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## Very Low Carbohydrate Diet Significantly Alters the Serum Metabolic Profiles in Obese Subjects

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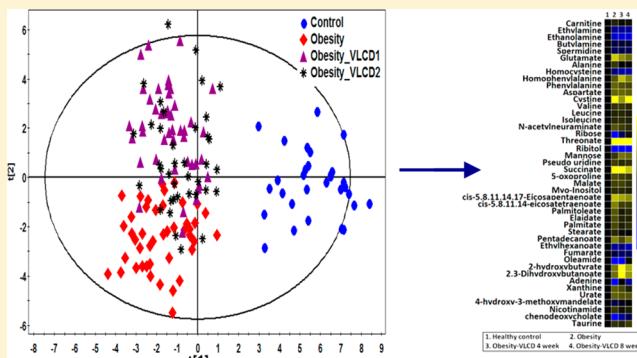
### Supporting Information

**ABSTRACT:** Emerging evidence has consistently shown that a very low carbohydrate diet (VLCD) can protect against the development of obesity, but the underlying mechanisms are not fully understood. Here we applied a comprehensive metabolomics approach using ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry and gas chromatography-time-of-flight mass spectrometry to study the effects of an 8-week dietary intervention with VLCD on serum metabolic profiles in obese subjects. The VLCD intervention resulted in a weight loss and significantly decreased homeostasis model assessment-insulin resistance. The metabolomics analysis identified a number of differential serum metabolites ( $p < 0.05$ ) primarily attributable to fatty acids, amino acids including branched chain amino acids, amines, lipids, carboxylic acids, and carbohydrates in obese subjects compared to healthy controls. The correlation analysis among time, VLCD intervention, and clinical parameters revealed that the changes of metabolites correlated with the changes of clinical parameters and showed differences in males and females. Fatty acids, amino acids, and carboxylic acids were increased in obese subjects compared with their normal healthy counterparts. Such increased levels of serum metabolites were attenuated after VLCD intake, suggesting that the health beneficial effects of VLCD are associated with attenuation of impaired fatty acid and amino acid metabolism. It also appears that VLCD induced significant metabolic alterations independent of the obesity-related metabolic changes. The altered metabolites in obese subjects post-VLCD intervention include arachidonate, *cis*-11,14-eicosadienoate, *cis*-11,14,17-eicosatrienoate, 2-aminobutyrate, acetyl-carnitine, and threonate, all of which are involved in inflammation and oxidation processes. The results revealed favorable shifts in fatty acids and amino acids after VLCD intake in obese subjects, which should be considered biomarkers for evaluating health beneficial effects of VLCD and similar dietary interventions.

**KEYWORDS:** very low carbohydrate diet, obesity, metabolomics, ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry, gas chromatography-time-of-flight mass spectrometry

## INTRODUCTION

Obesity has reached epidemic proportions worldwide<sup>1</sup> and is strongly linked to the development of diabetes, hypertension, cardiovascular disease, coronary heart disease, stroke, and several types of cancer.<sup>2</sup> Because of the severe comorbidities of obesity, people attempt to lose weight through several alternative diets. Recently, there has been a resurgence of interest in very low carbohydrate diet (VLCD) as a means of weight loss and metabolic improvements. Evidence from clinical studies and metaanalyses suggested that VLCD can decrease body weight, improve metabolic parameters, insulin resistance/sensitivity, and nonalcoholic fatty liver disease (NAFLD).<sup>3</sup> Alternative diets (high protein, low-carbohydrate,



high-fat) produce significantly greater weight loss in the short term (6 months) compared to the conventional fat-restricted diet<sup>4,5</sup> with no strict control of total energy intake. This significantly greater weight loss is likely due to spontaneous reduction in energy intake,<sup>5,6</sup> which may be linked to a lack of diet variety and changes in humoral satiety factors.<sup>7</sup> However, when energy intake is strictly controlled and reduced to a hypocaloric level, no difference in weight change is detectable between alternative and high-carbohydrate diets,<sup>8,9</sup> suggesting that primarily calorie restriction and not macronutrient

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**Table 1.** Demographics and Clinical Characteristics of Healthy Controls and Obese Human Subjects

characteristics	control	baseline	week 4	week 8
	<i>n</i> = 30	<i>n</i> = 45	<i>n</i> = 45	<i>n</i> = 38
age (year)	28.21 ± 5.35	31.87 ± 8.98 <sup>a</sup>	31.87 ± 8.98 <sup>a</sup>	32.33 ± 9.30 <sup>a</sup>
gender (male/female)	13/17	25/20	25/20	23/15
height (cm)	165.33 ± 7.88	170.88 ± 8.69 <sup>a</sup>	170.83 ± 8.64 <sup>a</sup>	171.54 ± 8.82 <sup>a</sup>
weight (kg)	58.37 ± 7.71	95.70 ± 18.67 <sup>a</sup>	89.83 ± 17.97 <sup>a</sup>	88.54 ± 18.01 <sup>a</sup>
BMI (kg/m <sup>2</sup> )	21.29 ± 1.75	32.58 ± 4.36 <sup>a</sup>	30.59 ± 4.21 <sup>a,b</sup>	29.88 ± 4.11 <sup>a,b</sup>
waist (cm)	77.04 ± 6.86	104.24 ± 11.44 <sup>a</sup>	100.69 ± 10.58 <sup>a</sup>	99.21 ± 9.85 <sup>a,b</sup>
hip (cm)	90.93 ± 3.93	110.56 ± 9.10 <sup>a</sup>	107.06 ± 8.93 <sup>a</sup>	106.84 ± 8.61 <sup>a</sup>
WHR	0.85 ± 0.06	0.94 ± 0.07 <sup>a</sup>	0.94 ± 0.05 <sup>a</sup>	0.93 ± 0.05 <sup>a</sup>
FPG (mmol/L)	4.76 ± 0.28	5.27 ± 0.88 <sup>a</sup>	4.99 ± 0.48 <sup>a,b</sup>	5.16 ± 0.42 <sup>a</sup>
2h PG (mmol/L)	5.54 ± 1.02	7.56 ± 1.73 <sup>a</sup>	NA	6.87 ± 1.69
FINS (μU/mL)	7.06 ± 3.00	22.67 ± 27.54 <sup>a</sup>	10.25 ± 5.94 <sup>a,b</sup>	11.89 ± 8.93 <sup>a,b</sup>
2h INS (μU/mL)	50.53 ± 23.81	136.56 ± 75.87 <sup>a</sup>	NA	71.88 ± 51.92 <sup>a,b</sup>
HOMA-IR	1.35 ± 0.77	6.16 ± 10.61 <sup>a</sup>	2.28 ± 1.48 <sup>a,b</sup>	2.74 ± 2.37 <sup>a,b</sup>
2h HOMA-IR	11.35 ± 7.55	49.19 ± 34.95	NA	21.51 ± 18.24

<sup>a</sup>*p* < 0.05, significantly different from healthy controls. <sup>b</sup>*p* < 0.05, significantly different from baseline.

