

# Plasma High-Resolution Metabolomics Differentiates Adults with Normal Weight Obesity from Lean Individuals

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**Objective:** This study explored underlying metabolism-related dysfunction by examining metabolomic profiles in adults categorized as lean, as having normal weight obesity (NWO), or as having overweight/obesity.

**Methods:** Participants ( $N=179$ ) had fasting plasma analyzed by liquid chromatography and high-resolution mass spectrometry for high-resolution metabolomics. Body composition was assessed by dual-energy x-ray absorptiometry. NWO was defined as BMI <25 and body fat >30% for women and >23% for men. Differentiating metabolomic features were determined by using linear regression models and likelihood ratio tests with false discovery rate correction. *Mummichog* was used for pathway and network analyses.

**Results:** A total of 222 metabolites significantly differed between the groups at a false discovery rate of  $q=0.2$ . Linoleic acid,  $\beta$ -alanine, histidine, and aspartate/asparagine metabolism pathways were significantly enriched (all  $P<0.01$ ) by metabolites that were similarly upregulated in the NWO and overweight/obesity groups compared with the lean group. A module analysis linked branched-chain amino acids and amino acid metabolites as elevated in the NWO and overweight/obesity groups compared with the lean group (all  $P<0.05$ ).

**Conclusions:** Metabolomic profiles of individuals with NWO reflected similar metabolic disruption as those of individuals with overweight/obesity. High-resolution metabolomics may help identify people at risk for developing obesity-related disease, despite normal BMI.

*Obesity* (2019) **27**, 1729-1737. doi:10.1002/oby.22654

## Introduction

Obesity is a leading risk factor for major diseases, including cardiovascular disease, type 2 diabetes, and cancer, and health conditions such as depression, obstructive sleep apnea, and decreased physical functioning (1). Individuals with obesity have excess fat mass and metabolic dysregulation resulting in increased all-cause mortality risk (1). BMI, calculated using simple anthropometric measures of height and weight, is used clinically to define obesity as a BMI greater than 30 kg/m<sup>2</sup>. Although BMI is useful for identifying individuals at extreme levels with very high or low adiposity, BMI values in more moderate ranges are not well correlated with body fatness (2,3). This is because BMI uses

total body weight and does not account for body composition components, such as lean mass and fat mass, which independently influence disease risk.

Within the range of intermediate BMI values is a group of individuals with a body composition phenotype termed normal weight obesity (NWO) (4). These individuals have BMI within the normal weight range (18.5-24.9 kg/m<sup>2</sup>) but exhibit excess fat mass. The current reported estimates for NWO are as high as 30% (4,5). Individuals with NWO were shown to have an increased risk of cardiometabolic disease and mortality compared with individuals who were normal weight and lean and individuals who were metabolically healthy with obesity (6). Previous studies have shown that individuals with NWO have elevated

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**Funding agencies:** This work is based on information from the Emory/Georgia Tech Predictive Health Institute and Center for Health Discovery and Well Being database, supported by the National Center for Advancing Translational Sciences of the NIH under award UL1 TR002378. Additional grant support includes NIH grants K01 DK102851 (JAA), R03 DK117246 (JAA), K24 DK096574 (TRZ), and P30 ES019776 (Health and Exposome Research Center at Emory University; DPJ and TRZ).

**Disclosure:** The authors declared no conflict of interest.

Additional Supporting Information may be found in the online version of this article.

**Received:** 28 May 2019; **Accepted:** 30 July 2019; **Published online** 5 November 2019. doi:10.1002/oby.22654

cardiometabolic disease risk factors, demonstrated by hyperlipidemia, hypertension, glucose intolerance, insulin resistance, increased inflammation, increased oxidative stress, and decreased physical functioning (7). Although there is a growing literature of metabolic dysregulation in NWO, there is a need to define the nutrition- and metabolism-related pathophysiology of NWO.

High-resolution metabolomics (HRM) is an innovative platform that is useful for exploring obesity-related disease from a systems-biology approach (8). HRM is a powerful tool for nutrition research because it enables the profiling of thousands of small-molecular-weight metabolites in human biological samples and allows the investigation of important questions regarding complex metabolite interactions that derive from diet, endogenous nutrient metabolism, the microbiome, and exogenous chemicals (8). Metabolomics has been used to identify specific metabolic signatures related to BMI and obesity (9,10). However, there is little known regarding the metabolomic profiles from HRM of individuals with NWO compared with other body composition subtypes. In this study, we used HRM to investigate differences in the plasma metabolome between three body composition subtypes: lean, NWO, and overweight/obesity. We hypothesized that individuals with NWO would have metabolomic profiles that were similar to participants with overweight/obesity and distinct from participants who were lean.

