of lipid or carnitine metabolism which would be reflected on plasma carnitine level. To test this hypothesis, we investigated the plasma metabolic profile, primarily based on the plasma carnitine levels in obese subjects.

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Herein, we presented the plasma metabolic profiles including carnitine and fatty acid levels for obese subjects and proposed possible causes of obesity based on plasma carnitine levels.

MATERIALS AND METHODS

Materials

L-carnitine hydrochloride, d_3-carnitine, C10 (decanoic acid), C12 (lauric acid), C14 (myristic acid), C16 (palmitic acid), C16:1 (palmitoleic acid), C18 (stearic acid), C18:1 (octadecenoic acid), and C18:2 (linoleic acid) fatty acids were supplied by TCI (Tokyo Chemical Industry Co., LTD., Tokyo, Japan). D_3-decanoic acid, D_3-lauric acid, C_{13}-stearic acid, and N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) were supplied by Sigma Chemical Co. (St. Louis, MO, USA) Organic solvents used were HPLC-grade solvents and all other chemicals were the highest grade commercially available.

Subjects

Sixty adolescent women subjects (14 to16 years of age) volunteered for this study. All subjects were receiving no medication and in good health. Each subject was classified into normal and obesity groups according to the Body weight index (BMI) values: >30, obesity (n=25); 18-25, normal (n=35). BMI was calculated using the following equation: BMI=Weight (kg) / Height^2 (m^2). All subjects enrolled in this study provided informed consent, and the study was approved by the Institutional Review Board of National Medical Center (Seoul, Korea).

Instrumentation

MS/MS was performed on a Applied Biosystems API 4000 triple quadrupole mass spectrometry equipped with turbo electrospray ion source and Harvard syringe pump. The LC system consisted of Agilent 1100 series binary pump and CTC-PAL autosampler. Samples were analyzed using C_8 guard cartridge column (4 cm×3 mm ID) operating at flow rate of 500 μl/min with a programmed gradient elution. The column temperature was 45°C and the injection volume was 5 μl. The ESI-MS/MS settings were as follows: the turbo ion-spray interface was maintained at 450°C with a nitrogen nebulization. The nitrogen was at a pressure of 40 psi. The turbo ion-spray drying gas (nitrogen) was at a pressure of 50 psi, the collision activated dissociation gas (CAD) was at a pressure of 4psi and curtain gas(CUR) was at a pressure of 10 psi. The turbo ion-spray voltage was 5,500 V. Carnitines were analyzed under multiple reaction monitoring. The multiple reaction monitoring

was performed in positive ionization mode; declustering potentials (DP), 110 V to 150 V; entrance potentials (EP), 10 V; collision energies (CE), 70 V to 88 V; collision cell exit potentials (CXP), 30 V to 34 V. The resolutions were adopted for low resolution (Q1 and Q3), respectively. The samples (10 μl) were loaded into the sample loop. The loop volume was 20 μl.

All GC-MS analyses were performed by a HP 6890A gas chromatography system coupled to a 5973 Network mass selective detector (Agilent Technologies, USA). A Hewlett-Packard 3365 chemstation, and a HP-5 column (5% Phenyl methyl siloxane, 30 m×0.25 mm I.D., and film thickness 0.33 μm) was used for the analyses of FFA. The following oven temperature program was used: 80°C for 5 min, 80° to 290°C at 5°C/min, and 290$^{·}$C for 5 min. The temperatures of the injection port and the interface were set at 280°C and 300°C, respectively. Final derivatized aliquot of 1.0 μl was injected with the split ratio of the injector being 1:10. The mass-selective detector was operated in electron impact mode at 70 eV. Helium was used as the carrier gas with a flow-rate of 0.8 ml/min. The mass fragment of the derivatives was confirmed by obtaining the data in full scan mode with a scan range from m/z 50 to 550. The dwell time was 100 ms for the ion selected from each compound for ion monitoring.

Clinical chemistry

AST (aspartate aminotransferase), ALT (alanine aminotransferase), fasting glucose, and total cholesterol levels were determined for all plasma samples. All measurements were performed using standard automated clinical chemistry instrumentation (COBAS INTEGRA 800, or ADVIA 120). Unpaired Student's t-tests were used to compare the clinical chemical data between groups.

Plasma carnitine levels

Plasma samples were prepared and plasma free and total carnitine concentrations for each sample were determined by electrospray-tandem mass spectrometry, as described in the previous report by Millington et al (2000).