composition is responsible for weight loss in hypocaloric diets.<sup>9,10</sup> It was reported that a weight-maintaining, high-protein diet was associated with improvements in overall glucose control, as postprandial blood-glucose concentrations and glycated hemoglobin decreased significantly compared to a conventional high-carbohydrate diet.<sup>11</sup> In contrast, a study showed that glycated hemoglobin and fasting plasma glucose decreased and insulin sensitivity increased in the high-carbohydrate but not the high-protein group, while weight loss in both groups was comparable.<sup>8</sup> We have shown that VLCD intervention induced rapid weight reduction with decreased total abdominal subcutaneous and visceral adipose tissue compartments, and liver fat content, increased skeletal muscle percentage of whole body weight, improved metabolic profile, and insulin resistance and sensitivity.<sup>12</sup> There is growing evidence that obesity and related conditions are characterized by a broad perturbation of metabolic physiology involving considerable changes in amino acid (branched chain amino acid (BCAA), and aromatic amino acids) and fatty acid metabolism<sup>13–15</sup> in addition to glucose.<sup>16</sup> This new evidence is prompting the application of methods monitoring a broad range of molecular species, i.e. metabolomics, to study the beneficial effects of potentially health-promoting diets.<sup>15,17</sup>

Metabonomics has been applied to investigate the effects of dietary carbohydrate modification on human serum metabolic profiles.<sup>18</sup> The application of metabonomics to well-designed controlled intervention studies can be a useful tool to elucidate the complex physiological effects of VLCD, which might help in understanding their beneficial effects on human health.<sup>19,20</sup>

Here we analyzed the serum metabolites in healthy controls and obesity subjects before and after VLCD intervention by ultraperformance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOFMS) and gas chromatography-time-of-flight mass spectrometry (GC-TOFMS) to determine metabolic differences between obese and healthy human subjects and investigate the effect of VLCD intervention on serum metabolic profiles in obese subjects.

## MATERIALS AND METHODS

### Study Populations

A number of 45 “healthy” obese (aged 17.8–52.0 years, mean BMI of 32.58 kg/m<sup>2</sup>) and 30 healthy control (aged 23.4–43.8

years, mean BMI of 21.29 kg/m<sup>2</sup>) subjects was recruited from the outpatient clinic of endocrinology and metabolism department of Shanghai Jiao Tong University affiliated Sixth People’s Hospital. The exclusion criteria were as follows:<sup>12</sup> pregnant or plan for pregnant; lactation or postmenopausal women; use of any prescription medication in previous 2 months; had any weight loss diet or pill during the past 6 months; consuming >20 g/day of alcohol; tobacco use within 6 months; cardiovascular or endocrine disease history; hypertension history or current elevated blood pressure (systolic blood pressure [SBP]: ≥ 150 mmHg; diastolic blood pressure [DBP] ≥ 90 mmHg; current treatment for hypertension); diabetes mellitus; acute or chronic infections; liver disease, kidney disease, gastrointestinal disease, or any other acute or chronic diseases requiring treatment.

The demographic information and clinical characteristics of all subjects are shown in Table 1. This study was approved by the Institutional Review Board of the Sixth People’s Hospital. All participants provided written informed consent.

### Experimental Protocol

One week before initiation of the study, all subjects were asked to maintain their habitual energy intake. At weeks 0, 4, and 8, serum samples were collected and anthropometric parameters, glucose concentration, and insulin resistance and sensitivity were measured. All study measurements were obtained before 10 a.m. after an overnight fast. Serum samples for metabolomics analysis were collected in the morning before breakfast and kept at –80 °C until analysis.

### Dietary Intervention

The obese subjects were subject to dietary intervention for two periods as reported in our previous study.<sup>12</sup> In brief, energy intake was restricted to less than 800 kcal/day (carbohydrate intake <20 g/day). All daily meals were replaced as follows: a cup of soybean milk (200 mL) and a boiled egg at breakfast; a diet nutrition bar (106 kcal: 2.8 g carbohydrate, 11.2 g protein, and 5.6 g fat; Nutrilease Health Technology Co., Ltd., Hangzhou, China), nonstarchy vegetables (<200 kcal), and 50 g protein from meat (i.e., beef, lean pork, skinned chicken, fish) at lunch and dinner. Supplementation of multivitamins and minerals was provided every day.

Subjects were also encouraged to drink at least 1.8 L of water per day, and asked to maintain their habitual level of physical

activity. Regular telephone contact to individual by nutritionists was provided for nutrient support.

### Anthropometric Measurements

Body weight and height were measured using standard methods for the calculation of BMI ( $\text{kg}/\text{m}^2$ ). Hip and waist circumferences were measured for the calculation of the waist/hip ratio (WHR).

### Glucose, Insulin, and Homeostatic Model Assessment of Insulin Resistance (HOMA-IR)

Plasma glucose concentrations (fasting glucose, and 2 h postprandial plasma glucose) were measured by the glucose oxidase-peroxidase method using commercial kits (Shanghai Biological Products Institution, Shanghai, China) according to the manufacturer's instructions. Serum insulin concentrations were measured using the radioimmunoassay method (Beijing North Institute of Biological Technology, Beijing, China). Insulin sensitivity was measured by HOMA, using the following formula:  $\text{HOMA} = (\text{fasting insulin in } \mu\text{U/mL} \times \text{fasting glucose in mM})/22.5$ .

### Serum Metabolomic Analysis

Serum samples were prepared and analyzed by UPLC-QTOFMS and GC-TOFMS following our previously published protocols<sup>21,22</sup> with minor modifications. Experimental details are provided in the Supporting Information.

### Data Analysis and Statistics

The acquired MS data from UPLC-QTOFMS and GC-TOFMS were analyzed according to our previously published work. The ESI positive and negative raw data generated from UPLC-QTOFMS was analyzed by the MarkerLynx applications manager version 4.1 (Waters, Manchester, U.K.).<sup>22,23</sup> The resulting data from the UPLC-QTOFMS platforms were subject to multivariate statistical analyses to establish characteristic metabolomic profiles associated with obesity before and after dietary intervention. For details, see Materials and Methods in the Supporting Information.

For GC-TOFMS generated data, the acquired MS files were analyzed according to our previous published work.<sup>21,22</sup> Briefly, the data generated in the GC-TOFMS instrument were analyzed by the ChromaTOF (v4.33, Leco Co., CA, USA). The resulting three dimension data set, including sample information, peak retention time, and peak intensities, was subject to multivariate statistical analyses to establish characteristic metabolomic profiles associated with obesity before and after dietary intervention. For details, see Methods in the Supporting Information.

For UPLC-QTOFMS generated data, compound annotation was performed by comparing the accurate mass ( $m/z$ ) and retention time (Rt) of reference standards and the accurate mass of compounds obtained from the web-based resources such as the Human Metabolome Database ([www.hmdb.ca](http://www.hmdb.ca)). For GC-TOFMS generated data, compound annotation was processed by comparing the mass fragments and Rt with the reference standards or mass fragments with NIST 05 Standard mass spectral databases in NIST MS search 2.0 (NIST, Gaithersburg, MD) software using a similarity of more than 70%.

The annotated metabolites in the two data sets resulting from UPLC-QTOFMS and GC-TOFMS were combined into a new data set for further statistical analysis by uni- and multivariate statistical methods. The combined data set was imported into the SIMCA-P+ 12.0 software package (Umetrics,

Umeå, Sweden). Principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were carried out to visualize the metabolic alterations between obese subjects and healthy controls. The metabolic effects of VLCD intervention were also investigated. In this study, the default 7-round cross-validation was applied with 1/ seventh of the samples being excluded from the mathematical model in each round in order to guard against overfitting. The variable importance in the projection (VIP) values of all of the peaks from the 7-fold cross-validated OPLS-DA model was taken as a coefficient for peak selection. VIP ranks the overall contribution of each variable to the OPLS-DA model, and those variables with  $\text{VIP} > 1.0$  are considered relevant for group discrimination.<sup>24</sup> In addition to the multivariate statistical method, the Mann–Whitney U test was also applied to measure the significance of each metabolite. Metabolites with both multivariate and univariate statistical significance ( $\text{VIP} > 1$  and  $p < 0.05$ ) were considered potential markers responsible for the differentiation of obesity subjects from healthy controls or obesity subjects before and after VLCD intervention.