## Methods

### Participants and study design

Emory University and Emory Healthcare employees were randomly invited to join the Emory/Georgia Tech Predictive Health Institute's Center for Health Discovery and Well Being (<http://predictivehealth.emory.edu>) cohort study between December 2007 and December 2010. Participants underwent extensive dietary, metabolic, and other phenotypic assessments, as described in detail elsewhere (11). All participants provided written informed consent, and the study was approved by the Emory University Institutional Review Board. Exclusion criteria included the addition of a new prescription medication for chronic disease treatment within the previous year (other than antihypertensive or antidiabetic agents), acute illness within 12 weeks of the study visit, hospitalization for an acute or chronic disease within the previous year, history of substance/drug or alcohol abuse, a current active malignant neoplasm, women who were pregnant or breastfeeding, or an uncontrolled (nonmedicated) or poorly controlled autoimmune, cardiovascular, endocrine, gastrointestinal, hematologic, infectious, inflammatory, musculoskeletal, neurologic, psychiatric, or respiratory disease (11). All data included in this analysis were collected at baseline visits. The current study included a subset of individuals with available baseline plasma HRM data. Demographic, education, and income information was self-reported. Participants were classified as having a history of chronic disease (yes or no) if they reported a current diagnosis of diabetes, hypertension, or hyperlipidemia or if they were currently taking antihypertensive, antidiabetic, or lipid-lowering medications.

### Clinical markers, physical fitness, and diet-quality scores

Fasting concentrations of glucose, insulin, and lipids were measured by Quest Diagnostics (Valencia, California). The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated according

to Matthews et al. (12). Systolic and diastolic blood pressure were measured using an automated machine (Omron, Kyoto, Japan). Physical fitness (maximum oxygen consumption [ $VO_{2max}$ ]) was assessed using a GE T2100 treadmill (GE Healthcare, Waukesha, Wisconsin), following a modified Balke protocol. Participants completed the Cross-Cultural Activity Participation Study (13) to determine whether individuals met the 2007 American College of Sports Medicine/American Heart Association physical activity and strength guidelines. Dietary intake was assessed using the 2005 Block Food Frequency Questionnaire (NutritionQuest, Berkeley, California). Any reported intakes less than 500 calories or greater than 5,000 calories were considered implausible and excluded. Three validated diet quality scores, the Alternate Healthy Eating Index (14), Dietary Approaches to Stop Hypertension diet score (15), and the Mediterranean Diet Score (16), were calculated from the Block Food Frequency Questionnaire output, as previously described (17).

### Body composition analysis and body composition subgroups

Whole and regional body composition was assessed with dual-energy x-ray absorptiometry using a Lunar iDXA densitometer and enCORE (version 12.2) with CoreScan software (GE Healthcare, Madison, Wisconsin). BMI was calculated from height and weight measured using an electronic scale and stadiometer (Tanita TBF-25; Tanita Health Management, Arlington Heights, Illinois). Participants were then classified into one of three body composition subtypes (lean, NWO, or overweight/obesity) based on sex-specific body fat percentage values and BMI. For men, body fat percentage > 23% was considered elevated, and for women, body fat percentage > 30% was considered elevated, based on published literature (18). Participants were categorized as having a lean body composition subtype if their BMI was between 18.5 and 24.9 and their body fat percentage was below the sex-specific cutoff values. NWO was defined as BMI between 18.5 and 24.9 and a body fat percentage above the sex-specific cutoff values. Lastly, overweight/obesity was categorized as BMI  $\geq$  25 and a body fat percentage above the sex-specific cutoff values. Waist circumference was measured three times by a health professional trained in anthropometry using a tape measure, and the average value is reported.

### Plasma HRM

Plasma HRM was performed on plasma samples from 179 fasted individuals using published methods (19) in the Emory University Clinical Biomarkers Laboratory. In brief, fasting plasma previously stored at  $-80^{\circ}\text{C}$  was treated with acetonitrile and an internal standard mixture using an established protocol (19). Following protein precipitation, fasting plasma samples were analyzed in triplicate with a Fourier transform mass spectrometer (Dionex UltiMate 3000, Q Exactive Orbitrap; Thermo Fisher, Waltham, Massachusetts) using C18 liquid chromatography and positive electrospray ionization to maximize the detection of low-molecular-weight chemicals. After analysis of all participant samples and quality control samples, liquid chromatography-mass spectrometry data were extracted using the R-based packages *apLCMS* (20) and *xMSanalyzer* (21) to provide a mass-to-charge feature table of detected ions denoted by relative retention time and accurate mass. Batch correction was completed by *ComBat* (22). Data preprocessing included (1) filtering of features based on the coefficient of variation, (2) filtering of samples based on Pearson correlation between averaged technical replicates and percentage of

missing values (features were retained only if there was a signal in at least 50% of samples), and (3) log<sub>10</sub> transformation, quantile normalization, and mean centering. A total of 9,967 metabolomic features were included in this analysis after data filtering.

## Metabolite identification

The R package xMSannotator was used for metabolite annotation, which uses multiple criteria to provide a score-based annotation (23). Identities of multiple endogenous metabolites, including the amino acids, have been confirmed by comparing coelution with an authentic standard (24) in the Emory Clinical Biomarkers Laboratory, and they are equivalent to a level 1 identification according to the Schymanski et al. criteria (25). Additional annotations were made with a high or medium confidence ( $\geq$  level 2) with a protonated adduct (M+H adducts). When identity confirmation was not available, metabolites were annotated by searching metabolite databases, such as the Human Metabolome Database (<http://www.hmdb.ca>) and METLIN (<https://metlin.scripps.edu>), for metabolite mass-to-charge matches. For selected features that could not be annotated based on mass spectrometry (MS1) data only, ion dissociation spectra (tandem mass spectrometry [MS/MS]) were collected on a Thermo Scientific Fusion Mass Spectrometer for MS/MS spectral library matching using the mzCloud database (<https://www.mzcloud.org>).