Plasma fatty acid levels

Each collected blood sample was immediately centrifuged at 3,000×g for 10 min and plasma was transferred into a microtube. The plasma samples were stored at -70°C until the analysis. Aliquots (100 μl) of plasma were spiked with 100 μl of internal standard working solution (each 100 μg/ml concentration of D$_3$-decanoic, D$_3$-lauric, and C$_{13}$-stearic acid) and 2 ml 0.4 M KOH-CH$_3$OH was added, vortex-mixed for 30 s and placed at room temperature for 10

min, and then extracted with 2 ml methylene chloride using a vortex mixer for 2 min followed by centrifugation at 2,000 rpm for 3 min. The organic phase was taken and aliquot of anhydrous sodium sulfate was added to remove traces of water. The organic phase was removed and evaporated to dryness under N_2 gas. Then, the residue was treated with 20 μl of MTBSTFA and 30 μl of toluene, reacted at 80°C water bath for 30 min, and transferred to GC-vial for analysis.

For calibration, standard mixture solution including C10:1, C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, and C18:2 fatty acids were prepared at various concentrations and analyzed as described above. For validation of the GC/MS method, intra- (n=5) and inter- (n=5) accuracy and precision were determined by repeated analysis of three QC samples at low, middle, and high concentrations. As a result, accuracy (as a percent difference from the nominal values) and precision (as a percent relative standard deviation) did not exceed ± 15 % for all QC samples tested.

Data analysis

All measured values including biochemical parameters, plasma carnitine levels, and plasma fatty acid levels were combined and used as a dataset for pattern recognition analysis. Subsequently, partial least squares - discriminant analysis (PLS-DA) was carried out for the dataset using SIMCA-P software (version 11.5 demo ver., Umetrics AB, Umeå, Sweden).

RESULTS

Clinical chemistry parameters

Several clinical chemistry parameters including fasting glucose, total cholesterol, AST, and ALT levels were measured for plasma samples of each group (Table I). Overall, the measured values in the normal and obese groups were within the normal ranges and significant differences between two groups were not observed except for ALT levels. Fasting glucose levels were 88.1 and 86.4 mg/dl for normal and obese group, respectively, and cholesterol levels were 169.5 and 176.3 mg/dl. AST and ALT levels were 20.1 and 25.8 IU/L and 10.8 and 26.2 IU/L for normal and obese groups, respectively. Obese group showed higher AST and ALT levels with statistically significant differences compared to normal group.

Fatty acid levels

Plasma concentration of C10:1, C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, and C18:2 fatty acids was determined and the results are summarized in Table II. Any difference in plasma free fatty acid levels was not observed between normal and obese groups.

Carnitine levels

Free and total carnitine concentrations were determined for plasma samples from 60 subjects. Acylcarnitine levels were calculated by subtracting the free carnitine concentration from total carnitine concentration. As shown in Table III, difference in free carnitine level was not observed between the two groups. However, total carnitine levels were 39.6 and 44.6 μg/ml for normal and obese groups, respectively. Obese group showed statistically significant difference compared to normal group. Consequently, obese group exhibited higher plasma acylcarnitine level than normal group.

Pattern recognition analysis for clinical chemistry parameters, fatty acid levels, and carnitine levels

PLS-DA analysis was conducted to investigate the differ-

Table I. Clinical chemistry parameters for the plasma samples from 60 subjects

Group	Fasting glucose (mg/dl)	Cholesterol (mg/dl)	AST (IU/L)	ALT (IU/L)
Normal	86.6 ± 9.6	169.5 ± 20.7	20.1 ± 2.4	10.8 ± 2.0
Obese	86.4 ± 9.3	176.3 ± 29.9	25.8 ± 11.9*	26.2 ± 20.3**

*$p < 0.05$, **$p < 0.001$ compared with normal group.

Table III. Carnitine levels for the plasma samples from 60 subjects

Group	Carnitines (μmol/L)		
	Free carnitine	Acylcarnitnine	Total carnitine
Normal	31.4 ± 8.3	8.0 ± 3.7	39.6 ± 8.0
Obese	32.9 ± 6.8	11.7 ± 2.9**	44.6 ± 8.3*

*$p < 0.05$, **$p < 0.001$ compared with normal group.

