Clinical Research Article

Metabolomic Profiling After a Meal Shows Greater Changes and Lower Metabolic Flexibility in Cardiometabolic Diseases

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Abbreviations: BCAA, branched-chain amino acid; BMI, body mass index; CMD, cardiometabolic disease; FDR, false discovery rate; HILIC, hydrophilic interaction liquid chromatography; INCAP, Institute of Nutrition of Central America and Panama; IQR, interquartile range; LC-FT-MS, liquid chromatography–Fourier transform mass spectrometry; m/z, mass-to-charge ratio; MSI, Metabolomics Standards Initiative; OPLS-DA, orthogonal partial least squares–discriminant analysis; PLS-DA, partial least squares–discriminant analysis; RT, retention time.

Abstract

Context: Metabolic flexibility is the physiologic acclimatization to differing energy availability and requirement states. Effectively maintaining metabolic flexibility remains challenging, particularly since metabolic dysregulations in meal consumption during cardiometabolic disease (CMD) pathophysiology are incompletely understood.

Objective: We compared metabolic flexibility following consumption of a standardized meal challenge among adults with or without CMDs.

Design, Setting, and Participants: Study participants (n = 349; age 37-54 years, 55% female) received a standardized meal challenge (520 kcal, 67.4 g carbohydrates, 24.3 g fat, 8.0 g protein; 259 mL). Blood samples were collected at baseline and 2 hours postchallenge. Plasma samples were assayed by high-resolution, nontargeted metabolomics with dual-column liquid chromatography and ultrahigh-resolution mass spectrometry. Metabolome-wide associations between features and meal challenge timepoint were assessed in multivariable linear regression models.

Results: Sixty-five percent of participants had ≥1 of 4 CMDs: 33% were obese, 6% had diabetes, 39% had hypertension, and 50% had metabolic syndrome. Log$_2$-normalized ratios of feature peak areas (postprandial:fasting) clustered separately among participants with

versus without any CMDs. Among participants with CMDs, the meal challenge altered 1756 feature peak areas (1063 reversed-phase [C18], 693 hydrophilic interaction liquid chromatography [HILIC]; all q < 0.05). In individuals without CMDs, the meal challenge changed 1383 feature peak areas (875 C18; 508 HILIC; all q < 0.05). There were 108 features (60 C18; 48 HILIC) that differed by the meal challenge and CMD status, including dipeptides, carnitines, glycerophospholipids, and a bile acid metabolite (all *P* < 0.05).

Conclusions: Among adults with CMDs, more metabolomic features differed after a meal challenge, which reflected lower metabolic flexibility relative to individuals without CMDs.

Key Words: cardiometabolic disease, metabolomics, metabolic health, meal challenge, postprandial state

Cardiometabolic diseases (CMDs) and other noncommunicable diseases account for over 70% of deaths globally [1]. More effectively reducing the disease burden from CMDs remains a major challenge [1]. One critical research gap is how to more effectively maintain cardiometabolic health, including metabolic flexibility in daily physiological processes, across the life course [2, 3]. Metabolic flexibility is broadly defined as the dynamic acclimatization that occurs to maintain energy homeostasis throughout heterogeneous physiologic needs and conditions [2, 3]. Healthy systems are considered those with metabolic resilience—the ability to effectively regulate—in response to differing energy states [4].

Impaired metabolic flexibility has been hypothesized to influence CMD pathophysiology [2, 3, 5]; however, specific contributions remain incompletely understood. One challenge is the lack of a single, standard definition of metabolic flexibility, particularly in light of numerous interrelated physiological processes involved in maintaining energy homeostasis [2, 3, 6]. Previous studies have begun characterizing different aspects of reduced metabolic flexibility, including the decreased ability to sense nutrients, maintain energy homeostasis, and shift between macronutrient fuels [6]. Few studies have evaluated activity metabolomics in response to meal consumption among people with versus without CMDs.

In this study, we defined metabolic flexibility as the changes in metabolomic profiles following a physiologic meal challenge, relative to while fasting. High-resolution, nontargeted metabolomic profiling provides greater granularity and metabolite coverage relative to standard diagnostic biomarkers [5, 7-10]. Our study objective was to compare the metabolomic profile response following a standardized meal challenge of adults with CMDs, relative to metabolically healthy participants. We hypothesized that: (i) there would be increased changes in metabolomic features after the meal challenge among people with CMDs, compared to those without CMDs; and (ii) metabolomic features in major energy, macronutrient, and bile acid pathways would be responsive in the meal challenge and differ by CMD status.

1. Materials and Methods

Study participants

We studied adult participants of the Institute of Nutrition of Central America and Panama (INCAP) Nutritional Supplementation Trial Longitudinal Study [11, 12]. The present analysis focuses on participants enrolled in the follow-up wave conducted from 2015 to 2017 [13].

Meal challenge

After an overnight fast, participants visited the research clinic in the morning. Baseline venous blood samples were drawn after confirming fasting. Individuals were excluded from the meal challenge if their fasting blood glucose was ≥180 mg/dL or they self-reported having diabetes. Study participants then received a standardized beverage, which was comprised of Incaparina (a vegetable protein mixture developed by INCAP), skim milk (lactose-free), safflower oil, and sugar. Each portion (259 mL) contained 520.0 kcal, 8.0 g protein, 24.3 g fat, and 67.4 g carbohydrate (Supplemental Table 1) [14]. This composition was intended to reflect a physiologic meal consisting of relatively higher fat and higher sugar content. Many geographic settings undergoing the nutrition transition have greater availability of higher fat and higher sugar-containing diets, which have been hypothesized to contribute to CMDs [15]. Hyperglycemia and hyperlipidemia are components of diabetes and metabolic syndrome, and often co-occur with obesity and hypertension [16-19].

Data collection

Trained phlebotomists collected venous blood samples at baseline (fasting) and 120 minutes after the meal challenge (postprandial). Plasma samples for metabolomic analysis

were collected in heparin tubes. Samples were maintained on ice prior to centrifugation at 3000 revolutions per minute for 10 minutes, and subsequently at –80 °C until assay. Blood samples were assayed for plasma glucose, insulin, and lipids (further details regarding assay methodology and instruments in Supplemental Table 2) [14].