## Statistical analyses and bioinformatics

Descriptive statistics (mean [SD]) are presented for clinical variables. Distributions were assessed for normality, and any non-normally distributed clinical variables were natural log transformed for use in parametric statistics and back transformed for data presentation. ANCOVA tests, with adjustment for age, race, sex, and history of chronic disease (yes or no), were used to test for overall group differences in clinical, body composition, and lifestyle factors. Post hoc comparisons between specific groups were assessed with Tukey honestly significant difference tests. Fisher exact tests were used for comparison of categorical variables because of small numbers in the variable levels. HRM bioinformatics analyses were performed using R. For HRM analyses, we used multiple linear regression analyses with likelihood ratio tests, adjusting for age, sex, race, and history of chronic disease, to determine differences between the three body composition groups (lean, NWO, and overweight/obesity). False discovery rate was controlled for with the Benjamini-Hochberg procedure ( $q=0.2$ ). Metabolites that significantly differed between the groups were analyzed by the *mummichog* pathway enrichment and module analysis program (26). Significantly enriched metabolic pathways that included less than four metabolites were excluded from findings. Module analyses were also produced from *mummichog*, which are unbiased from established biological pathways and which construct independent networks of highly correlated metabolites (26). To test for differences in significantly enriched pathways and module metabolites between body composition subtypes, intensity values for individual metabolites within each pathway and network were compared, with adjustment for age, race, sex, and history of disease. In post hoc analyses of differing metabolites, we also controlled for group differences in  $VO_{2max}$ . In a subset of the cohort ( $n=86$ ), sensitivity analyses were performed on metabolites of interest between individuals classified as having NWO and overweight/obesity using Student *t* tests. Individuals in the subset were matched by age (within 2 years), race, and sex. Of 43 individuals classified as having NWO, 32 were matched to individuals classified as having overweight/obesity on all three criteria, and 11 were matched on two of the three criteria.

Statistics comparing clinical variables and individual metabolite intensity values were performed in JMP Pro (version 13, SAS Institute Inc., Cary, North Carolina).

## Results

Demographic and clinical characteristics for all participants are shown in Table 1. Distributions of age and race did not significantly differ between the three groups ( $P=0.07$  and  $P=0.3$ , respectively). There were significantly more women in the NWO group ( $P<0.05$ ) compared with the lean and overweight/obesity groups. The overweight/obesity group had a significantly higher proportion of individuals with a history of chronic disease compared with the lean and NWO groups ( $P=0.01$ ). In general, the population was highly educated and participants reported a high annual household income, which was similar between all groups ( $P>0.05$  for both). Fasting plasma glucose, total cholesterol, low-density lipoprotein cholesterol, and diastolic blood pressure levels did not significantly differ between the groups ( $P>0.05$ ). Fasting insulin, HOMA-IR, and triglyceride values were similar between the lean and NWO groups ( $P>0.05$ ) but were significantly higher in the overweight/obesity group ( $P<0.05$ ). Systolic blood pressure levels differed only between the NWO and overweight/obesity groups ( $P<0.05$ ). High-density lipoprotein cholesterol levels did not differ between the lean and NWO groups but were significantly lower in the overweight/obesity group ( $P<0.05$ ). The proportion of participants in each group with adverse clinical biomarkers is shown in Supporting Information Table S1.

## Body composition, diet quality, and physical fitness

Body composition and lifestyle variables are presented in Table 2. Per the body composition subtype classification, BMI was similar between the lean and NWO groups ( $P>0.05$ ) but was significantly higher in the overweight/obesity group ( $P<0.05$ ). Body fat percentage increased significantly from participants classified as lean to having NWO to having overweight/obesity ( $P<0.05$ ). Although lean body mass did not differ between the lean and overweight/obesity groups, it was significantly lower in the NWO group ( $P<0.05$ ). Visceral adipose tissue increased significantly with each group, whereas waist circumference was significantly higher only in the overweight/obesity group ( $P<0.05$ ).  $VO_{2max}$  was highest in the lean group and significantly lower in the NWO and overweight/obesity groups ( $P<0.05$ ). Based on self-reported data, a greater proportion of the lean group completed moderate-to-vigorous aerobic activity ( $P<0.05$ ), but there were no differences between groups for strength training ( $P>0.05$ ). The Mediterranean Diet Score and the Alternate Healthy Eating Index were similar across all groups ( $P>0.05$ ). The Dietary Approaches to Stop Hypertension diet quality score was significantly higher in the lean group compared with the overweight/obesity group ( $P<0.05$ ).

