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Impact of pre-analytic blood sample collection factors on metabolomics

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Abstract

Background—Many epidemiologic studies are using metabolomics to discover markers of carcinogenesis. However, limited data are available on the influence of pre-analytic blood collection factors on metabolite measurement.

Methods—We quantified 166 metabolites in archived plasma from 423 Health Professionals Follow-up Study and Nurses' Health Study participants using liquid chromatography–tandem mass spectrometry (LC-MS). We compared multivariable-adjusted geometric mean metabolite LC-MS peak areas across fasting time, season of blood collection, and time of day of blood collection categories.

Results—The majority of metabolites (160 of 166 metabolites) had geometric mean peak areas that were within 15% comparing samples donated after fasting 9-12 versus ≥13 hours; greater differences were observed in samples donated after fasting ≤4 hours. Metabolite peak areas generally were similar across season of blood collection, although levels of certain metabolites (e.g., bile acids; purines/pyrimidines) tended to be different in the summer versus winter months. After adjusting for fasting status, geometric mean peak areas for bile acids and vitamins, but not other metabolites, differed by time of day of blood collection.

Conclusion—Fasting, season of blood collection, and time of day of blood collection were not important sources of variability in measurements of most metabolites in our study. However,

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considering blood collection variables in the design or analysis of studies may be important for certain specific metabolites, particularly bile acids, purines/pyrimidines, and vitamins.

Impact—These results may be useful for investigators formulating analysis plans for epidemiologic metabolomics studies, including determining which metabolites to *a priori* exclude from analyses.

Keywords

Blood specimen collection; Cohort studies; LC-MS; Metabolomics; Tandem mass spectrometry

Introduction

Metabolomic profiling of archived biospecimens from participants enrolled in epidemiologic studies has the potential to facilitate discovery of early biological markers of carcinogenesis as well as markers of cancer prognosis (1). However, conducting a large-scale biospecimen collection can be challenging, particularly when participants are spread across a wide geographic area, and standardizing certain aspects of the blood collection protocol across participants may be particularly difficult. For example, in an uncontrolled setting, some participants may not be able to follow strict protocols with regard to fasting prior to blood draw or time of day of blood collection. Additionally, blood collections in large populations may span several months, resulting in different seasons of blood collection across participants, and thus seasonal differences in exposures (e.g., diet or light exposure) that may affect biomarker levels.

Few studies have evaluated whether levels of plasma metabolites measured by metabolomics profiling platforms vary by pre-analytic blood sample collection factors, such as fasting time, time of day of blood collection, or season of blood collection (2-9). Such information is necessary to understand the importance of controlling for these variables in the design of metabolomics studies (e.g., matching cases and controls on season of blood draw or restricting the study population to those fasting at least 8 hours) or during data analysis (e.g., excluding certain metabolites likely to be inaccurately measured based on pre-analytic variables). Failure to adequately consider these sources of measurement error could lead to decreased power of statistical tests and biased association measures (10). We evaluated differences in plasma metabolite peak areas across participants in two large, prospective cohort studies with different fasting times at blood draw, times of day of blood draw, and seasons of blood draw. Our analyses considered data on 166 metabolites, measured by a liquid chromatography–tandem mass spectrometry (LC-MS) metabolomics platform, that performed well in our previous pilot testing (11).

Materials and Methods

Study Population

The Nurses' Health Study (NHS) was established in 1976 when 121,700 female nurses aged 30-55 years completed a mailed questionnaire. The Health Professionals Follow-up Study (HPFS) was initiated in 1986 when 51,529 men aged 40-75 years and working in health professions completed a mailed questionnaire. Both cohorts enrolled only adults living in the

United States and have followed participants since enrollment via mailed biennial questionnaires. Blood samples were collected in heparin tubes from 32,826 women in NHS from 1989-1990 and in EDTA tubes from 18,225 men in HPFS from 1993-1995. Participants arranged to have their blood collected and shipped the samples via overnight mail with an icepack to a central laboratory. On arrival, the samples were processed and aliquots of plasma, white blood cells, and red blood cells were stored in liquid nitrogen freezers; >95% of samples arrived within 24 hours of collection (12, 13). Participants provided information on the date of blood collection, time at which blood was drawn, and number of hours since last eating before blood draw on a questionnaire returned with the samples.