Pearson correlation analysis was made to evaluate the relation of metabolite change along different time points (baseline, 4 and 8 weeks after VLCD intervention) versus the change of BMI, serum glucose level, and insulin sensitivity, giving a value ranging from 1.0 (maximum positive correlation) to -1 (maximum anticorrelation) and 0 (no correlation). More specifically, using the ratio of metabolite change over baseline (FC value), we also evaluated the correlation of this ratio versus the corresponding change of BMI, serum glucose level, and insulin sensitivity at 4 weeks and 8 weeks within the subgroups of female and male participants, correspondingly.

## RESULTS

### Demographics and Clinical Characteristics

The demographics and clinical characteristics of healthy control and obese human are shown in Table 1. A significant difference was observed in age ( $P < 0.05$ ) between the obese and healthy subject groups (mean age: 28 vs 32 years, respectively). As expected, obese subjects were heavier and had higher WHR than healthy controls. The mean BMI of obese subjects was  $32.58 \text{ kg/m}^2$ , whereas the mean BMI was  $21.29 \text{ kg/m}^2$  for healthy controls.

Obese subjects had higher levels of insulin ( $p < 0.05$ ), HOMA-IR ( $p < 0.05$ ), and blood glucose ( $p < 0.05$ ) than healthy controls (Table 1).

As shown in Table 1, after the VLCD intervention, the BMI was significantly reduced from  $32.58 \text{ kg/m}^2$  to  $30.59 \text{ kg/m}^2$  ( $p < 0.05$ ) at week 4 and further reduced to  $29.88 \text{ kg/m}^2$  ( $p < 0.01$ ) at week 8. Similarly, significant reductions in fasting insulin (FINS) and 2h postprandial insulin (2h INS) were observed (all  $P$  values  $<0.05$ ). However, the change of fasting plasma glucose (FPG,  $P = 0.503$ ) and 2h postprandial plasma glucose (2h PG,  $P = 0.079$ ) was not statistic significant at the end of the study. HOMA-IR and 2h HOMA-IR significantly improved with a 2-fold reduction (all  $P$  values  $<0.05$ ), respectively.

### Serum Metabolite Profiles of Obese and Healthy Control Subjects

A total of 113 metabolites were annotated from the detected spectral features from GC-TOFMS and UPLC-QTOFMS using reference standards as well as the available database (NIST library 2005 and HMDB). The peak intensity comparison of

**Table 2.** List of Serum Differential Metabolites in Obese Subjects Relative to Healthy Controls, Obese Subjects after VLCD Intervention Relative to Controls, and Obese Subjects after VLCD Intervention Relative to Obese Subjects