## HRM

Of the 9,967 filtered metabolomic features, 1,533 features were significantly associated with the body composition subtypes at  $P<0.05$  (Figure 1A). Following false discovery rate correction, there were 222 significantly associated metabolites ( $q=0.2$ ), which were used as input for *mummichog* (26) pathway enrichment and module analyses. Significantly enriched pathways are shown in Figure 1B. There were 10 significantly enriched pathways predominantly related to

TABLE 1 Demographic and clinical characteristics

	Lean (n = 26)	NWO (n = 43)	Overweight/obesity (n = 110)
Age, y	47.3 ± 2.0	47.8 ± 1.6	50.6 ± 1.0
Female sex	15 (58)	35 (81)*	66 (60)
White	23 (88)	35 (81)	79 (72)
Education			
Less than high school	1 (4)	—	—
Completed high school	1 (4)	—	4 (4)
Some college	1 (4)	5 (12)	21 (19)
4 years of college	6 (23)	12 (28)	25 (23)
Any graduate school	17 (65)	26 (60)	60 (55)
Annual household income, \$			
≤ 50,000	1 (4)	1 (3)	14 (14)
> 50,000-100,000	6 (24)	6 (15)	33 (32)
> 100,000-200,000	10 (40)	21 (53)	31 (30)
> 200,000	8 (32)	12 (30)	25 (24)
Chronic disease (yes)	5 (19)	6 (14)	38 (35)*
Plasma glucose, mg/dL	95.7 ± 4.8	93.1 ± 4.0	94.9 ± 3.1
Plasma insulin, $\mu$ IU/mL*	2.7 ± 0.5*	3.6 ± 0.5*	5.5 ± 0.6
HOMA-IR*	0.6 ± 0.1*	0.8 ± 0.1*	1.3 ± 0.1
Total cholesterol, mg/dL	193.9 ± 9.4	201.4 ± 8.0	197.8 ± 6.1
LDL-C, mg/dL	105.3 ± 8.2	117.8 ± 7.0	118.4 ± 5.3
HDL-C, mg/dL	72.7 ± 3.8*	63.5 ± 3.3*	55.5 ± 2.5
Triglycerides, mg/dL	81.1 ± 11.4*	101.2 ± 9.7*	117.2 ± 7.4
Systolic blood pressure, mm Hg	119.4 ± 3.6*	118.1 ± 3.1*	126.1 ± 2.3
Diastolic blood pressure, mm Hg	74.7 ± 2.5	75.5 ± 2.1	79.9 ± 1.6

Values are mean ± SE or n (%). Results of Tukey post hoc analyses are denoted by capital letters. Values not connected by the same letter are significantly different at  $P < 0.05$ . Plasma variables were adjusted for age, sex, race, and history of chronic disease.

\*Variables were natural-log transformed for analyses and back transformed for data presentation; reported as geometric mean ± SE.

HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; LDL-C, low-density lipoprotein cholesterol; NWO, normal weight obesity.

lipid and amino acid metabolism. Representative metabolites within the significantly enriched pathways are shown in Figure 2. All metabolites included in Figure 2 were matched by an M+H adduct and have a level 1 or level 2 annotation with high or medium confidence (23,27). Metabolites within the linoleic acid metabolism pathway, such as linoleic acid and oxidized linoleic acid-related metabolites, were higher in the NWO and overweight/obesity groups compared with the lean group ( $P < 0.05$  for all). Metabolites within  $\beta$ -alanine, histidine, and aspartate/asparagine metabolism were significantly elevated in the NWO and overweight/obesity groups compared with the lean group. Glutathione and glutamate metabolism contained metabolic features that were similarly elevated in the NWO and overweight/obesity groups compared with the lean group as well as metabolite levels that were elevated in only the overweight/obesity group compared with the lean group. The significantly enriched pathways lysine metabolism, glycine and serine metabolism, and the urea cycle contained metabolite levels that were higher in the overweight/obesity group compared with the lean group. Following further adjustment for  $VO_{2max}$ , lysine levels were similar between all three groups. No other findings in pathway analyses changed after adjustment for  $VO_{2max}$ , as shown in Figure 2. Significantly enriched pathways with all tentatively annotated metabolic features are shown in Supporting Information Table S2.

Figure 3 depicts a module analysis of metabolites significantly differing between the body composition subtypes ( $P < 0.05$  for all metabolites). The module was predominantly composed of amino acids and amino acid-related metabolites (17 of 21 metabolites), including the branched-chain amino acids (BCAAs) leucine/isoleucine, cystine, pyruvate, histidine, 5-oxoproline, ornithine, and putrescine, which had significantly elevated levels in the NWO and overweight/obesity groups compared with the lean group. Additional amino acid metabolite intensities, such as the BCAA valine, 3-methyl-2-oxobutanoic acid (a valine-related metabolite), the aromatic amino acids (AAAs) tyrosine and threonine, glutamate, and phenylpyruvate (a phenylalanine-related metabolite), were higher in the overweight/obesity subtype compared with the lean subtype, but these metabolite intensity levels in the NWO group did not differ from either of the other groups. Following additional adjustment for  $VO_{2max}$ , 5-oxoproline levels were significantly elevated in the overweight/obesity group compared with the lean group but did not differ significantly in the NWO group. All of the metabolic features tested followed the same pattern after adjustment for  $VO_{2max}$ , as noted in Figure 3.