Table II. Fatty acids levels for the plasma samples from 60 subjects

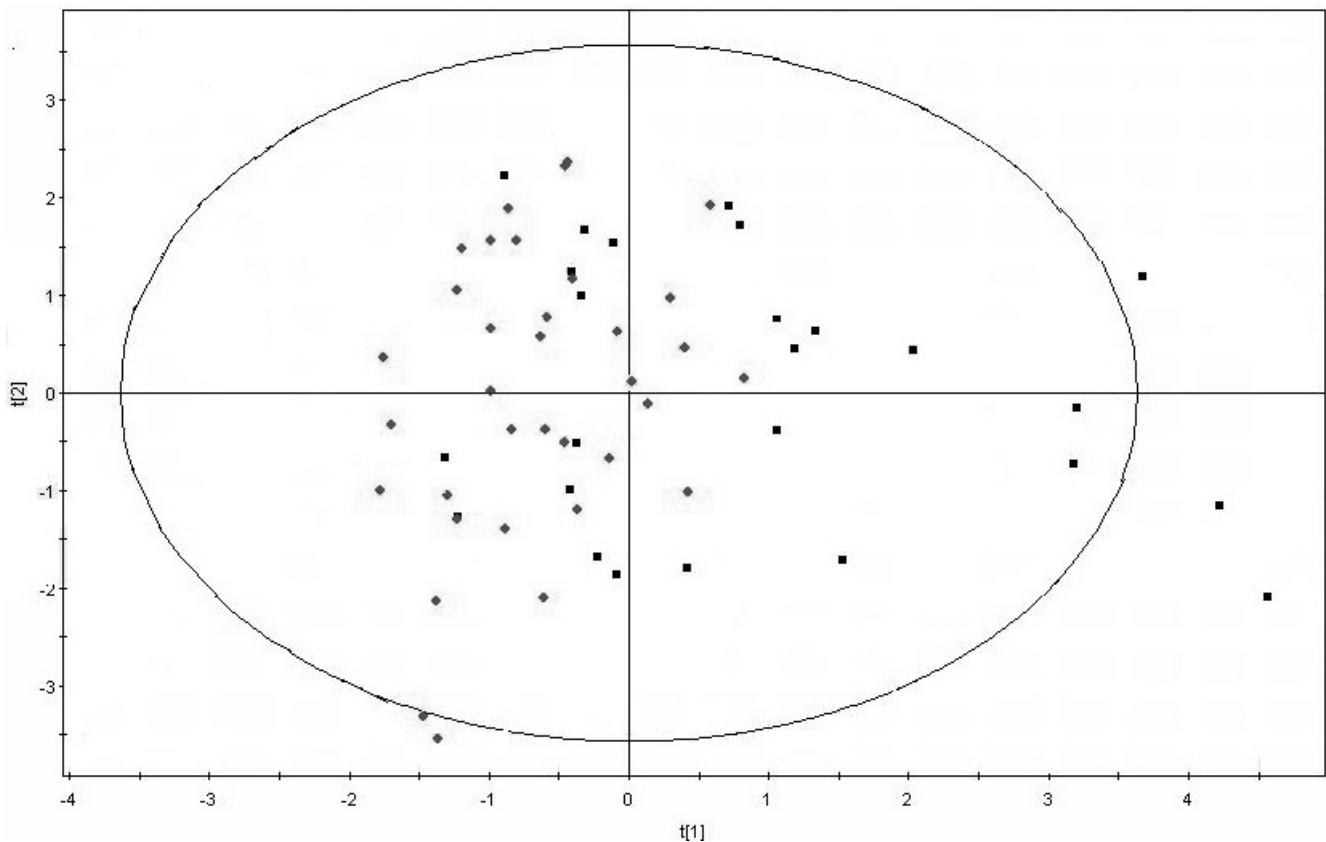| Group	Fatty acids (μg/ml)					
	C10:0	C12:0	C14:0	C16:0	C18:1	C18:0
Normal	0.50 ± 0.03	1.81 ± 0.84	22.9 ± 14.9	206 ± 122	0.82 ± 0.36	406 ± 338
Obese	0.50 ± 0.14	2.07 ± 1.79	20.4 ± 14.0	212 ± 231	0.89 ± 0.46	415 ± 344

Fig. 1. PLS-DA scatter plot for the 60 plasma samples. X- and Y-axes represent scores of component 1 (t[1]) and component 2 (t[2]) resulted from PLS-DA analysis, respectively. Diamond, normal group; Square, obese group.

ence in the patterns of various biochemical parameters including plasma carnitine and fatty acid levels between normal and obese groups. The score plot resulted from PLS-DA analysis are shown in Fig. 1. On the score plot, normal and lean groups were mixed together, whereas obese group was separated from the other two groups and distributed mainly in the positive region for the component 1 axis. This suggests that obese group shows a slight but clear difference in the profile of biochemical parameters. The loading values of the variables associated with the component 1 and 2 are displayed in Fig. 2. Most variables were positioned around the center of the two axes, but total carnitine, acylcarnitine, AST, and ALT were located in the positive region on the component 1 axis. This indicated that total carnitine, acylcarnitine, AST, and ALT levels are the most contributive factors distinguishing the obese group from normal group.