compd	class	obesity-control				obesity_VLCD 4 wk-control				obesity_VLCD 8 wk-control				obesity_VLCD 8 wk-obesity			
		VIP	FC	P	VIP	FC	P	VIP	FC	P	VIP	FC	P	VIP	FC	P	VIP
carnitine	alkylamines	1.14	1.25	$2.26 \times 10^{-4}$	0.57	1.12	$3.40 \times 10^{-2}$	0.90	1.16	$4.45 \times 10^{-3}$							
ethylamine	alkylamines	2.10	0.73	$6.42 \times 10^{-12}$	2.33	0.74	$1.87 \times 10^{-11}$	2.32	0.74	$2.36 \times 10^{-11}$							
ethanolamine	alkylamines	1.94	0.62	$3.25 \times 10^{-10}$	2.17	0.62	$2.03 \times 10^{-10}$	2.22	0.61	$1.52 \times 10^{-10}$							
butylamine	alkylamines	1.38	0.88	$1.97 \times 10^{-8}$	2.09	0.89	$9.32 \times 10^{-9}$	1.99	0.89	$6.72 \times 10^{-8}$							
sperrmidine	alkylamines	1.78	0.84	$2.88 \times 10^{-8}$	1.97	0.85	$6.79 \times 10^{-8}$	1.89	0.85	$6.13 \times 10^{-7}$							
glutamate	amino acid	1.74	2.19	$6.56 \times 10^{-10}$	1.49	1.85	$4.41 \times 10^{-7}$	1.58	1.64	$1.47 \times 10^{-5}$	1.08	0.84	$2.79 \times 10^{-2}$	1.82	0.75	$3.40 \times 10^{-3}$	
alanine	amino acid	1.18	1.34	$2.73 \times 10^{-5}$	0.24	1.06	$5.67 \times 10^{-1}$	0.28	1.06	$3.64 \times 10^{-1}$	1.79	0.79	$1.89 \times 10^{-4}$	1.76	0.79	$1.32 \times 10^{-3}$	
homocysteine	amino acid	1.10	0.77	$1.41 \times 10^{-3}$	1.59	0.70	$3.78 \times 10^{-5}$	1.48	0.70	$1.13 \times 10^{-4}$							
homophenylalanine	amino acid	1.07	1.51	$4.42 \times 10^{-5}$	1.22	2.10	$3.14 \times 10^{-6}$	0.95	1.73	$1.55 \times 10^{-2}$							
phenylalanine	amino acid	1.88	1.59	$5.70 \times 10^{-10}$	1.78	1.49	$6.02 \times 10^{-8}$	1.72	1.44	$1.85 \times 10^{-6}$							
aspartate	amino acid	1.81	1.60	$5.32 \times 10^{-10}$	1.84	1.60	$1.28 \times 10^{-8}$	1.80	1.56	$2.52 \times 10^{-7}$							
cystine	amino acid	1.81	2.58	$4.94 \times 10^{-11}$	1.59	1.90	$1.07 \times 10^{-6}$	1.95	2.57	$1.58 \times 10^{-8}$	1.45	0.74	$2.82 \times 10^{-3}$				
valine	amino acid	1.39	1.32	$6.36 \times 10^{-6}$	1.35	1.34	$7.56 \times 10^{-6}$	1.16	1.27	$1.23 \times 10^{-3}$							
leucine	amino acid	1.16	1.25	$1.22 \times 10^{-4}$	1.15	1.26	$5.17 \times 10^{-4}$	1.00	1.21	$2.38 \times 10^{-3}$							
isoleucine	amino acid	1.05	1.27	$4.27 \times 10^{-4}$	1.02	1.28	$2.05 \times 10^{-3}$	1.00	1.24	$4.45 \times 10^{-3}$							
N-acetylneuraminate	carbohydrates	1.43	1.51	$7.43 \times 10^{-6}$	1.11	1.38	$4.76 \times 10^{-3}$	1.25	1.29	$2.95 \times 10^{-4}$							
ribose	carbohydrates	1.22	0.51	$2.45 \times 10^{-6}$	0.43	0.83	$8.75 \times 10^{-2}$	0.31	1.15	$9.81 \times 10^{-1}$	1.42	1.64	$1.77 \times 10^{-3}$	2.21	2.26	$2.21 \times 10^{-5}$	
threonate	carbohydrates	1.83	2.73	$6.55 \times 10^{-9}$	1.83	2.81	$1.89 \times 10^{-10}$	1.95	2.98	$2.79 \times 10^{-9}$							
ribitol	carbohydrates	1.37	0.40	$5.69 \times 10^{-4}$	1.70	0.36	$6.90 \times 10^{-5}$	1.56	0.39	$4.90 \times 10^{-4}$							
mannose	carbohydrates	1.28	1.34	$4.21 \times 10^{-5}$	2.03	1.68	$9.29 \times 10^{-10}$	1.91	1.60	$3.17 \times 10^{-8}$	1.78	1.25	$2.22 \times 10^{-4}$	1.59	1.19	$1.15 \times 10^{-2}$	
pseudouridine	carbohydrates	1.02	1.39	$1.90 \times 10^{-4}$	0.86	1.28	$1.29 \times 10^{-2}$	1.22	1.31	$1.46 \times 10^{-3}$							
succinate	carboxylic acids	1.77	3.15	$1.99 \times 10^{-12}$	1.99	2.48	$2.70 \times 10^{-11}$	1.74	2.17	$7.21 \times 10^{-9}$	1.17	0.79	$3.22 \times 10^{-2}$	1.73	0.69	$4.00 \times 10^{-4}$	
S-oxoproline	carboxylic acids	1.75	1.47	$7.97 \times 10^{-9}$	1.32	1.40	$2.85 \times 10^{-5}$	1.42	1.33	$6.82 \times 10^{-5}$							
malic acid	carboxylic acids	1.39	1.41	$4.41 \times 10^{-6}$	1.25	1.41	$2.79 \times 10^{-4}$	0.66	1.31	$1.65 \times 10^{-2}$							
myoinositol	cyclic alcohols	1.35	1.29	$1.51 \times 10^{-5}$	1.05	1.28	$8.32 \times 10^{-3}$	1.02	1.22	$7.47 \times 10^{-3}$							
cis-5,8,11,14,17-eicosapentaenoate	fatty acid	1.65	2.13	$1.67 \times 10^{-6}$	1.02	1.98	$8.06 \times 10^{-3}$	1.33	1.83	$9.71 \times 10^{-5}$	1.38	0.93	$4.98 \times 10^{-2}$				
cis-5,8,11,14-eicosatetraenoate	fatty acid	1.33	1.75	$1.34 \times 10^{-7}$	0.99	1.38	$1.04 \times 10^{-2}$	1.25	1.37	$9.71 \times 10^{-5}$							
Palmitoleate	fatty acid	0.99	1.51	$5.69 \times 10^{-4}$	0.44	1.19	$3.41 \times 10^{-1}$	0.80	1.26	$1.45 \times 10^{-2}$	1.12	0.79	$2.56 \times 10^{-2}$	1.13	0.84	$1.95 \times 10^{-1}$	
elaidate	fatty acid	1.19	1.48	$9.29 \times 10^{-5}$	0.62	1.24	$9.37 \times 10^{-2}$	0.89	1.24	$8.32 \times 10^{-3}$							
palmitate	fatty acid	1.19	1.43	$5.86 \times 10^{-5}$	0.58	1.22	$1.50 \times 10^{-1}$	1.15	1.29	$1.59 \times 10^{-4}$							
stearate	fatty acid	1.16	1.27	$2.07 \times 10^{-4}$	0.54	1.11	$1.50 \times 10^{-1}$	0.19	1.03	$1.00 \times 10^{-1}$	1.31	0.87	$1.05 \times 10^{-2}$				
Pentadecanoate	fatty acid	1.28	1.76	$2.72 \times 10^{-5}$	0.84	1.43	$3.59 \times 10^{-2}$	1.43	1.76	$1.72 \times 10^{-5}$	1.06	0.81	$2.51 \times 10^{-2}$				
ethylhexanoate	fatty acid	1.25	0.73	$2.60 \times 10^{-5}$	1.11	0.78	$2.05 \times 10^{-3}$	1.46	0.68	$4.07 \times 10^{-5}$							
fumarate	fatty acids	1.02	0.88	$2.38 \times 10^{-3}$	1.64	0.82	$3.88 \times 10^{-6}$	1.37	0.84	$5.36 \times 10^{-4}$	1.11	0.93	$6.60 \times 10^{-2}$				
oleamide	fatty amides	1.63	0.13	$2.88 \times 10^{-6}$	0.98	0.40	$1.53 \times 10^{-4}$	0.17	1.12	$9.90 \times 10^{-1}$							
2-hydroxybutyrate	hydroxy acids	1.60	1.70	$5.78 \times 10^{-7}$	1.84	2.86	$2.34 \times 10^{-10}$	1.42	2.00	$3.13 \times 10^{-5}$	2.24	1.68	$1.27 \times 10^{-5}$				
2,3-dihydroxybutanoate	hydroxy acids	1.23	1.65	$4.85 \times 10^{-5}$	1.77	2.49	$1.99 \times 10^{-8}$	1.37	1.88	$1.02 \times 10^{-4}$	1.93	1.51	$6.22 \times 10^{-4}$				
adenine	imidazopyrimidines	1.17	0.42	$5.07 \times 10^{-5}$	0.02	0.97	$3.30 \times 10^{-1}$	0.45	0.73	$1.65 \times 10^{-2}$	1.61	2.30	$7.32 \times 10^{-4}$				
xanthine	imidazopyrimidines	1.51	1.56	$2.20 \times 10^{-6}$	1.06	1.44	$1.32 \times 10^{-3}$	0.74	1.24	$3.03 \times 10^{-2}$	1.68	0.80	$5.39 \times 10^{-3}$				