In sensitivity analyses of matched participants classified as having NWO and overweight/obesity, there were no changes in statistical findings from the results reported above and shown in Figures 2 and 3;

**TABLE 2** Body composition variables, physical fitness, and diet quality scores

	Lean (n = 26)	NWO (n = 43)	Overweight/obesity (n = 110)
BMI	23.9 ± 0.9*	24.3 ± 0.7*	30.8 ± 0.6 <sup>‡</sup>
Total body mass, kg	69.7 ± 2.9*	68.5 ± 2.5*	87.6 ± 1.9 <sup>‡</sup>
Lean body mass, kg	50.3 ± 1.5*	44.4 ± 1.2 <sup>‡</sup>	51.1 ± 1.0*
Fat mass, kg	16.5 ± 1.8*	21.5 ± 1.5 <sup>‡</sup>	33.6 ± 1.1
Total body fat, %	23.1 ± 1.0*	31.3 ± 0.8 <sup>‡</sup>	37.7 ± 0.6
Visceral adipose tissue, kg*	0.22 ± 0.04*	0.5 ± 0.1 <sup>‡</sup>	1.3 ± 0.2
Waist circumference, cm			
Men	82.3 ± 3.2*	85.8 ± 3.2*	97.6 ± 2.6 <sup>‡</sup>
Women	77.0 ± 2.9*	77.5 ± 2.1*	91.7 ± 1.7 <sup>‡</sup>
VO <sub>2max</sub> , mL/min/kg	42.9 ± 2.3*	34.6 ± 2.0 <sup>‡</sup>	34.2 ± 1.5 <sup>‡</sup>
Met MVPA guidelines <sup>‡</sup>	16 (64)*	14 (33) <sup>‡</sup>	45 (41) <sup>c</sup>
Met strength guidelines <sup>‡</sup>	10 (38)	8 (19)	23 (21)
Mediterranean Diet Score	4.7 ± 0.4	4.1 ± 0.4	3.9 ± 0.3
DASH diet score	5.5 ± 0.3*	5.0 ± 0.2* <sup>‡</sup>	4.9 ± 0.2 <sup>‡</sup>
Alternate Healthy Eating Index	48.6 ± 2.6	46.8 ± 2.2	45.0 ± 1.7

Values are mean ± SE or n (%). Results of Tukey post hoc analyses are denoted by capital letters. Values not connected by the same letter are significantly different at  $P < 0.05$ . <sup>†</sup>The variable visceral adipose tissue was natural-log transformed for analyses and back transformed for data presentation and is reported as geometric mean ± SE. <sup>‡</sup>Met 2007 guidelines of 30 minutes of moderate-activity exercises at least 5 times/week, 20 minutes of vigorous-activity exercises 3 times/week, or a combination of both. Participants reported meeting strength guidelines by performing muscular strengthening exercises at least twice weekly. DASH, Dietary Approaches to Stop Hypertension; MVPA, moderate-to-vigorous physical activity; NWO, normal weight obesity.

all metabolite intensity levels remained similar between the NWO and overweight/obesity groups.

## Discussion

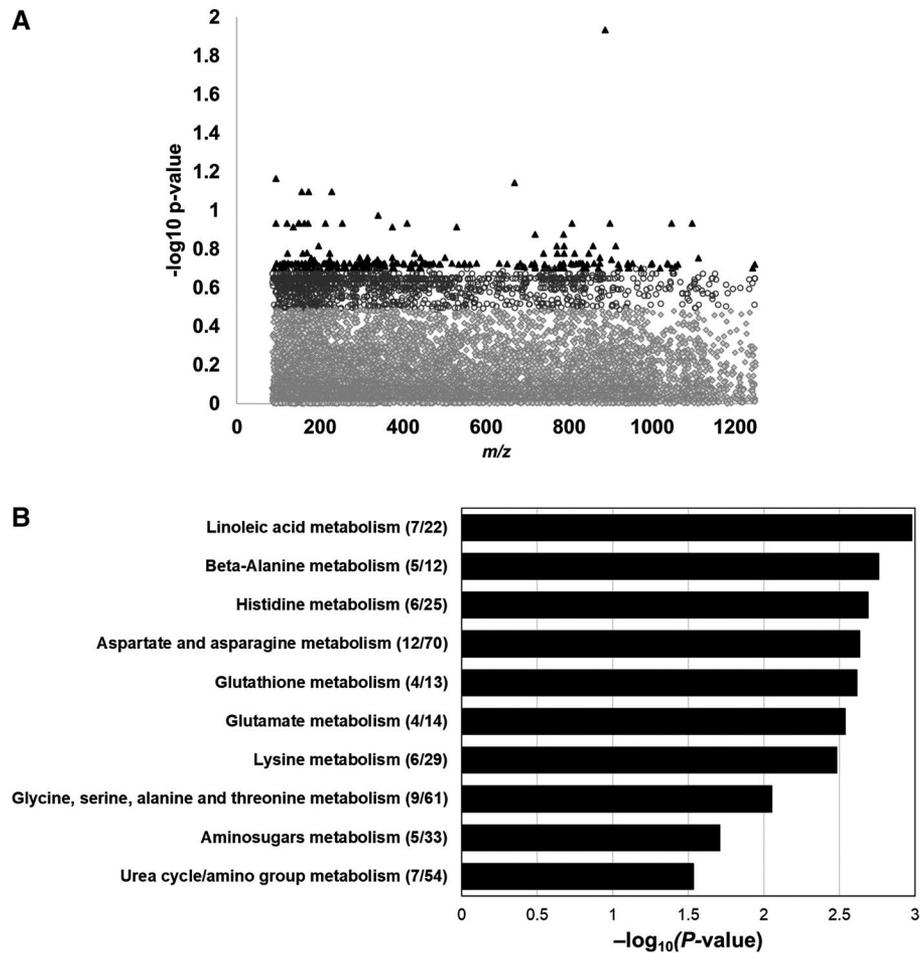
In this Atlanta, Georgia-based cohort, we found that adults with a NWO phenotype had metabolomic profiles that were similar to those of individuals with overweight/obesity and distinct from those of lean individuals. In particular, linoleic acid, β-alanine, histidine, and aspartate/asparagine metabolism, as well as some BCAAs, were upregulated in the NWO and overweight/obesity subtypes compared with the lean subtype. We also found dysregulation of amino acid metabolism related to valine, tyrosine, and phenylalanine in the overweight/obesity group compared with the lean group.