Trained study staff interviewed study participants regarding sociodemographic and clinical information, including use of medications [13]. Standard protocols were used to obtain anthropometric measurements [13].

Random selection of analysis subset

From the 1139 individuals who participated in the 2015-2017 data collection wave, we sampled a random subset (n = 401; Supplemental Fig. 1) [14]. Exclusion criteria included: (i) pregnancy or lactation (n = 4) or missing key clinical variables (n = 4); (ii) incomplete metabolomic data (<2 timepoints of blood samples; n = 44 for reversed-phase [C18], n = 47 for hydrophilic interaction liquid chromatography [HILIC]); and (iii) failed quality check between technical replicates (n = 0 for C18; n = 3 for HILIC). After these exclusions, 349 participants with C18 data and 343 participants with HILIC data were included in the analysis.

High-resolution metabolomics

Pairs of plasma samples (fasting, postprandial state) were randomly ordered for nontargeted metabolomic assay. Each plasma sample (fasting or postprandial state) was assayed in triplicate for high-resolution metabolomic data. After frozen samples were thawed, acetonitrile (2:1, v/v; HPLC grade; Millipore, MA, USA; Sigma Aldrich, MO, USA) was added to precipitate protein [20]. Samples were maintained on ice for 30 minutes, centrifuged (14 000g for 10 minutes at 4 °C), and the supernatant was stored at 4 °C in a refrigerated autosampler prior to assay [20, 21].

The following commercially available, stable isotope-labeled internal standards were included: [$^{13}C_6$]-D-glucose, [^{15}N]-indole, [2-^{15}N]-L-lysine dihydrochloride, [$^{13}C_5$]-L-glutamic acid, [$^{13}C_7$]-benzoic acid, [3,4-$^{13}C_2$]-cholesterol, [^{15}N]-L-tyrosine, [trimethyl-$^{13}C_3$]-caffeine, [$^{15}N_2$]-uracil, [3,3-$^{13}C_2$]-cystine, [1,2-$^{13}C_2$]-palmitic acid, [$^{15}N,^{13}C_5$]-L-methionine, [^{15}N]-choline chloride, and 2'-deoxyguanosine-$^{15}N_2,^{13}C_{10}$-5'-monophosphate [20, 21]. Human reference plasma from the National Institute of Standards and Technology (standard reference material 1950) and a pooled reference plasma (Q-std3) prepared from commercial human plasma samples (Equitech Bio, TX, USA) were included for quality control [22]. Q-std3 was included

before and after every 20 samples; specifically, triplicates of 2 Q-Std3 samples were included as the beginning, middle, or end of every batch of 40 samples.

Liquid chromatography mass spectrometry

Plasma samples were assayed by liquid chromatography–Fourier transform mass spectrometry (LC-FT-MS) with 2 chromatographic columns: C18 (Higgins Analytical, Targa, 2.1 × 50 mm) with negative electrospray ionization, and HILIC (Waters BEH Amide 2.1 × 50 mm) with positive electrospray ionization [21, 23]. A Switchos control valve (LC Packings) allowed for alternation between the 2 columns [21]. Data acquisition occurred by mass spectrometer (Orbitrap Fusion mass spectrometer; Thermo Fisher Scientific, MA, USA) with specifications as previously described [21]. The scan range for the detection of mass-to-charge ratio (m/z) scan was between 85 and 1250, and mass resolution was 120 000.

Data extraction

Raw data (.raw files) were collected throughout the chromatographic separation and converted to .cdf files (Xcalibur software; Thermo Fisher Scientific, CA, US). apLCMS [24] and xMSanalyzer [25] were used for the extraction and initial preprocessing of chromatographic data. Preprocessing included noise reduction, peak identification, retention time correction, peak alignment, feature quantification, weak signal detection, and batch effect adjustment with ComBat [24-26]. A feature was defined as a unique combination of m/z and retention time. The ion intensity (peak area) of each feature was integrated.

Standard operating procedures and quality control were based on prior studies, including via xMSanalyzer [25]. For each sample, pairwise Pearson correlations between replicates were assessed and averaged. Prior to exclusions of participants and data filtering of features (Supplemental Fig. 1 [14]), the median averaged pairwise Pearson correlation between replicates was 98.7% (interquartile range [IQR], 97.0%, 99.5%) across samples from C18 data, and 99.5% (IQR, 98.6%, 99.8%) across all samples from HILIC data. Samples (n = 0 for C18; n = 3 for HILIC) with mean Pearson correlation coefficients <0.75 across technical replicates were excluded.

Features observed in <80% of study participant samples in each column (C18, HILIC) were filtered [27]. Any feature peak area values that were missing or zero were subsequently assigned as half of the lowest observed value (limit of detection) of the feature peak area across all samples for each column.

Definitions

CMD definitions were based on guidelines of the World Health Organization, American Diabetes Association, American College of Cardiology, American Heart Association, and National Cholesterol Education Program (Adult Treatment Panel III report; Supplemental Table 2) [14, 16-19]. Participants were characterized as being metabolically unhealthy if they had any of 4 CMDs (obesity, hypertension, diabetes, metabolic syndrome).

Statistical analysis

Statistical analysis was conducted utilizing R (version 3.5.1; R Foundation for Statistical Computing; Vienna, Austria) and SAS (version 9.4; SAS Institute Inc.; Cary, NC, US). We used a complete case approach (Supplemental Fig. 1) [14]. Prior to analysis, we converted feature intensities (peak areas) to $\log_2$-normalized values. For each feature, we calculated the peak area ratio as the postprandial timepoint divided by the fasting timepoint. Statistical significance was based on 2-sided hypothesis tests, and α value <0.05. After feature-by-feature multivariable regressions with metabolomic data, the *P* values were collectively adjusted for a false discovery rate (FDR), which controls the proportion of selected features that incorrectly reject the null hypothesis. FDR-adjusted $q < 0.05$ was considered significant for Stages 1 and 2A. In regressions with interaction terms (Stages 2B), $P < 0.05$ was considered significant.