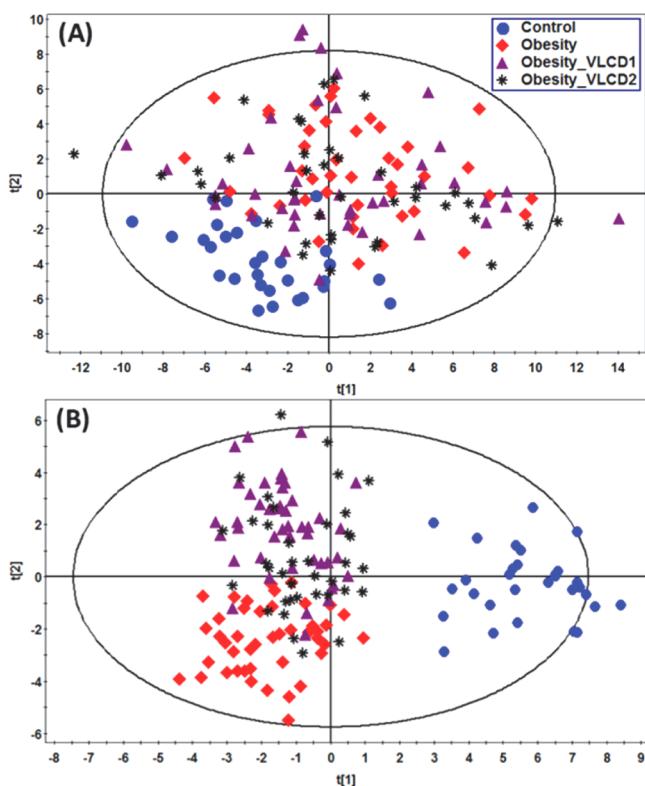
Table 2. continued

compd	class	obesity-control			obesity_VLCD 4 wk-control			obesity_VLCD 8 wk-control			obesity_VLCD 4 wk-obesity		
		VIP	FC	P	VIP	FC	P	VIP	FC	P	VIP	FC	P
uric acid	imidazopyrimidines	1.04	1.57	4.83 × 10 <sup>-4</sup>	1.02	1.50	1.91 × 10 <sup>-3</sup>	1.01	1.50	6.00 × 10 <sup>-3</sup>	1.29	1.16	1.34 × 10 <sup>-2</sup>
4-hydroxy-3-methoxymandelate	phenols	1.22	0.87	3.27 × 10 <sup>-2</sup>	0.81	0.94	3.52 × 10 <sup>-1</sup>	1.09	1.00	6.28 × 10 <sup>-1</sup>			
nicotinamide	pyridines	1.57	1.33	5.78 × 10 <sup>-7</sup>	0.90	1.15	1.33 × 10 <sup>-2</sup>	1.18	1.20	2.58 × 10 <sup>-3</sup>	1.69	0.86	7.67 × 10 <sup>-4</sup>
chenodeoxycholate	steroids	1.11	0.45	1.52 × 10 <sup>-3</sup>	0.51	0.73	1.17 × 10 <sup>-1</sup>	0.31	0.85	8.09 × 10 <sup>-1</sup>			
taurine	sulfonic acids	1.22	1.32	1.06 × 10 <sup>-4</sup>	1.18	1.27	1.32 × 10 <sup>-3</sup>	1.48	1.37	2.81 × 10 <sup>-5</sup>			
2-aminobutyrate	amino acid										2.74	1.64	2.79 × 10 <sup>-7</sup>
proline	amino acid										2.37	0.73	1.86 × 10 <sup>-6</sup>
ornithine	amino acid										1.82	0.68	1.60 × 10 <sup>-4</sup>
tryptophan	amino acid										1.48	0.84	5.01 × 10 <sup>-3</sup>
methionine	amino acid										1.18	0.81	5.01 × 10 <sup>-3</sup>
threonine	amino acid										1.36	0.88	1.10 × 10 <sup>-2</sup>
theanine	amino acid										1.31	1.36	1.75 × 10 <sup>-2</sup>
cis-11,14-eicosadienoate	fatty acid										2.05	0.83	3.64 × 10 <sup>-2</sup>
cis-11,14,17-eicosatrienoate	fatty acid										1.04	0.75	2.75 × 10 <sup>-5</sup>
arachidonate	fatty acid										1.35	1.17	1.51 × 10 <sup>-2</sup>
acetyl/carnitine	fatty acid esters										2.21	1.47	6.89 × 10 <sup>-6</sup>
3-hydroxybutyrate	hydroxy acids										2.32	6.33	3.78 × 10 <sup>-7</sup>
p-cresol	phenols										2.18	2.54	2.95 × 10 <sup>-5</sup>
3-aminophenol	phenols										1.23	0.62	3.16 × 10 <sup>-2</sup>
threitol	sugar alcohols										1.80	0.76	2.01 × 10 <sup>-4</sup>
urea	ureas										1.13	1.19	1.55 × 10 <sup>-2</sup>
glyceraldehyde	alcohols										1.44	1.26	0.83
glycerol	sugar alcohols										2.30	1.35	7.86 × 10 <sup>-4</sup>
											1.36	0.81	6.92 × 10 <sup>-3</sup>

<sup>a</sup>The metabolites responsible for the differentiation of metabolic profiles of obese subjects; or obese subjects with 4 weeks of intervention; or obese subjects with 8 weeks of intervention, from controls and metabolic profiles of obese subjects with 4 weeks of intervention; or obese subjects with 8 weeks of intervention from obese subjects were obtained using a univariate statistical analysis, Mann–Whitney U test. The corresponding fold change shows how these selected differential metabolites varied in the obese subjects before and after VLCD intervention from those of normal controls or in the obese subjects after VLCD intervention from those of obese subjects. VIP was obtained from OPLS-DA with a threshold of 1.0.

the differentially expressed metabolite levels between control and obese subjects before and after VLCD intervention is summarized in Table 2.

To distinguish healthy controls from obese subjects at baseline as well as after VLCD intervention, principal component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed with the 113 metabolites generated (Figure 1). There appears

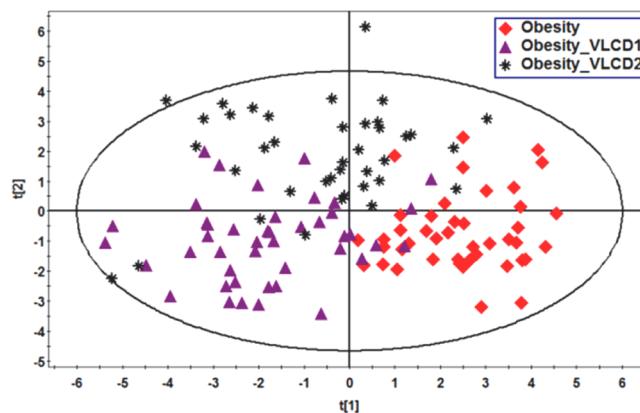


**Figure 1.** (A) PCA and (B) OPLS-DA scores plot constructed with the 113 annotated metabolites.

to be a separation between healthy controls and obese subjects, reflecting the pathophysiological variations of obesity. Notably, the metabolic patterns of obese subjects deviate from baseline after 8 weeks of VLCD intervention and the metabolic pattern is still distinct from that of the healthy controls. Analogously, distinct separation was seen among the metabolite profiles of the obese subjects before and after VLCD intervention, indicative of the effects of dietary intervention on health pathology (Figure 2 and Supporting Information Figure S1).

Based on VIP value ( $>1$ ) and Mann–Whitney U test  $p$  value ( $<0.05$ ), a total of 44 metabolites were significantly altered in obese subjects compared to healthy controls (Table 2), and the results were summarized as a Venn diagram exhibiting the commonly and specifically affected metabolites (Figure 3B). These metabolites represent key metabolic pathways involving amino acid metabolism, fatty acid metabolism, lipid metabolism, carbohydrates metabolism, and TCA cycle (Figure 3C).

**Beneficial Effects of VLCD Intervention in Obese Subjects.** The variations of differential metabolites listed in Table 2 upon obesity were investigated in obesity subjects after VLCD intervention, and as a result, not all the metabolites were attenuated or normalized. Metabolomic response profiles of representative metabolites listed in Table 2 upon obesity, and obesity after VLCD intervention was depicted as a heat map in