An analysis of classic clinical measures showed similar profiles between individuals with NWO and lean individuals for lipid levels, insulin resistance (via HOMA-IR), and blood pressure. Whereas other studies have shown elevated clinical measures in individuals with NWO compared with lean individuals (7,28), we did not find those distinctions in this cohort. Only triglyceride concentrations were similar between individuals with NWO and overweight/obesity. All other clinical variables were comparable between the NWO and lean groups. The combined results of the clinical measures and HRM in individuals with NWO show that HRM may be a more sensitive measure to detect metabolism-related dysfunction prior to altered clinical measures in middle-aged adults.

Linoleic acid is an essential omega-6 polyunsaturated fatty acid, and its effects on cardiometabolic health have been debated (29,30). In our study, linoleic acid metabolism was significantly upregulated in the NWO and

overweight/obesity groups compared with the lean group, indicating disruption of this metabolic pathway with elevated adiposity. Additional studies have shown increased total linoleic acid levels in participants with BMI > 30 (31), whereas others have reported decreased levels of linoleic acid but increased levels of linoleic acid-related metabolites (32,33). Because it can be converted to arachidonic acid, linoleic acid has been suggested to promote proinflammatory pathways (34,35). Evidence has suggested that individuals with obesity may have a greater proinflammatory response to linoleic acid consumption compared with lean individuals (34,36). Previous studies have shown that individuals with NWO have increased circulating proinflammatory biomarkers (7). Through their actions on peroxisome proliferator-activated receptor gamma activation (37), the oxidized linoleic acid metabolites 9-HODE and 13-HODE may promote both inflammation and adipocyte differentiation (34). In addition, through competition with the shared Δ6 desaturase enzyme, a high intake of linoleic acid may blunt the anti-inflammatory effects of α-linolenic acid (a precursor to docosahexaenoic acid and eicosapentaenoic acid) (34,38). In aggregate, upregulated linoleic acid metabolism may be indicative of increased inflammation in settings of excess adiposity.

Previous studies have reported elevated amino acid concentrations in individuals with obesity. Our findings show similarly increased levels of amino acids and related metabolites, including histidine, in individuals with NWO compared with individuals classified as lean. Studies using principal component analyses to investigate relationships between cardiometabolic health and the plasma metabolome have identified histidine as a significantly associated metabolite (39,40), although others have found a negative association with, or no relationship between, histidine, BMI, and obesity (9,31,41). Metabolites that are enriched within histidine and β-alanine overlap with glutamate metabolism and may represent anaplerotic substrates (31). Lysine metabolism was upregulated in the overweight/obesity