DISCUSSION

In the present study, 60 plasma samples collected from adolescent Korean women were analyzed and the rela-

tionship between plasma carnitine level and obesity was investigated. The obese group showed elevated AST, ALT and acylcarnitine levels compared to normal group and pattern recognition analysis with biochemical parameters revealed that obese group has a different metabolic pattern from normal group based on plasma acylcarnitine, AST, and ALT levels. However, significant differences in plasma fatty acid levels were not observed.

For the investigated clinical chemistry parameters, obese group did not show abnormalities in mean data, although AST and ALT levels of the obese group were found to be somewhat higher than that of normal group. However, five samples from the obese group showed higher ALT levels more than 50 IU/L, exceeding the normal range (8-35 IU/L). This indicated the high risk of non-alcoholic fatty acid disease in obese subjects as reported previously (Quiros-Tejeira *et al.*, 2007).

In our data, obese group exhibited no difference in free carnitine level, whereas it showed higher total carnitine levels compared to normal group. Elevated plasma acylcarnitine levels in the obese group might have some correlation with biochemical problems, which are related to car-
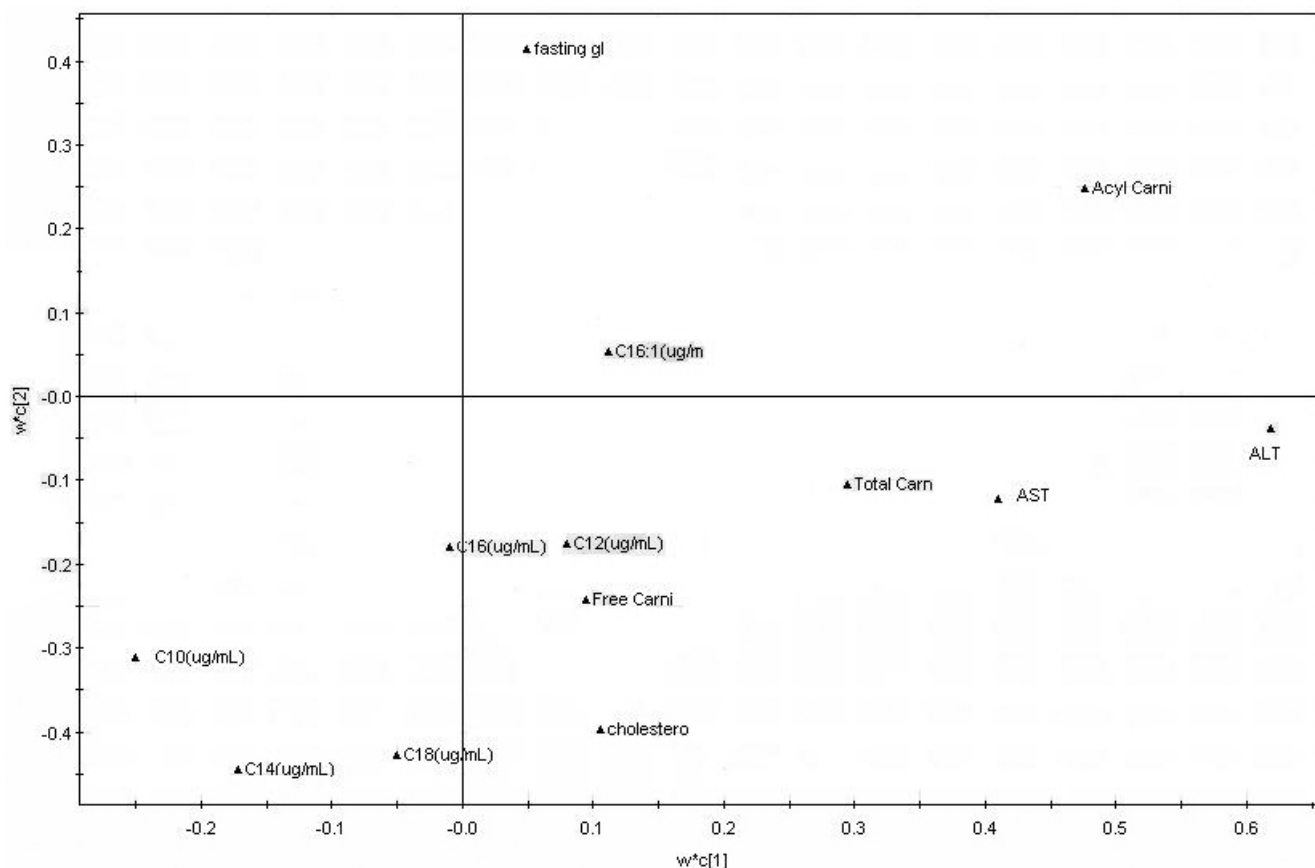
Fig. 2. PLS-DA loading plot for the 13 variables. X- and Y-axes represent scores of component 1(t [1]) and component 2(t [2]) resulted from PLS-DA analysis.