Our analyses included 423 participants (252 from NHS and 171 from HPFS) selected as controls for a previous study of plasma metabolomics and pancreatic cancer (14). All participants provided implied consent by returning the questionnaires and blood samples. The study was approved by the Human Research Committee at Brigham and Women's Hospital (Boston, MA).

Metabolite Profiling

Profiles of 231 endogenous polar metabolites and lipids were obtained using LC-MS at the Broad Institute of the Massachusetts Institute of Technology and Harvard University (Cambridge, MA). A detailed description of the metabolite profiling methods has been previously published (14). For the current analysis, we excluded 35 metabolites that were not reasonably reproducible in samples processed up to 24 hours after blood draw (due to the design of our collections) in previous pilot tests (11, 14). Reasonable reproducibility was defined as an intraclass correlation (ICC) ≥0.75 comparing samples processed immediately versus 24 hours later (15). In addition, we excluded 15 metabolites with inter-assay coefficients of variation >25%, as measured in blinded quality control samples, and 15 metabolites with undetectable levels in >10% of participants. Thus, 166 metabolites were retained for these analyses.

Statistical Analysis

Metabolite levels were reported as measured LC-MS peak areas, which are proportional to metabolite concentration. We grouped metabolites into 9 categories: amines (n=5); amino acid derivatives (n=13); amino acids (n=28); bile acids (n=5); lipids and lipid metabolites (n=88); organic acids (n=11); purines, pyrimidines, and derivatives (n=3); vitamins (n=3); and other (n=10) (see Supplemental Table S1 for list of metabolites within each category). To quantify the strength of the relationship between metabolites in each category, after adjusting for potential differences between participants that might affect metabolite levels, we calculated Spearman partial correlation coefficients. The covariates included: age at blood draw (years, continuous), race (white, non-white), body mass index at blood draw (continuous, kg/m^2), physical activity (metabolic equivalent hours/week, continuous), sex/menopausal status/hormone use (male, female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use), and total caloric intake (kcal, continuous). Data on covariates were obtained from the cohort questionnaire closest to the date of blood collection.

For the analyses of metabolite levels according to blood sample collection factors, each metabolite peak area was log-transformed to improve the normality of its distribution and included as the dependent variable in a robust variance linear regression model (PROC MIXED, version 9.3; SAS Institute, Cary, NC). The primary independent variable in the model was the pre-analytic blood collection variable of interest: fasting time (≤4 [referent], 5-8, 9-12, ≥13 hours), season of blood draw (February-April [referent], May-July, August-October, November-January), or time of day of blood draw (6-8am [referent], 9-10am, 11am-12pm, 1-2pm, 3pm-12am). We calculated the mean log-transformed metabolite peak area in each category of the blood collection variable after adjusting for the covariates (i.e., marginal means). We then calculated multivariable-adjusted geometric mean peak areas by taking the anti-log of each marginal mean. We calculated the percent difference in geometric mean metabolite peak area between each category of the blood collection variable and the referent category (e.g., fasting 9-12, 5-8, or ≤4 hours versus ≥13 hours) by subtracting the referent geometric mean peak area from the geometric mean peak area of the category of interest and dividing by the referent geometric mean peak area.

Within each metabolite category, we calculated the median as well as the 10^{th} and 90^{th} percentiles of the percentage differences in geometric mean metabolite peak area for each blood collection variable category (except the referent group). We also calculated the percentage (95% confidence interval) of metabolites in each metabolite category whose geometric mean peak areas were ±15% of the referent category geometric mean and considered alternate cutpoints (10%, 20%) in sensitivity analyses.

Because fasting time and time of day of blood draw are highly correlated, analyses of time of day of blood draw were further adjusted for fasting time (≤4, 5-8, 9-12, ≥13 hours). Also, to further explore the impact of fasting time on associations between time of day of blood draw and metabolite concentration, we conducted separate analyses among individuals fasting ≤8 hours and those fasting >8 hours. Due to the smaller sample size, time of day of blood draw was evaluated in 3 categories in these analyses (6-8am, 9am-12pm, 1pm-12am).