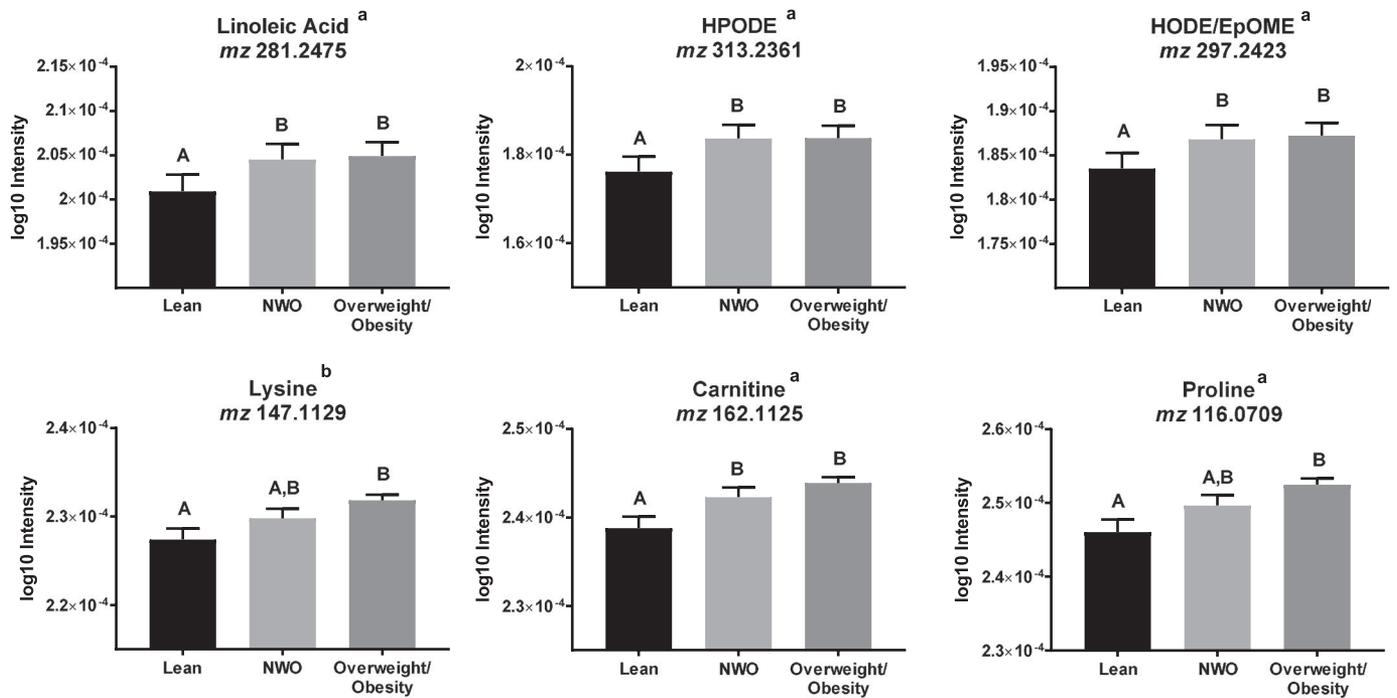


**Figure 1** (A) Manhattan plot of metabolites significantly different between body composition subtypes. There were 1,533 metabolic features that were significant at  $P < 0.05$  (grey open circles) and 222 metabolic features that were significant at a false discovery rate (FDR) of  $q < 0.2$  (black triangles). (B) Pathway enrichment analysis of the 222 metabolites significantly associated with the body composition subtypes at an FDR of  $q < 0.2$ . Numbers in parentheses denote the number of significant metabolic features (numerator) out of the number of detected metabolic features within that pathway (denominator). *m/z*, mass-to-charge.

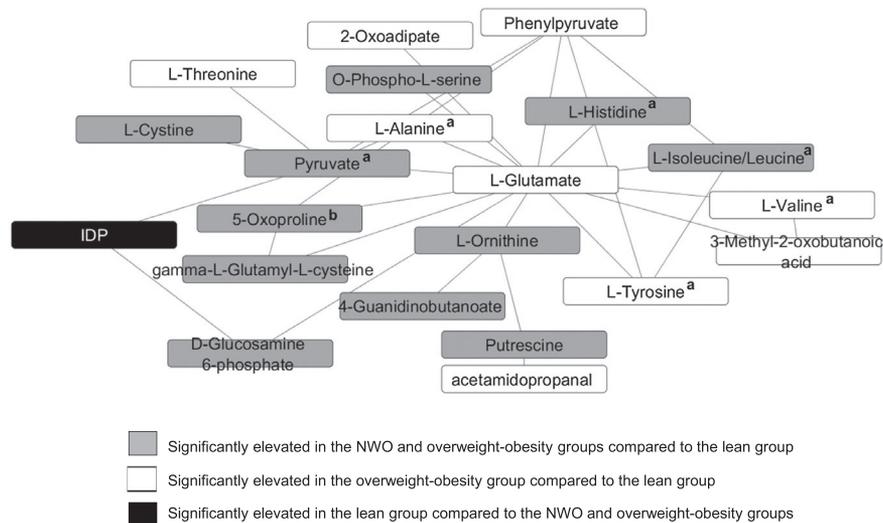
group. Lysine is an essential amino acid that is needed to synthesize carnitine for fatty acid transport into the mitochondria for oxidation. Both carnitine and lysine levels were shown to be elevated in obesity (41), and here we report higher levels of carnitine in participants with NWO and overweight/obesity compared with lean participants. Acylcarnitine levels, especially C3 and C5 acylcarnitine levels (42), have been found to be elevated in obesity, perhaps as a result of incompletely oxidized BCAAs. Finally, pathways related to nitrogenous waste excretion and aspartate/asparagine metabolism were dysregulated in the NWO and overweight/obesity groups compared with the lean group, in line with other obesity and cardiometabolic disease research (43). Our findings of altered amino acid metabolism are in accordance with published reports regarding obesity pathophysiology, and they represent new findings for individuals with NWO.