nitine or fatty acid metabolism in the obese group.

Excessively accumulated acyl-CoA esters in mitochondria are apt to be released into plasma as a form of acyl-carnitine after transesterification with carnitine. Therefore, plasma acylcarnitine profiling will theoretically reflect the intramitochondrial accumulation of acyl-CoA esters (Costa et al., 1998; Speikerkotter et al., 2000; Speikerkotter et al., 2004). This phenomenon can be observed in the disorders of acyl-CoA dehydrogenases. However, in this case, plasma free carnitine level would be reduced since the intramitochondrial formation and subsequent loss of acylcarnitines lead to a secondary carnitine deficiency. This is not totally agreed with our observation in the plasma samples from obese group and not enough to explain our data showing the elevated plasma acylcarnitine levels.

Another key enzyme regulating carnitine metabolism is carnitine palmitoyltransferase (CPT). It is known that two types of CPT existed: CPT 1 which catalyzes the formation of acylcarnitine from carnitine and acyl-CoA, and CPT 2 which catalyzes the formation of acyl-CoA from acylcarnitine and CoA in the inner and outer mitochodiral membranes, respectively. It has been well established that the regulation of mitochondrial fatty acid oxidation (FAO) mainly involves CPT 1, whereas the possible role of CPT 2 in the control of FAO remained to be defined (McGarry and Brown, 1997; Bonnefont et al., 1999; Bonnefont et al., 2004).

CPT has been extensively discussed as a plausible target enzyme for obesity (Ronnett et al., 2005; Bobitaille et al., 2007; Kuhajda and Ronnett, 2007; Robitaille et al., 2007) and it is reported that genetic variations for the CPT 1A and CPT 1B genes are modestly associated with some obesity phenotype and an increased risk of obesity. Kuhajda and Ronnett (2007) suggested the possibility for the treatment of obesity by modulation of CPT 1 activity. However, CPT 1 deficiency is characterized to be an elevated level of plasma carnitine due to excessive free carnitine (Stanley et al., 1992; Bonnefont et al., 1999; Longo et al., 2006), which is contradictory to our results showing the elevated plasma acylcarnitine level in the obese group. Unfortunately, we did not perform individual acylcarnitine profile analyses which could induce increase or decrease

in the production of individual carnitine species (C2-C18 acylcarnitines profile). Although continued to be studied, present investigations give a possibility whether CPT 1 has a minimal effect on obese group.

Meanwhile, CPT 2 deficiency also leads to a disorder of lipid metabolism by blocking the recycling of carnitine and fatty acid oxidation, which is characterized by abnormally high level of plasma acylcarnitines (Bonnefont et al., 1999; Thuillier et al., 2003; Minkler et al., 2005; Longo et al., 2006). The experimental of clinical evidences directly supporting the effect of CPT 2 modulation on obesity is still not available. But the up-regulation of CPT 2 gene in the obesity resistant mouse model (Castro-Chavez et al., 2003) indirectly suggests that the modulated CPT 2 activity can, possibly, lead to obesity. Furthermore, the improvement of obesity by feeding L-carnitine can be understood in this context. Therefore, the possibility of obesity caused by the alteration of CPT 2 activity can not be excluded.

In conclusion, the obese subjects exhibited altered plasma carnitine profile with elevation of the acylcarnitine level. This result suggests a new possibility of involvement of CPT 2 enzyme in obesity. Nevertheless, before drawing any conclusion from this study, it should be considered that the present data represented the metabolic status for the relatively restricted subjects of obese Korean adolescent women. Identification of the type of acylcarnitines elevated in the obese group is in progress. Further investigation on the effect of carnitine load or under strict diet control with complementary metabolic and genomic analysis will be followed for clarification of the correlations between clinical chemistry parameters, acylcarnitine profiles, free fatty acids profiles and alteration of the related enzyme activities in obesity.

ACKNOWLEDGMENTS

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