Descriptive analysis and visualizations

Continuous variables were reported as mean (standard deviation) or median (IQR), and categorical variables were reported as n (%). Subgroups were compared by Kruskal-Wallis, Wilcoxon rank-sum, or Mantel-Haenszel chi-square test statistics. Correlations between $\log_2$-normalized feature peak areas and their ratios (postprandial/fasting), and CMD biomarkers were evaluated by Spearman rank correlation coefficients. We compared differences of $\log_2$-normalized feature peak area ratios between participants with versus without any CMDs with MetaboAnalystR [28]. As examples, we visualized the clustering of feature peak area ratios with unsupervised dimensionality reduction (principal components analysis) and supervised discriminant analysis approaches (eg, partial least squares–discriminant analysis [PLS-DA], orthogonal partial least squares–discriminant analysis [OPLS-DA]) [28].

Feature selection approach via regressions

We used a 2-stage feature selection approach based on multivariable linear mixed regressions with repeated measurements (Supplemental Fig. 2) [14]. In Stage 1, we assessed whether the peak areas of each feature differed by the meal challenge timepoint, adjusting for age and sex. The model equation was:

$$Y \text{ } (\log_2\text{-normalized feature peak area})_{i,j,t}$$
$$= \beta_{0i,j,t} + \beta_1 X_1 \text{ (timepoint [postprandial, fasting])}_{i,j,t}$$
$$+ \beta_2 X_2 \text{ (age at study visit)}_i + \beta_3 X_3 \text{ (sex)}_i$$
$$\text{where study participant} = i, \text{ feature} = j, \text{ and timepoint} = t.$$

Features with beta coefficients of the meal challenge (β_1) with FDR-adjusted $q < 0.05$ were annotated, considered in functional pathway analysis, and eligible for Stage 2. In Stage 2A, we assessed whether feature peak areas responded to the meal challenge, accounting for age and sex, among participants either with or without CMDs. The same Stage 1 regression equation was used among these 2 stratified groups of participants. In Stage 2B, we additionally included CMD status and the 2-way multiplicative interaction term between the meal challenge timepoint (postprandial, fasting) as independent variables in the Stage 1 regressions. The Stage 2B regression equation was:

$$Y \text{ } (\log_2\text{-normalized feature peak area})_{i,j,t}$$
$$= \beta_{0i,j,t} + \beta_1 X_1 \text{ (timepoint [postprandial, fasting])}_{i,j,t}$$
$$+ \beta_2 X_2 \text{ (any CMD)}_i + \beta_3 X_3 \text{ (age at study visit)}_i$$
$$+ \beta_4 X_4 \text{ (sex)}_i + \beta_5 X_5 \text{ (timepoint*anyCMD)}_i$$
$$\text{where study participant} = i, \text{ feature} = j, \text{ timepoint} = t.$$

Features with beta coefficients of the interaction term (β_5) with $P < 0.05$ were annotated and considered in functional pathways.

Feature annotations

We used annotations from xMSannotator, which considers multiple criteria in its algorithm [29] and incorporates Human Metabolome Database reference database information [30] for putative identification of features (Supplemental Fig. 3) [14]. We considered feature annotation confidence and assigned identification confidence scores to some features, based on the 5-level system proposed by the Metabolomics Standards Initiative (MSI; Supplemental Fig. 3) [14, 31]. Annotations of select metabolites in Stage 2B regressions were compared to identities by co-elution and MS/MS fragmentation patterns relative to authentic standards.

Ethical conduct of research

The Institutional Review Boards at Emory University (Atlanta, Georgia, US) and INCAP (Guatemala City, Guatemala) approved the study protocol. All participants provided their written informed consent.

2. Results

Sociodemographic and cardiometabolic health characteristics of the 349 study participants are in Table 1. Briefly, among study participants (37-54 years of age), 116 (33%) had body mass index (BMI) considered obese, 22 (6%) had diabetes, 135 (39%) had hypertension, and 173 (50%) had metabolic syndrome (Table 2). One hundred twenty-three adults were considered metabolically healthy insofar as they had none of these 4 CMDs (35%; Table 2). A lower proportion of men (51%) had at least 1 CMD compared with women (76%; $P < 0.01$; Table 2). Relative to men, greater proportions of women had obesity (43% vs 21%), metabolic syndrome (65% vs 31 %), and any CMD (76% vs 51%; all $P < 0.01$; Table 2). Several CMD indicators (postprandial glucose, glycated hemoglobin, cholesterol [total, HDL, non-HDL]) differed between men and women (all $P < 0.01$; Table 2).

We observed 9849 C18 and 13 908 HILIC metabolomic features in total (Supplemental Fig. 1) [14]. After data filtering, 5085 C18 and 7444 HILIC features remained eligible for the feature selection process, (Supplemental Fig. 1) [14]. Among all participants, 2090 features (C18: 1288; HILIC: 802) had peak areas that were altered following the meal challenge, compared to fasting (all q < 0.05; Table 3).

Among these features, 1180 (56.5%) had putative annotations (49.2% C18; 68.1% HILIC, Table 3).

Comparing metabolomic response to meal challenge among participants with versus without CMDs

Among participants with CMDs (n = 226), 1063 C18 and 693 HILIC features had differential peak areas pre– versus post–meal challenge (all q < 0.05; Fig. 1A; Table 3). Of these C18 features, 747 increased and 316 decreased after the meal challenge. Among the HILIC features, 400 increased and 293 decreased following the meal challenge.

In individuals without the 4 CMDs (n = 123), 875 C18 and 508 HILIC features had peak areas that differed after the meal challenge (all q < 0.05; Fig. 1A; Table 3). Of these C18 features, 613 increased and 262 decreased after the meal challenge. Among these HILIC features that were responsive to the meal challenge, 325 features increased and 183 decreased.