Results

The mean age at blood draw was 65 years among men in HPFS and 60 years among women in NHS (Table 1). Among both men and women, a large majority were white (94% in HPFS; 99% in NHS). The most common season of blood collection was late summer/early autumn among men (46% in August-October) and late winter/early spring among women (36% in February-April). The majority of participants in both cohorts had fasted for more than 8 hours at blood collection and had their blood drawn in the morning.

The distribution of inter-assay coefficients of variation for metabolites in each category is shown in Table 2. Spearman partial correlation coefficients among amines, amino acid derivatives, and purines and pyrimidines were weak to moderate (Supplemental Table S2). Correlations among amino acids, bile acids, lipids, and organic acids ranged from nearly 0 to >0.8.

Metabolite peak areas were similar across categories of fasting time for the majority of metabolites, particularly among people who had last eaten >4 hours prior to blood draw (Table 3). Specifically, geometric mean metabolite peak areas measured in samples collected after 9-12 or 5-8 hours fasting were within 15% of geometric mean metabolite peak areas in samples collected after fasting at least 13 hours for 91-100% of amino acid derivatives, amino acids, lipids and lipid metabolites, organic acids, and purines and pyrimidines. Metabolite peak areas were less similar when comparing samples from participants fasting ≤4 versus ≥13 hours. In particular, geometric mean peak areas comparing participants fasting ≤4 versus ≥13 hours were within 15% for only 1 of 5 bile acids and none of the 3 vitamins. Supplemental Table S1 shows the percentage differences in geometric mean peak areas by fasting time for each metabolite. Results for the 10% and 20% cut-offs were similar to the 15% cut-off (Supplemental Table S3).

Geometric mean peak areas of most metabolites were similar across seasons of blood collection (Table 4 and Supplemental Table S4). Among participants whose blood was collected in May-July, August-October, or November-January, geometric mean peak areas for at least 80% of amines, amino acid derivatives, amino acids, and lipids and lipid metabolites were within 15% of those among participants whose blood was collected between February and April. However, levels of certain types of metabolites appeared to vary by season. For example, geometric mean metabolite peak areas in samples collected during peak sun months (August-October) were within 15% of those collected during low sun months (February-April) for just 67% of purines/pyrimidines, 55% of organic acids, and none of the bile acids. When using the more conservative criterion of percentage difference ≤10%, these figures were 33%, 55%, and 0%, respectively (Supplemental Table S5).

We investigated percentage differences in geometric mean metabolite peak areas by time of day of blood collection adjusting for fasting time (Table 5 and Supplemental Tables S6-S7). Geometric mean levels of amino acid derivatives, amino acids, lipids and lipid metabolites, organic acids, and purines and pyrimidines generally were similar across individuals whose blood was collected at different times during the day. However, levels of certain metabolites, such as bile acids and vitamins, appeared to be more variable during the day. To further examine these patterns, we conducted additional analyses separately among participants fasting ≤8 hours and participants fasting >8 hours (Supplemental Table S8). Differences in geometric mean metabolite peak areas for bile acids by time of day of blood draw were evident among both participants fasting >8 and ≤8 hours. Among participants fasting >8 hours, bile acid levels tended to be lower in those whose time of blood draw was 9am-12pm or 1pm-12am versus 6-8am (percentage differences, 10^{th} to 90^{th} percentiles = −25%, −32% to −14%, and −15%, −52% to −4%, respectively). Differences in vitamin peak areas by time of day of blood draw were mainly apparent among participants fasting <8 hours; vitamin levels tended to be higher among those who blood was drawn between 9am and 12am versus 6-8am.