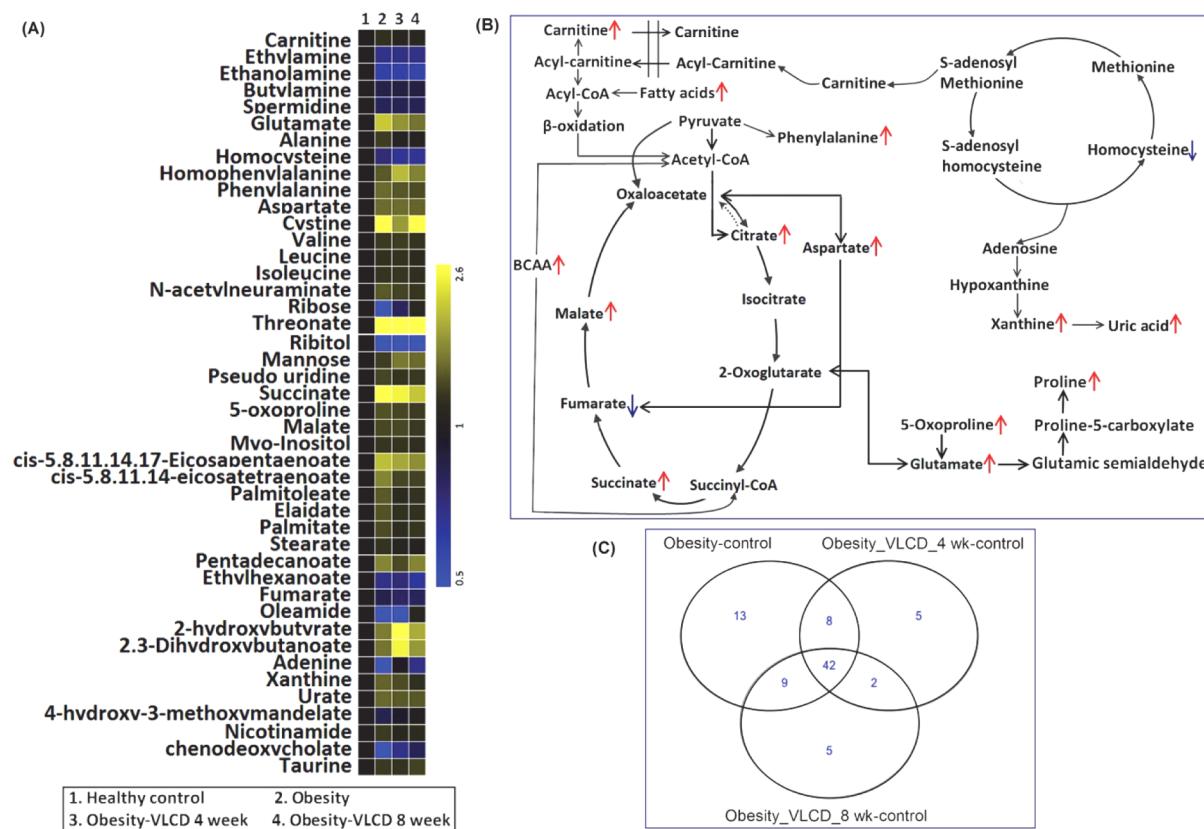
**Figure 2.** OPLS-DA scores plot constructed with the 113 annotated metabolites.

Figure 3A. The heat map generated indicates less significant fluctuation of metabolite levels (in fold change, relative to healthy control) in obesity after VLCD intervention, suggesting that VLCD could attenuate the metabolic perturbation in obese subjects.

The concentrations of carnitine, fatty acids including *cis*-5,8,11,14,17-eicosapentaenoate, *cis*-5,8,11,14-eicosatetraenoate, palmitoleate, elaidate, palmitate, and stearate, succinate, 5-oxoproline, xanthine, urate, nicotinamide, chenodeoxycholate, oleamide, 2-hydroxybutyrate, *N*-acetylneuraminate, ribose, glutamate, and alanine were altered in obese subjects. After VLCD intervention, however, the levels of the above metabolites were altered to a less significant degree, suggesting that VLCD can attenuate the metabolic alteration in obesity.

We further selected the differentially expressed serum metabolites before and after 4 weeks or 8 weeks of VLCD intervention in the obese subjects based on the VIP values ( $VIP > 1$ ) by one predictive component and two orthogonal component OPLS-DA models ( $R^2X = 0.293$ ,  $R^2Y = 0.772$ ,  $Q^2(\text{cum}) = 0.591$ ;  $R^2X = 0.293$ ,  $R^2Y = 0.783$ ,  $Q^2(\text{cum}) = 0.561$ ), respectively (Supporting Information Figure S1). A list of differential metabolites (Table 2) including 2-aminobutyrate, proline, ornithine, tryptophan, methionine, threonine, theanine, *cis*-11,14-eicosadienoate, *cis*-11,14,17-eicosatrienoate, arachidonate, acetyl-carnitine, 3-hydroxybutyrate, *p*-cresol, 3-amino-phenol, threitol, urea, glutamate, alanine, cysteine, ribose, mannose, succinate, *cis*-5,8,11,14,17-eicosapentaenoate, palmitoleate, stearate, pentadecanoate, fumarate, 2-hydroxybutyrate, 2,3-dihydroxybutanoate, adenine, and nicotinamide was identified after 4 weeks of VLCD intervention and proline, ornithine, tryptophan, threonine, *p*-cresol, threitol, urea, glutamate, alanine, ribose, mannose, succinate, palmitoleate, elaidate, stearate, oleamide, xanthine, 4-hydroxy-3-methoxy-mandelate, nicotinamide, chenodeoxycholate, glyceraldehyde, and glycerol after 8 weeks of VLCD intervention. Most of them are different from those differential metabolites in obese subjects relative to healthy controls.

**Correction of Metabolite Change along Different Time Points (Baseline and 4 Weeks and 8 Weeks after VLCD Intervention) versus the Change of BMI, Plasma Glucose Level, and Insulin Sensitivity.** A correlation analysis was performed among the 44 differential metabolites (Figure 4 and Supporting Information Figure S2), which revealed a wide range of correlation coefficients among the interactions between time (baseline and 4 and 8 weeks after



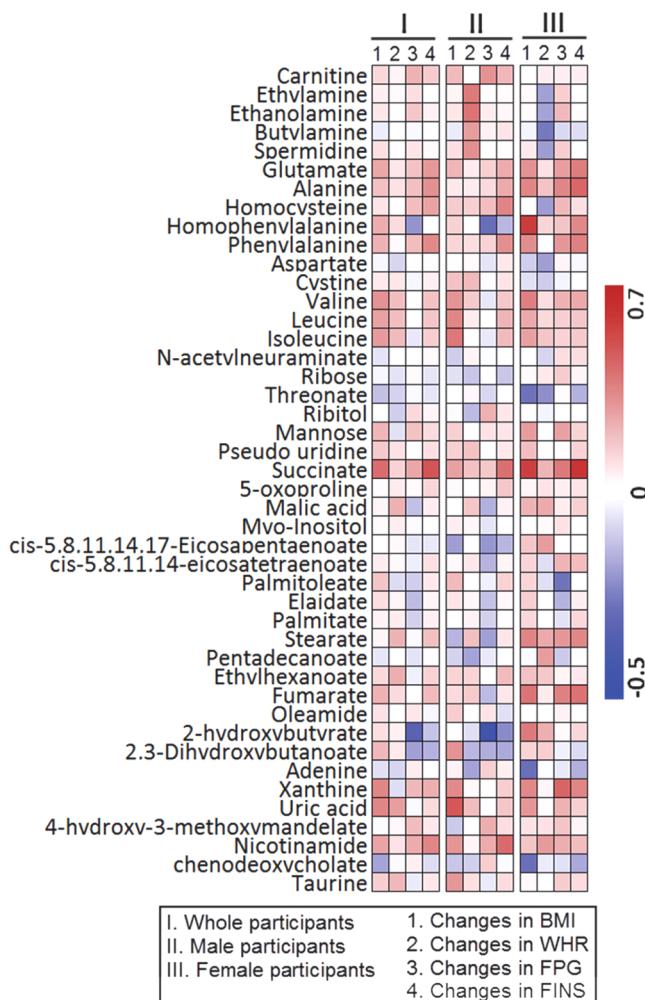
**Figure 3.** (A) Heat map showing changes in metabolites compared to healthy control at obesity subjects and at obesity subjects after VLCD intervention at weeks four and eight. Shades of yellow and blue represent fold increase and fold decrease of a metabolite, respectively, in obesity subjects, obesity subjects after 4 weeks of VLCD intervention, or obesity subjects after 8 weeks of VLCD intervention relative to healthy controls (see color scale). (B) Metabolic pathways being affected by VLCD intervention. (C) Venn diagram exhibiting the commonly and specifically affected metabolites. ↑ higher level in obesity subjects compared to healthy controls; ↓ lower level in obesity subjects compared to healthy controls.