Subtracting the overlapping differential features observed in both groups, 658 feature peak areas (369 C18; 289 HILIC) were altered following the meal challenge only among individuals with CMDs (Fig. 1A). In participants

Table 1. Sociodemographic and Clinical Characteristics of Adult Study Participants (n = 349)

	n	Overall 349	n	Males 157	n	Females 192	P^a
		Median (IQR)		Median (IQR)		Median (IQR)	
Sociodemographics							
Age at follow-up (years)[b]	349	44.0 (40.0, 47.0)	157	44.0 (40.0, 47.0)	192	44.0 (41.0, 47.5)	0.79
Anthropometry							
BMI (kg/m^2)	349	27.8 (24.8, 31.1)	157	26.5 (24.3, 29.4)	192	28.9 (25.9, 32.4)	<0.01
Biochemical indicators							
Glucose profile							
Fasting blood glucose (mg/dL)	349	98.6 (93.1, 105.2)	157	97.5 (93.2, 104.0)	192	99.3 (93.0, 105.6)	0.65
Postprandial glucose (mg/dL)	349	109.3 (95.6, 123.6)	157	100.7 (90.8, 116.4)	192	116.6 (102.9, 128.4)	<0.01
Glycated hemoglobin (%)	348	5.8 (5.5, 6.0)	157	5.7 (5.5, 5.9)	191	5.8 (5.6, 6.0)	<0.01
Lipid profile							
Triglycerides (mg/dL)	349	144.0 (100.0, 207.0)	157	142.0 (98.0, 213.0)	192	144.5 (101.5, 197.0)	0.89
Total cholesterol (mg/dL)	349	175.0 (151.0, 198.0)	157	167.0 (143.0, 189.0)	192	182.0 (158.0, 208.0)	<0.01
HDL-cholesterol (mg/dL)	349	40.7 (36.3, 46.8)	157	37.8 (33.7, 44.6)	192	42.5 (37.9, 48.9)	<0.01
Non-HDL-cholesterol (mg/dL)[c]	349	132.1 (110.7, 154.3)	157	124.4 (104.0, 149.6)	192	136.9 (116.7, 159.1)	<0.01
Clinical							
Systolic blood pressure (mm Hg)	349	122.0 (113.5, 132.0)	157	123.0 (114.5, 132.0)	192	120.5 (112.0, 131.8)	0.24
Diastolic blood pressure (mm Hg)	349	73.5 (67.5, 80.5)	157	73.5 (67.5, 79.5)	192	73.3 (67.5, 80.5)	0.60

Data values are either median (IQR) or n (%). Among study participants with available metabolomic data at both timepoints and key variables of interest (CMDs, Atole exposure).

Abbreviations: BMI, body mass index; CMDs, cardiometabolic diseases; HDL, high-density lipoprotein; IQR, interquartile range.

[a]P values based on Wilcoxon rank-sum tests.

[b]At study visit date (of biological sample collection) in 2015–2017 data collection.

[c]Non-HDL-cholesterol (mg/dL) calculated as the difference between total (mg/dL) and HDL-cholesterol (mg/dL) plasma concentrations.

Table 2. CMDs Among Guatemalan Adults (n = 349)

	Overall	Males	Females	P^a
	N = 349	n = 157	n = 192	
	n (%)	n (%)	n (%)	
CMDs				
Obesity[b]	116 (33%)	33 (21%)	83 (43%)	<0.01
Diabetes[c]	22 (6%)	8 (5%)	14 (7%)	0.40
Pre-diabetes[c]	130 (37%)	52 (33%)	78 (41%)	0.15
Hypertension[d]	135 (39%)	59 (38%)	76 (40%)	0.70
Pre-hypertension[d]	67 (19%)	36 (23%)	31 (16%)	0.11
Metabolic syndrome[e]	173 (50%)	48 (31%)	125 (65%)	<0.01
Central obesity[e]	210 (60%)	35 (22%)	175 (91%)	<0.01
High fasting blood glucose or medication use[e]	151 (43%)	60 (38%)	91 (47%)	0.09
High triglycerides or statin use[e]	167 (48%)	76 (48%)	91 (47%)	0.85
Low HDL-cholesterol[e]	241 (69%)	91 (58%)	150 (78%)	<0.01
High blood pressure or medication use[e]	113 (32%)	48 (31%)	65 (34%)	0.52
Any CMD[f]	226 (65%)	80 (51%)	146 (76%)	<0.01

Data values are either median (IQR) or n (%). Among study participants with available metabolomic data at both timepoints and key variables of interest (CMDs, Atole exposure).

Abbreviations: BMI, body mass index; CMDs, cardiometabolic diseases; HDL, high-density lipoprotein; IQR, interquartile range.

[a] P values based on Mantel-Haenszel chi-square tests.

[b] According to World Health Organization categorization [16].

[c] Defined by recommendations of the American Diabetes Association [17] (See Supplemental Table 2 [14]).

[d] Per hypertension diagnosis cutoff values from the 2017 American College of Cardiology/American Heart Association Guideline for the Prevention, Detection, Evaluation, and Management of High Blood Pressure in Adults [18] (See Supplemental Table 2 [14]).

[e] Metabolic syndrome was defined as having 3 or more of the 5 criteria, based on the National Cholesterol Education Program guidelines [19] (See Supplemental Table 2 [14]).

[f] Metabolically unhealthy defined as having any of the 4 assessed CMDs. In other words, presence of obesity, hypertension, diabetes, and/or metabolic syndrome, including comorbidities.

without CMDs, 285 unique features (181 C18; 104 HILIC) changed in the meal challenge and were not observed in the CMD subgroup (Fig. 1A). Log$_2$-normalized postprandial:baseline peak area ratios clustered separately among metabolically healthy versus unhealthy participants, based on OPLS-DA (Fig. 1B) and PLS-DA (Supplemental Fig. 4) [14].

We also assessed whether the response of each metabolomic feature peak area to the meal challenge varied by CMD status by examining the 2-way multiplicative interaction between CMD status and the meal challenge timepoint. A total of 108 feature peak areas (60

Table 3. Summary of Metabolomic Features Differing by Meal Challenge and CMD Status[a]

Differing features (#)	Meal Challenge		Any CMD	
LC-FT-MS columns	C18	HILIC	C18	HILIC
n[b,c]	349	343	349	343
Feature selection				
Stage 1: Overall linear regression[d]				
q < 0.05 [e]	1288	802	---	---
Stage 2A: Stratified linear regression[f,g]				
CMDs subgroup[g]				
q < 0.05[e]	---	---	1063	693
No CMDs subgroup[g]				
q < 0.05[e]	---	---	875	508
Stage 2B: Overall linear regression[g,h]				
Interaction term (subgroup × time)[g]				
P < 0.05[e]	---	---	60	48
q < 0.05	---	---	0	0
Annotations	Stage 1		Stage 2B	
Annotated features	634	546	27	32
Total annotations	3406	5238	153	281

Abbreviations: CMD, cardiometabolic disease; FDR, false discovery rate; HILIC, hydrophilic interaction liquid chromatography; LC-FT-MS, liquid chromatography–Fourier transform mass spectrometry.