Similar to previous obesity-related research (42,44), we found dysregulation of BCAAs, AAAs, and related metabolites, which was associated

with greater adiposity in the module analysis. There is now a well-established metabolic signature of obesity, including elevated BCAA and AAA concentrations (particularly tyrosine and phenylalanine), related to insulin resistance, mitochondrial oxidative capacity overload (42), and, ultimately, increased risk of developing type 2 diabetes (44). The altered flux of BCAA catabolism exceeds mitochondrial oxidative capacity and ultimately leads to the release of BCAAs into the blood (42). The increase in AAA levels may be due to competition for the same cellular transport protein used by large neutral amino acids. Elevated levels of glutamate, alanine, and pyruvate in individuals with obesity, which we also show in participants with NWO, may also be linked to altered BCAA metabolism and overload of the Krebs cycle (42). Glutamate is produced in the first step of BCAA catabolism, and increased concentrations of glutamate may shift pyruvate toward conversion to alanine (42). In summary, we found altered BCAA and AAA metabolism in participants with NWO and overweight/obesity, which may reflect the underlying pathophysiology of insulin resistance and mitochondrial energy metabolism overload.



**Figure 2** Representative metabolites within significantly enriched metabolic pathways. All metabolites were matched by an M+H adduct in positive electrospray ionization mode. Results of Tukey post hoc analyses are denoted by capital letters. Values not connected by the same letter are significantly different at  $P < 0.05$ . <sup>a</sup>Findings were confirmed in post hoc analyses with further adjustment for  $VO_{2max}$  and in a subset of the cohort ( $n=86$ ) in which participants were categorized as having NWO or overweight/obesity and were matched by age, race/ethnicity, and sex. <sup>b</sup>Following further adjustment for  $VO_{2max}$ , metabolite levels were similar between all three groups. EpOME, epoxy-octadecenoic acid (a peroxidation product of linoleic acid); HODE, hydroxyoctadecadienoic acid (a derivative of linoleic acid); HPODE, hydroperoxy-octadecadienoic acid (intermediate of linoleic acid metabolism and precursor for the oxidized metabolite octadecadienoic acid); *mz*, mass-to-charge; M+H, protonated adduct; NWO, normal weight obesity.



**Figure 3** Module analysis of correlated metabolic features that were significantly associated with the three body composition subtypes. Results of Tukey post hoc analyses are denoted by box color and significantly different at  $P < 0.05$ . <sup>a</sup>Findings were confirmed in post hoc analyses with further adjustment for  $VO_{2max}$  and in a subset of the cohort ( $n=86$ ) in which participants were categorized as having NWO or overweight/obesity and matched by age, race/ethnicity, and sex. <sup>b</sup>Following further adjustment for  $VO_{2max}$ , metabolite levels were significantly elevated in the overweight/obesity group compared with the lean group. IDP, inosine diphosphate.

In this study, individuals with NWO had significantly lower lean body mass compared with those in the lean and overweight/obesity groups, and individuals with NWO had significantly higher visceral adipose tissue compared with lean individuals. Furthermore, individuals with NWO and overweight/obesity had significantly lower fitness levels compared with lean individuals. Relevant to our metabolomics findings, resistance and aerobic training in insulin-resistant adults with overweight showed reductions in the whole-plasma molar sum of the BCAAs and improved clearance of acyl groups (45). Thus, the plasma metabolomic differences observed between individuals with NWO and lean individuals may reflect a combination of differences in body composition and fitness, although further adjustment for  $VO_{2max}$  did not alter our main findings. While physical fitness is important for metabolic health, differences described here were more likely due to differences in body composition subtypes or in other variables that were not assessed.

To our knowledge, this is the first study to examine the plasma metabolomic profiles of individuals with NWO, and it fills an important gap in knowledge about this population. This novel approach allowed for the comparison of detailed health profiles between groups beyond classic clinical laboratory assessments. Furthermore, the use of pathway enrichment analysis provides context to associations of disease with metabolic pathways instead of single metabolites. Pathway analysis also provides the advantage of being downstream from genetic changes and allows insight into products of genetic or epigenetic alterations. A limitation of the study was its cross-sectional nature, which impedes our ability to infer causality in the results. Health status, education, and income were collected by self-report and therefore may be subject to recall bias. This cohort was predominantly composed of individuals who reported a high education and income, which may not be reflective of the general US population. Our power to determine differences in outcomes between groups might have been limited by small numbers. For example, several metabolites in participants with NWO had intermediate values that were between the metabolite values in lean participants and participants with overweight/obesity but were not statistically significantly different. This might have been due to small numbers between groups or heterogeneity in the metabolic health of individuals with NWO. Finally, there are no established cut points to define obesity based on body fat percentage, and applying another threshold to define obesity in this population might have yielded different results.

This study reports novel findings in this adult population that individuals with NWO have altered metabolomic profiles, denoting underlying metabolic dysfunction similar to individuals with overweight/obesity, despite having normal BMI and generally normal clinical biomarkers. Specifically, linoleic acid and amino acid pathways were dysregulated in the NWO and overweight/obesity subtypes compared with the lean subtype. Thus, the plasma metabolome may be a useful measure of health status to detect perturbations that predict early metabolic changes. Larger, prospective studies are needed to determine whether HRM can identify normal weight individuals at risk for obesity-related diseases and whether targeted interventions in individuals with NWO can reduce such risks. **O**

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