VLCD intervention), treatment (VLCD intervention), and clinical parameters (the change of BMI, serum glucose level, and insulin sensitivity), ranging from 1.0 (maximum positive correlation) to -0.5 (maximum anticorrelation) and 0 (no correlation, see color bar scale in Figure 4). Figure 4 and Supporting Information Figure S2 illustrate that several high positive (dark red and red regions) or negative (blue regions) correlations were observed among several metabolites among male and female participants. From the correlation, difference matrix, succinate, alanine, and fumarate were positively correlated with changes of BMI, FPG, and FINS in females, but with no correlation in males. Similarly, glycochenodeoxycholic acid, methionine, uric acid, leucine, and isoleucine were positively correlated with changes of BMI in male participants, but with no correlation in females. Glalactose, arabinofuranose, and thymine were positively correlated with changes of FPG and FINS in both males and females.

Using the ratio of metabolite change over baseline (FC value), we also evaluated the correlation of this ratio versus the corresponding change of BMI, serum glucose level, and insulin sensitivity at 4 weeks and 8 weeks within the subgroups of female and male participants, correspondingly. As shown in Figure 5, fatty acids including *cis*-5,8,11,14,17-eicosapentaenoate, *cis*-5,8,11,14-eicosatetraenoate, palmitoleate, elaidate, palmitate, myristate, and linoleate were positively correlated with the changes of BMI in female after 8 weeks of VLCD intervention, but with no correlation in males. The change of BMI was positively correlated with the changes of FINS in

males after 8 weeks of VLCD intervention but with less correlation in females. The change of *cis*-5,8,11,14,17-eicosapentaenoic acid was negatively correlated with the change of PFG and FINS in females after 4 weeks of VLCD intervention, but this correlation became positive after 8 weeks of VLCD intervention. The changes of oleamide, 2-hydroxybutyrate, and 2,3-dihydroxybutanoate were negatively correlated with the change of BMI, FPG, and FINS in both males and females after 4 weeks of VLCD intervention, which became more negative after 8 weeks of VLCD intervention. BCAAs, leucine, isoleucine, and valine were positively correlated with the changes of BMI, FPG, and FINS in both males and females after 4 or 8 weeks of VLCD intervention.

**Metabolite Markers Associated with Age.** Because there is a statistically significant difference in age between obese subjects and healthy controls, we tried to identify metabolite markers associated with age among the subjects in the control group. We first established the PCA scores plot (three component,  $R^2X = 0.400$ ,  $Q^2(\text{cum}) = 0.108$ , Supporting Information Figure S3) of all the healthy controls, and there's no clear separation among the healthy controls with different ages. We then compared all the metabolites between the subjects of older ages (>29 years old) and the younger subjects (<25 years old) and one metabolite, tocopherol (vitamin E) of statistical significance ( $p < 0.05$ ) was removed from the list of potential markers.



**Figure 4.** Heat map showing interactions between time (metabolite change from baseline, 4 weeks, and then 8 weeks), VLCD intervention (4 weeks and 8 weeks), and clinical parameters. Shades of red and blue represent positive correlation and negative correlation, respectively (see color scale).

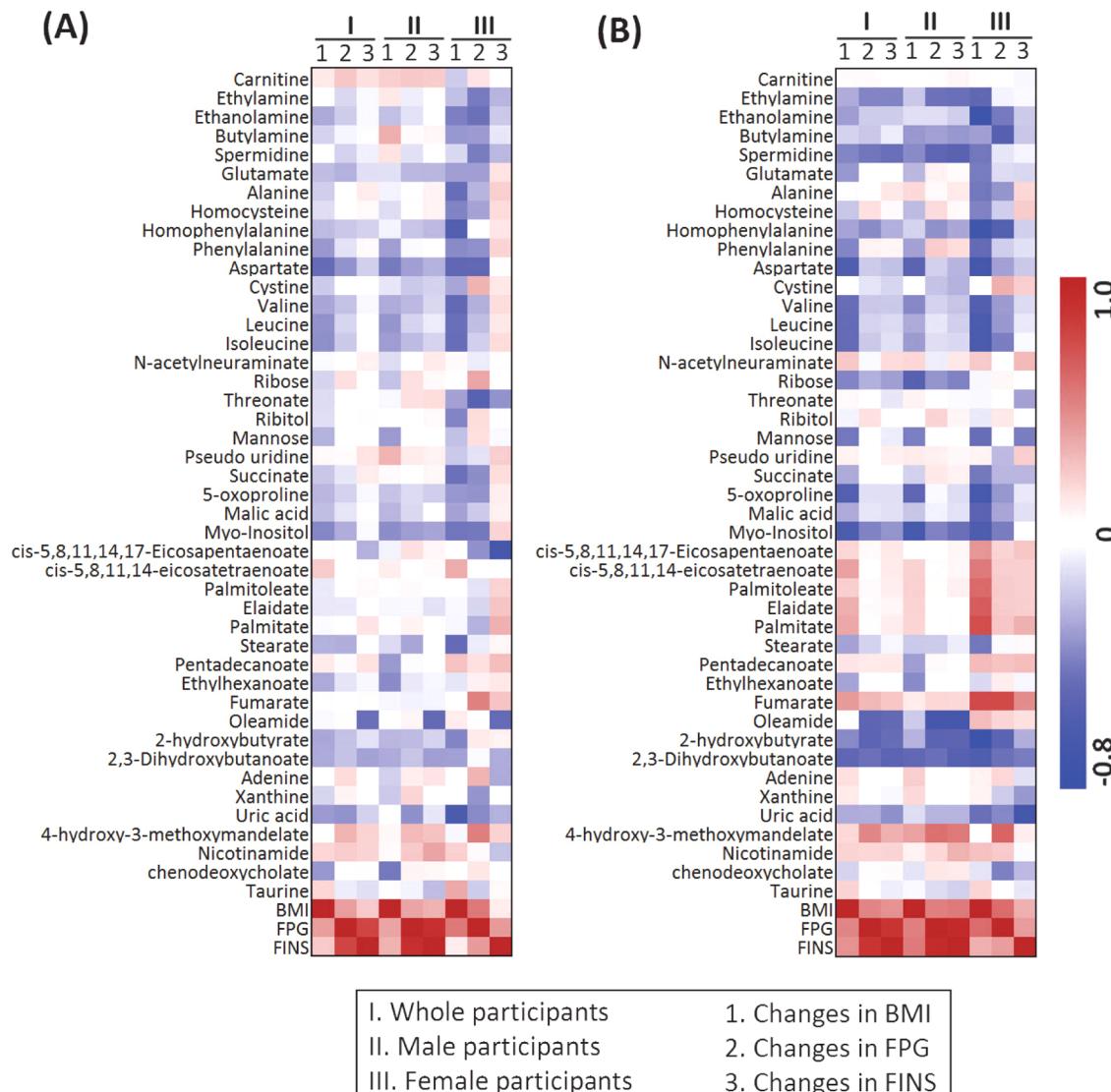
## DISCUSSION

In the present study, we applied a comprehensive metabolomics approach to understand the metabolic differences between obese and healthy control subjects and determine how those metabolic profiles were impacted by VLCD intervention. Several obesity-related changes described herein confirm prior studies, including the higher levels of blood glucose, insulin, and HOMA-IR in obese subjects compared to healthy controls.<sup>12</sup> The OPLS-DA analysis of healthy controls and obese subjects revealed that obese subjects had a clearly distinct metabolic profile from healthy controls. Several metabolites associated with amino acid metabolism, fatty acid metabolism, lipid metabolism, carbohydrate metabolism, and TCA cycle were altered, and changes in these metabolic profiles contributed to the difference between healthy controls and obese subjects (Table 2). These results were consistent with previously published metabolomic studies analyzing healthy obese and control subjects, where several key metabolic pathways, including BCAA metabolism, fatty acid metabolism, bile acid metabolism, and gut microbial-host co-metabolism were significantly altered in association with the obese phenotype.<sup>13,14</sup> VLCD intervention resulted in a global