[a] Values in this table indicate the number of features with log$_2$-normalized peak areas, which differed by the key subgroups of interest (meal challenge, any CMDs).

[b] The total features observed were 9849 (C18) and 13 908 (HILIC). After data filtering, 5085 (C18) and 7444 (HILIC) features remained eligible for the feature selection approach. A complete case approach was utilized in multivariable regressions.

[c] All regressions with C18 data were available among 349 participants with 2 samples (fasting, postprandial) of metabolomic data and key variables of interest (utilized to define CMDs). HILIC regressions were among 343 participants, based on data availability of key variables.

[d] Stage 1 feature selection was based on multivariable regressions. For each feature, a linear model with repeated measurements was utilized (Proc Mixed in SAS); the model equation was: Y (log$_2$-normalized feature peak area)$_{i,j,t}$ = $\beta_{0i,j,t}$ + $\beta_1 X_1$ (timepoint [postprandial, baseline]) $_{i,j,t}$ + $\beta_2 X_2$ (age at study visit)$_i$ + $\beta_3 X_3$ (sex)$_i$, where each study participant was denoted as $_i$, feature was $_j$, and timepoint was $_t$. Features remained eligible for Stage 2 feature selection if the beta-coefficient of the meal challenge timepoint (β_1) had FDR-adjusted P-value (q) < 0.05.

[e] These features were subsequently eligible for visualizations, annotations, and pathway analysis.

[f] Stage 2A feature selection utilized the same regression equation as in Stage 1, except these sets of regressions were stratified by CMD status (any versus none). In each subgroup, the total number of features with q < 0.05 of the beta-coefficient (β_1) of the meal challenge timepoint are included in this table.

[g] Among 1288 C18 features, or among 802 HILIC features

[h] Among features eligible from Stage 1 selection, these were also considered with multivariable regressions that additionally considered subgroups of interest and their interaction terms with meal challenge as independent variables (Stage 2B). A linear model with repeated measurements was utilized (Proc Mixed in SAS); the model equation was: Y (log$_2$-normalized feature peak area)$_{i,j,t}$ = $\beta_{0i,j,t}$ + $\beta_1 X_1$ (timepoint [postprandial challenge, baseline]) $_{i,j,t}$ + $\beta_2 X_2$ (any CMDs)$_i$ + $\beta_3 X_3$ (age at study visit)$_i$ + $\beta_4 X_4$ (sex)$_i$ + $\beta_5 X_5$ (anyCMDs*timepoint)$_i$ where each study participant was denoted as $_i$, feature was $_j$, and timepoint was $_t$. The number of features with P < 0.05 and q < 0.05 the beta-coefficient (β_5) of the interaction term were included in this table.

C18; 48 HILIC) were associated with this interaction term (all $P < 0.05$; Table 3). Among these features, 59 (54.6%) had putative annotations (45.0% C18; 66.7% HILIC; Table 3). Excluding features with low xMSannotator confidence scores (MSI Level 4), delta parts per million (ppm) ≤5, there were 13 features from C18 data and 9 features from HILIC data with annotations (Supplemental Table 3) [14]. The interaction term was respectively associated with increased carnitines (trans-2-dodecanoylcarnitine) and dipeptides (histidinyl-tryptophan or tryptophyl-histidine) peak areas (all $P < 0.05$; MSI Levels 2 and 3 [31]; Supplemental Table 3 [14], Table 4). The interaction term was also associated with phospholipids, specifically increased phosphatidic acid (PA) 34:2 and decreased phosphatidylcholine (PC) 38:3 peak areas (all $P < 0.05$; Supplemental Table 3 [14], Table 4). Decreased peak area of a bile acid metabolite (chenodeoxycholic acid glycine conjugate or glycoursodeoxycholic acid) was associated with

the interaction term ($P < 0.05$; Table 4). Increased 3b-17-b-dihydroxyetiocholane (a steroid hormone) and 1H-indole-3-carboxaldehyde (a microbiome-derived product) peak areas were respectively associated with the interaction term (both $P < 0.05$; MSI Levels 2 and 3 [31]; Table 4).

Correlations between $\log_2$ feature peak area ratios (postprandial/fasting) and a panel of CMD biomarkers are shown in Fig. 2. The peak area ratio of isovalerylcarnitine or valerylcarnitine (m/z 246.1697, retention time [RT] 28) was positively correlated with BMI, diastolic blood pressure, and triglycerides (all $P < 0.05$), and negatively correlated with HDL-cholesterol ($P < 0.01$). The peak area ratio of phosphatidylcholine (38:3; m/z 834.5970, RT 27) was negatively correlated with BMI and fasting plasma glucose (both $P < 0.05$). The peak area ratio of histidinyl-tryptophan or tryptophyl-histidine (m/z 346.1367, RT 60) was positively correlated with postprandial glucose ($P = 0.03$).
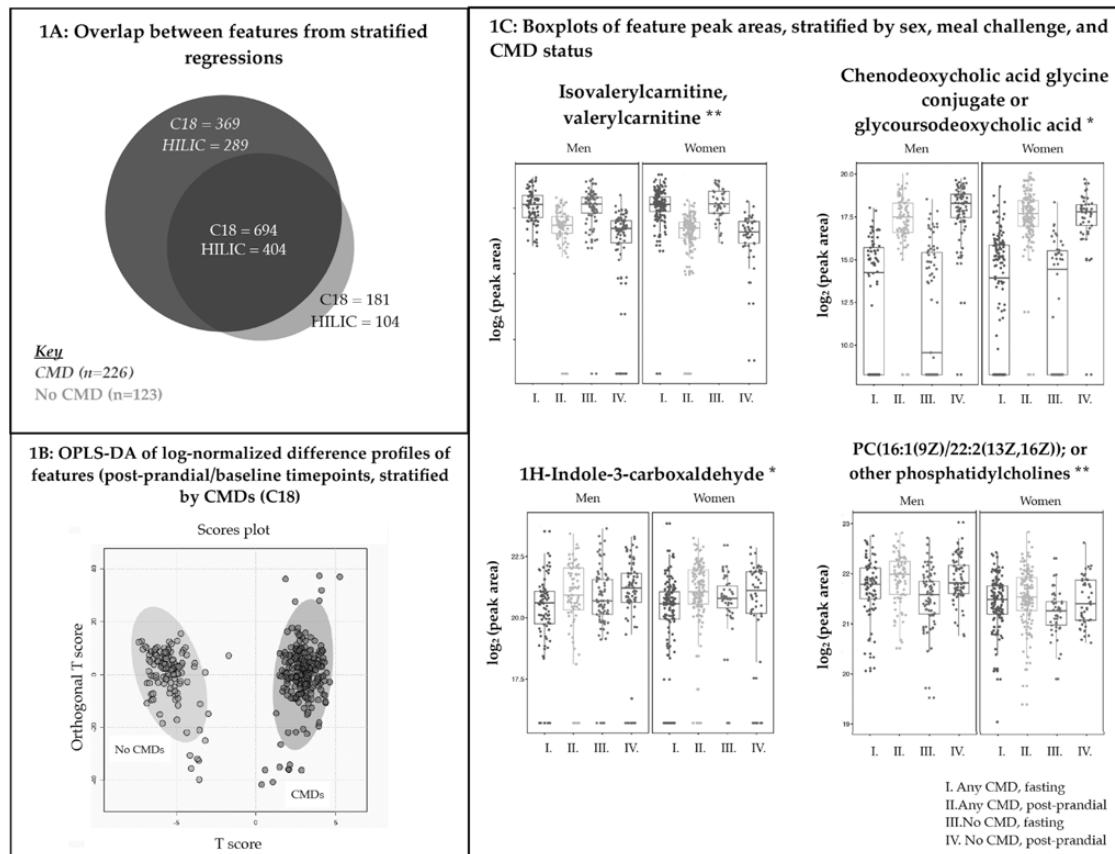
Figure 1. Metabolomic feature peak area changes following standardized meal challenge differed between metabolically healthy versus unhealthy individuals. **1A:** In this Venn diagram, each circle represents the numbers of features with beta coefficients with FDR-adjusted *P* values (q) < 0.05 in stratified regressions (Stage 2A in feature selection approach) in each subgroup (metabolically healthy or unhealthy). Circle diameters were proportionally scaled by the number of features (C18, HILIC) represented. **1B:** OPLS-DA was used to compare whether feature peak area ratios (postprandial/fasting) clustered in distinct patterns among metabolically healthy versus unhealthy participants. Each circle represents an individual (metabolically healthy—light gray, metabolic unhealthy—dark gray). Data are from LC-FT-MS (C18, negative electrospray ionization). **1C:** Examples of C18 (*) and HILIC (**) feature peak areas with putative annotations (from xMSannotator results among features selected in Stage 2B regressions with *P* < 0.05) are represented in boxplots, stratified by sex, meal challenge, and CMD status (Supplemental Table 4 [14]). Abbreviations: CMD, cardiometabolic disease; FDR, false discovery rate; HILIC, hydrophilic interaction liquid chromatography; LC-FT-MS, liquid chromatography Fourier transform mass spectrometry; OPLS-DA, orthogonal partial least squares–discriminant analysis.

3. Discussion

Our results showed that a greater number of metabolomic feature peak areas differed following a standardized meal challenge among adults with CMDs (1756 features), compared with those without CMDs (1383 features). This finding reflected reduced metabolic flexibility, or worsened ability to acclimatize to the standardized meal challenge, among adults with CMDs. Metabolomic features that differed by the meal challenge and CMD status included dipeptides, glycerophospholipids, carnitines, and a bile acid metabolite.

Metabolic flexibility in CMDs

Our finding that more features changed after a meal challenge among adults with CMDs was consistent with the increased metabolic dysregulation in CMDs that has been previously described [2-4]. Healthy metabolism has been described as having metabolic resilience, or the ability to maintain stability in energy homeostasis during dynamic changes, such as caloric availability in a postabsorptive state or deficit in a fasting state [2-4]. One hypothesis is that during CMD pathophysiology, physiologic challenges result in short-term compensatory mechanisms of the body that gradually create an "allostatic load" or an inability to adequately respond, such as abnormal accumulation of lipids [32]. Different facets of metabolic inflexibility, including dysregulation of substrate availability ("push") and requirement ("pull"), are associated with elevated or reduced biomarkers in obesity, insulin resistance, and deficiency [2, 3]. Increased triglycerides in skeletal muscle are often observed with insulin resistance and are hypothesized to be explained by abnormal muscle glucose oxidation (increased in a basal state, decreased with insulin administration) [33]. Other evidence has also shown metabolomic profiles associated with CMD status although many prior studies have been cross-sectional [5, 7-10, 34] and difficult to directly compare to our findings.

Protein metabolism in CMDs

We found that the meal challenge response of dipeptides and carnitines differed among participants with versus without CMDs. Prior evidence has corroborated that protein catabolites differed in CMDs [5, 7-10]. Murine studies have provided potential mechanisms of how catabolic protein metabolites affect systemic insulin resistance and obesity [35, 36]. Decreased relative concentrations of histidine and tryptophan were found in urine of rats with diabetes, compared with healthy controls [37]. One explanation is that large neutral amino acids, including tryptophan, phenylalanine, leucine, isoleucine, and valine, compete for cellular transport in mammalian cells [10]. In a dysregulated state, an imbalance of amino acids could result in decreased activities involving the other amino acids [10]. For example, it has been hypothesized that excess branched-chain amino acids (BCAAs) could result in decreased neurotransmitters such as serotonin, which is derived from tryptophan [10]. Other issues arising from an imbalance of amino acids include: (i) a BCAA catabolite increases trans-endothelial fatty acid transport; (ii) greater BCAAs activate the mammalian target of rapamycin (mTOR), which can cause the uncoupling of insulin signaling; and (iii) mitotoxic metabolites result in dysregulated beta-cell mitochondrial function [35, 36]. In vivo human studies have found that plasma tryptophan and histidine concentrations differed by type 2 diabetes [8, 37] and obesity [38] status.