metabolite alteration in obese subjects, leading to an attenuated metabolite perturbation in obese subjects, suggesting that VLCD could attenuate the obesity induced metabolic perturbation. Amino acids are well-known to stimulate the endogenous release of glucoregulatory hormones and might thereby modulate glucose metabolism.<sup>25,26</sup> Interestingly, ingestion of VLCD actually decreases blood glucose level in obesity participants. Changes of amino acids were positively correlated with the changes in BMI, FPG, and FINS. The decreased BMI, FPG, and FINS was consistent with the results of the decreased levels of amino acids after VLCD intervention. However, not all the differentially expressed metabolites in obese subjects were attenuated or normalized by VLCD intervention. Therefore, a list of differential metabolites (Table 2) responsible for the separation between obese subjects before and after VLCD intervention was identified. These "VLCD-induced" markers are different from those differential metabolites in obese subjects relative to the healthy controls.

Consistent with the results presented by Newgard et al.,<sup>13</sup> we found that key compounds of fatty acid synthesis and oxidation (carnitine) were significantly increased in obese subjects. Fatty acids are an important energy source in the body and provide energy through  $\beta$ -oxidation. Research has shown that obesity and diabetes are related to the decreased fatty acid oxidation capacity, and as a result, excessive fatty acids will accumulate in the body as triglycerides, thereby causing lipotoxicity. Many reports have revealed that fatty liver, adipose tissue, and pancreatic fat accumulation are directly related to insulin resistance.<sup>27-29</sup> Serum fatty acid composition may modulate insulin action, and increased serum fatty acid concentrations are known to impair glucose metabolism, potentially causing diabetes.<sup>30</sup> Consistent with the previous report by Kim et al.,<sup>14</sup> *cis*-5,8,11,14,17-eicosapentaenoic acid, *cis*-5,8,11,14-eicosatetraenoic acid, palmitoleic acid, elaidic acid, stearic acid, palmitic acid, and pentadecanoic acid were found to be significantly increased in obese subjects compared to healthy controls, representing fatty acids that circulate as triglycerides or other esterified species. These findings are consistent with the strong increase in triglyceride levels in obese subjects. It was reported that hypocaloric diets with VLC (12% energy) in obese subjects lead to greater increases in markers of lipolysis (ketones and free fatty acids) together with greater decreases of markers of lipogenesis and deposition of body fat (leptin) when compared to low-fat (56% energy as CHO) hypocaloric diets.<sup>31</sup> As a product of fat lipolysis and oxidation, 3-hydroxybutyrate was of 6.33-fold increase at week 4 and subsequently decreased in week 8, with its concentration remaining significantly higher than that at baseline. The changes of 3-hydroxybutyrate were negatively correlated with the change of BMI, FPG, and FINS in both males and females after 4 or 8 weeks of VLCD intervention (Supporting Information Figure S2), which was consistent with the results of the increased 3-hydroxybutyrate level and the decreased clinical parameters after VLCD intervention. A study reported that fatty acids significantly increased in the first 7 days but decreased in week 8 in diabetic patients who participated in an 8-week VLCD intervention.<sup>32</sup> Considering the changes of fatty acids and ketone body together, the changes might reflect the process of fat burning during the 8-week period, leading to the significant weight loss.

The changes in amino acid metabolism have also been reported for obese people.<sup>33,34</sup> Of particular interest, the physiological effects of BCAA have been noted in various aspects of energy metabolism, including the stimulation of



**Figure 5.** Heat maps showing the correlation of the change of metabolite with baseline (A) after 4 weeks of VLCD intervention and (B) after 8 weeks of VLCD intervention versus the changes in BMI, FPG, and FINS. Shades of red and blue represent positive correlation and negative correlation, respectively; in the change of metabolite in obesity subjects after 4 weeks of VLCD intervention or obesity subjects after 8 weeks of VLCD intervention relative to the changes of clinical parameters (see color scale).

insulin secretion in pancreatic  $\beta$  cells<sup>35</sup> and the control of appetite through the mTOR pathway in hypothalamic neurons.<sup>36</sup> It is therefore likely that, in the future, BCAA will be used clinically as a marker of insulin resistance. Emerging evidence suggests BCAA could be a marker for obesity and combined with phenylalanine and tyrosine could be used for the prediction of diabetes risks.<sup>13,37</sup> The higher BCAA levels on obese subjects may reflect an overload of BCAA catabolism in obese subjects.<sup>13</sup> However, the levels of BCAA in obese subjects did not change significantly after VLCD intake, which indicates that the VLCD is unlikely affecting dietary intake or plasma concentrations of BCAs. BCAs, leucine, isoleucine, and valine were positively correlated with the changes of BMI, FPG, and FINS in both males and females after 4 or 8 weeks of VLCD intervention, which was consistent with the decreased BCAs level after VLCD intervention, although no significant changes were found. The gut microbiota has recently been suggested to contribute to the development of obesity. The composition of the gut microbiota has been shown to differ in

lean and obese humans and animals and to change rapidly in response to dietary factors.<sup>38</sup> Interestingly, there is direct evidence that changing the amount and/or type of carbohydrate over periods of up to four weeks has a profound and rapid influence on the composition of the gut microbiota and its metabolic products in adult human volunteers.<sup>39–42</sup> Some metabolites including bile acids, phenolic, benzoyl, and phenyl derivatives and lipids that regulate host–microbiota interactions are believed to be involved in gut microbiota–host co-metabolism.<sup>43</sup> Four or eight weeks of VLCD intervention clearly altered the metabolites involved in gut microbiota, which supports the reported findings of altered gut microbiota after diet intervention.

The aim of this study was to determine metabolic differences between obese and healthy human subjects and investigate the effect of VLCD intervention on serum metabolic profiles in obese subjects by a metabonomic approach. However, there are several limitations in the current MS-based study. First, the relatively small size used in this study may not be sufficiently

large to detect all diet-associated metabolic changes. Second, being a short-term designed study, the evidence of the long-term effects of VLCD was unclear. Longer-term and further studies are needed to investigate the effects and to reveal the underlying mechanisms of VLCD intervention on weight loss and weight maintenance, as well as on glucose prevention in the future.

## CONCLUSION

In conclusion, we identified comprehensive metabolic shifts in obese subjects including altered fatty acid metabolism, amino acid metabolism, carbohydrate metabolism, and TCA cycle. VLCD is able to attenuate metabolic perturbation in obese subjects, characterized by the less significant metabolic alterations. It also appears that VLCD induced significant metabolic alterations independent of the obesity induced metabolic changes. These VLCD-induced alterations include a panel of metabolites that are involved in inflammation and oxidation processes, suggesting that VLCD exerts the beneficial effects associating with the anti-inflammatory and antioxidant mechanisms in addition to the attenuation of obesity-induced metabolic perturbation.

## ASSOCIATED CONTENT

### Supporting Information

Supplementary methods, tables, and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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