We found that trans-2-dodecanoylcarnitine peak areas differed by CMD status and the meal challenge. There is limited previous evidence that can be directly compared, however prior studies with other study designs have highlighted the plausibility of our result. Dodecanoylcarnitine (C12) and other acylcarnitine peak intensities were increased among overweight participants after being randomly assigned a low-calorie diet for 12 weeks [39]. Several previous studies have reported the association between acylcarnitines and diabetes indicators, however the directionality of findings have been inconsistent [40-42]. One study found that lower concentrations of acylcarnitines were associated with improved diabetes indicators such as insulin sensitivity [40]. A separate study found that arachidonoyl-carnitine was associated with greater plasma glucose concentration [41]. A supplementation study among participants with impaired glucose tolerance showed that 2 g/day of L-carnitine increased acylcarnitine concentrations in skeletal muscle but had no effects on whole-body insulin sensitivity [42].

Putative mechanisms have been hypothesized to explain the observed links between acylcarnitines, obesity, and diabetes [43]. Bariatric surgery has been associated with improved type 2 diabetes and major metabolic alterations, including fatty acid metabolism [43, 44]. Medium- and long-chain fatty acids are transported as acylcarnitines across mitochondrial membranes for β-oxidation [6, 45]. Dodecanoylcarnitine and other acylcarnitines have a key role in fatty acid β-oxidation and have been hypothesized to indicate metabolic flexibility [43, 44].

Three dietary supplementation studies have shown that L-carnitine, a natural form found in red meats, improved metabolic flexibility [42, 46, 47]. A randomized, placebo-controlled, double-blind crossover trial showed that L-carnitine supplementation restored metabolic flexibility (evaluated by hyperinsulinemic-euglycemic clamp

Table 4. Associations Between Metabolomic Response to Meal Challenge and CMD Status

		Feature[a]		Monoisotopic mass	Adduct	Adjusted association with interaction term (meal challenge × any CMDs)[b]			Technical column
M/Z	RT	Annotation(s)	HMDB			β	SE	P	
671.4670	275	PA (34:2)	HMDB07860	672.4730	M-H	0.54	0.23	0.02	C18
834.5970	27	PC (38:3) [c]	HMDB08020 [c]	811.6091 [c]	M+Na [c]	-0.10	0.04	0.01	HILIC
246.1697	28	Isovalerylcarnitine, valerylcarnitine	HMDB00688, HMDB13128	245.1627	M+H	-0.19	0.06	<0.01	HILIC
343.2669	23	Trans-2-dodecenoylcarnitine	HMDB13326	---	M+H [+1]	0.60	0.29	0.03	HILIC
364.1367	60	Histidinyl-tryptophan, tryptophyl-histidine	HMDB28896, HMDB29085	341.1488	M+Na	1.80	0.79	0.02	HILIC
386.1198	57	Histidinyl-tryptophan, tryptophyl-histidine	HMDB28896, HMDB29085	341.1488	M+2Na-H	0.86	0.40	0.03	HILIC
159.0652	282	Succinylacetone	HMDB00635	158.0579	M+H	-0.19	0.09	0.04	HILIC
292.2369	232	3b,17b-dihydroxyetiocholane	HMDB00369	---	M-H [-1]	0.73	0.35	0.04	C18
450.3143	26	Chenodeoxycholic acid glycine conjugate, glycoursodeoxycholic acid	HMDB00637, HMDB00708	---	M-H [+2]	-0.87	0.42	0.04	C18
144.0454	240	1H-indole-3-carboxaldehyde	HMDB29737	145.0528	M-H	0.72	0.27	0.01	C18

Abbreviations: CMD, cardiometabolic disease; HILIC, hydrophilic interaction liquid chromatography; LC-FT-MS, liquid chromatography–Fourier transform mass spectrometry; m/z, mass-to-charge ratio; OPLS-DA, orthogonal partial least squares–discriminant analysis; PA, phosphatidic acid; PC, phosphatidylcholine; RT, retention time.

[a] In this table, features were selected in Stage 2B regressions among all participants (n = 349), based on associations with interaction terms between any CMDs and meal challenge (P < 0.05). Among annotated features, those with energy, macronutrient metabolism, excretory or bile acids, and microbiome-derived metabolites included in this table.

[b] For each feature peak area, a linear model with repeated measurements was utilized (Proc Mixed in SAS; Stage 2B regressions). The model equation was: Y (log$_2$-normalized feature peak area)$_{i,j,t}$ = β$_{0i,j,t}$ + β$_1$X$_1$ (timepoint [postprandial, baseline])$_{i,j,t}$ + β$_2$X$_2$ (any CMDs)$_i$ + β$_3$X$_3$ (age at study visit)$_i$ + β$_4$X$_4$ (sex)$_i$ + β$_5$X$_5$ (any CMDs*timepoint)$_i$ where each study participant was denoted as $_i$, feature was $_j$, and timepoint was $_t$. In this table, the beta-coefficient, SE, and P value of the interaction term in each regression were included.

[c] Details regarding other phosphatidylcholines in Supplemental Table 3 [14].

	BMI (kg/m²)	Fasting plasma glucose (mg/dL)	Postprandial plasma glucose (mg/dL)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Triglycerides (mg/dL)	HDL-cholesterol (mg/dL)
A. C18							
PA 34:2 (m/z 671.4670, RT 275)	0.13	0.15	0.06	0.01	0.05	0.07	-0.01
Glycoursodeoxycholic acid** (m/z 450.3143, RT 26)	-0.19	-0.13	-0.11	-0.11	-0.20	-0.10	<0.01
3b, 17b-dihydroxyetiocholane (m/z 292.2369, RT 232)	<0.01	0.05	0.03	0.10	0.07	0.22	-0.01
1H-indole-3-carboxaldehyde (m/z 144.0454, RT 240)	0.06	0.07	0.07	0.04	-0.05	-0.03	0.04

-0.20 -0.09 0.01 0.10 0.20

	BMI (kg/m²)	Fasting plasma glucose (mg/dL)	Postprandial plasma glucose (mg/dL)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Triglycerides (mg/dL)	HDL-cholesterol (mg/dL)
B. HILIC							
Phosphatidylserine *** (m/z 386.1198, RT 57)	-0.06	-0.05	-0.08	-0.01	-0.05	-0.07	-0.05
Histidinyl-tryptophan, tryptophyl-histidine (m/z 364.1367, RT 60)	0.09	0.04	0.11	-0.07	-0.03	0.06	0.02
Trans-2-dodecenoylcarnitine (m/z 343.2669, RT 23)	0.05	-0.01	0.04	-0.05	-0.06	-0.02	0.01
Isovalerylcarnitine, valerylcarnitine (m/z 246.1697, RT 28)	0.12	0.04	-0.05	0.08	0.13	0.25	-0.20
PC(38:3) (m/z 834.5970, RT 27)	-0.16	-0.11	-0.08	-0.03	-0.06	-0.02	-0.03
Succinylacetone (m/z 159.0652, RT 282)	-0.08	-0.01	-0.14	0.03	0.04	-0.02	0.01

-0.20 -0.09 0.02 0.10 0.20

(Left axis label: Feature peak areas ratios (post-prandial/fasting)*)

Figure 2. Correlations between CMD biomarkers and metabolomic feature peak area ratios (postprandial/fasting), stratified by column. *All values are Spearman rank correlations of each bivariate associations between a log_2-normalized feature peak area ratio (postprandial/fasting) and CMD biomarker. ** Chenodeoxyclic acid glucine conjugate was another potential annotation. *** Other potential annotations included histidinyl-tryptophan and tryptophyl-histidine. Abbreviations: m/z, mass-to-charge ratio; RT, retention time.

and high-energy meal challenge) among individuals with impaired glucose tolerance [42]. Murine studies have also confirmed the links between carnitines and metabolic flexibility [48, 49]. One study found that mice with a gene deletion of carnitine acetyltransferase had lower whole-body carbohydrate oxidation after a meal challenge [49]. L-carnitine supplementation resulted in increased average daily respiratory exchange ratio, which reflected systemic carbohydrate oxidation, among mice with obesity and insulin resistance [46].

Phospholipids in CMDs

Our findings showed that 2 glycerophospholipids (phosphatidylcholine and phosphatidic acid) were associated with the interaction between CMD status and meal challenge timepoint. Prior literature has established links between phospholipids with mitochondrial function [50], which is linked with metabolic flexibility [6]. Mitochondria have a central role in energy homeostasis at the cellular level, and the ability to alternate between converting different carbon sources (fatty acids, glucose, amino acids) to acetyl-CoA,

which is substrate for the tricarboxyclic acid cycle [6]. Phosphatidylcholines and phosphatidylethanolamines account for the majority of phospholipids in mitochondrial membranes [51]. Fasting plasma insulin was positively associated with membrane lipids (phosphatidylcholine, phosphatidylethanolamine, sphingomyelin; all $P < 0.05$) among women with obesity and no diabetes [52].

Bile acids in dysregulated metabolism

In our study, the response of bile acid metabolite (chenodeoxycholic acid glycine conjugate or glycoursodeoxycholic acid) in a meal challenge differed by CMD status. Prior studies have found associations between glycoursodeoxycholic acid and diabetes treatment (metformin) [53] as well as outcomes (gestational diabetes) [54]. Metformin upregulates conjugated bile acids such as glycoursodeoxycholic acid, which is hypothesized to explain the lower circulating cholesterol concentrations observed with metformin treatment [53].

Bile acids have nonclassical functions in regulating energy homeostasis, including glucose and lipid metabolism

[55-57]. Bile acid sequestration reduces plasma glucose and glycated hemoglobin among people with type 2 diabetes mellitus [56]. Putative mechanisms include the ability of bile acids to activate a cell surface receptor (G-protein–coupled receptor [GPCR] TGR5 [GPR131]) to improve glucose tolerance [58], and nuclear receptors (pregnane X receptor, constitutive androstane receptor, vitamin D receptor) that can subsequently affect transcriptional activities of bile acid, lipid, and glucose metabolism [59-61].

Limitations and strengths

We were unable to consider each of the 4 CMDs separately, given the limited power to detect differences across the respective subsets of people with each individual CMDs (eg, diabetes). Causal inferences could not be made based on our findings, given that CMD status was assessed during the same study visit as the meal challenge response. Our interpretation that a greater number of feature changes could reflect lower metabolic flexibility was based on the assumption that most metabolomic features have homeostatic regulation between the study timepoints among healthy individuals. There are still unresolved challenges in nontargeted metabolomics, particularly in resolving the major bottleneck of accurately identifying large numbers of known and unknown features [62]. Interpretations of our findings should account for the methodological limitations of nontargeted metabolomics, including that feature peak areas are relative quantifications. Future studies with orthogonal methods, including MS/MS, are needed for validation and confirmation. Features could not be converted to concentrations by reference standardization [22] as known concentrations of the calibrated reference were not available for annotated features selected by Stage 2B regressions. We did not assess the potential influences of genetics, the gut microbiome, or adipose or skeletal tissues on our associations of interest [3, 63].

Strengths of our study included the standardized meal challenge, which addresses the heterogeneity of meal challenge (eg, macronutrient composition, intake frequency, follow-up duration) in prior literature considering metabolomics. To our knowledge, our sample size was larger than other metabolomic studies with meal challenges among individuals with metabolic diseases [64, 65]. Nontargeted high-resolution metabolomic data have greater coverage of metabolites compared with other approaches [5]. The utilization of dual columns (C18, HILIC) and different electrospray ionization modes (positive, negative) additionally provided increased feature coverage [21]. Stable isotope-labeled internal standards and 2 sets of quality controls were included in the metabolomic assays, in order to monitor and correct potential sources of bias (eg, instrument drift).

4. Summary

Following a standardized meal challenge, a greater number of metabolomic feature peak areas differed among participants with CMDs, relative to those without CMDs, which could reflect increased metabolic dysregulation and decreased flexibility. Features that were altered by the meal challenge and CMD status included dipeptides, glycerophospholipids, carnitines, and a bile acid metabolite.

Acknowledgments

Author Contributions: A.D.S. conceived and designed this study. D.P.J. developed the high-resolution metabolomic assay protocol and workflow. M.R.Z. directed all field activities and supervised the glucose assays. T.Y. designed the bioinformatic workflow for the initial processing of mass spectrometry data. E.A.Y. and A.D.S. analyzed data, and E.A.Y. wrote the initial manuscript draft. All authors (E.A.Y., T.Y., D.P.J., M.R.Z., and A.D.S.) contributed to critically revising the paper. A.D.S. had primary responsibility for final content. All authors read and approved the final manuscript.

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Data availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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