Discussion

Overall, our results suggest that, after excluding metabolites that are not stable with delays in processing up to 24 hours, fasting time, time of day of blood draw, and season of blood

draw are not important sources of variability for the majority of metabolites measured by our metabolomics profiling platform, particularly amines, amino acids, amino acid derivatives, and lipids and lipid metabolites. However, our results also suggest that careful attention to pre-analytic variables related to blood collection may be necessary in analyses focused on certain types of metabolites. Specifically, we observed variability in levels of organic acids, purines and pyrimidines, bile acids, and vitamins by fasting status, especially when comparing participants who ate relatively recently (fasting time ≤4 hours) to those whose last meal was more distant (fasting time ≥13 hours). Regarding differences by season of blood draw, we observed a pattern in which measurements of bile acids, organic acids, and purines and pyrimidines were most variable between months with peak sun (May-October) versus low sun (February-April) in the Northern Hemisphere, where the large majority of participants in our study reside. Finally, after adjusting for fasting time, having blood drawn in the late morning, afternoon, or evening compared with the early morning (6-8am) was associated with variability in measured peak areas for bile acids and vitamins, although variation in vitamin levels by time of day of blood draw was restricted mainly to individuals fasting <8 hours.

Few previous studies have investigated differences in metabolite profiles by pre-analytic variables related to blood collection. Brauer et al. conducted amino acid metabolite profiling using electrospray tandem-mass spectrometry in whole blood collected from 10 adults after fasting >5 hours as well as 3 and 5 hours after eating a standardized meal (5). They observed changes in amino acid levels 3 and 5 hours postprandial, suggesting that a fasting period longer than 5 hours should be considered for measurement of amino acids. Similarly, in our analysis, fasting time between 5 and 12 hours compared with ≥13 hours appeared to have minimal influence on levels of amino acids. We observed more differences in amino acid levels when comparing samples from participants fasting ≤4 hours versus ≥13 hours, although percentage differences in geometric mean peak areas remained within 15% for the large majority (86%) of amino acids. In our study, bile acids and vitamins were the most variable types of metabolites across fasting time categories and tended to be higher in participants fasting ≤4 hours versus ≥13 hours. This pattern likely reflects the intake of nutrients and reabsorption of bile acids from the intestine following ingestion of food (16). In general, our results regarding variability in metabolite levels by fasting status are congruent with the conclusion of Sampson et al. that fasting accounts for little of the variability in metabolites and, therefore, metabolomics can be a useful tool even in studies without strict guidelines on fasting before blood draws (4).

Few data have been published on potential seasonal differences in metabolite profiles. The differences we observed in levels of bile acids, organic acids, and purines and pyrimidines across individuals with different seasons of blood draw might reflect seasonal variation in diet or other exposures, such as sunlight. Interestingly, the pattern we observed of higher urate levels among participants who donated blood in the summer versus winter months has been previously reported, although the mechanism underlying this pattern is not clear (17). Overall, season did not appear to be an important source of variability for most types of metabolites in our study.

Several studies have reported on diurnal variation in biomarker concentrations. Ang et al. observed significant 24-hour variation in six amino acids (leucine, lysine, methionine, phenylalanine, proline, tyrosine) in a laboratory study of 8 men who consumed a nutritional drink every hour while awake (3). Similarly, in our analysis of individuals who fasted ≤8 hours before blood draw, the percentage difference in geometric mean metabolite peak area comparing individuals who donated blood between 1pm and 12am with those who donated blood between 6am and 8am was >15% for four of the six amino acids highlighted by Ang et al (data not shown). However, we did not observe large differences in the 6 amino acids by time of day of blood draw among participants who had fasted more than 8 hours at blood draw (all percentage differences <10%, data not shown). The differences we observed in bile acids by time of day of blood draw among participants fasting ≤8 hours are consistent with known changes in concentrations of circulating bile acids following meal ingestion. In addition, serum levels of bile acids have been shown to decrease during the day among fasting individuals (18), consistent with our results among individuals fasting >8 hours. The differences we observed in levels of vitamins by time of day of blood draw, only among participants fasting <8 hours, also likely reflect food ingestion. However, it should be noted that the 3 vitamins included in our analysis were B-complex vitamins, which are water-soluble and not stored in the body. Thus, our results likely do not reflect variability in levels of fat-soluble vitamins during the day.

Several limitations of our study should be considered. First, we examined differences in metabolite levels across participants with different blood collection characteristics, rather than differences within individuals observed under different conditions. However, we adjusted the mean metabolite peak areas for a variety of individual-level characteristics (e.g., sex, age, body mass index) that might influence metabolite levels and our findings are generally consistent with those observed in the few clinic-based studies. Still, it is possible that some of our novel findings, particularly those related to season of blood draw, are due to chance and should be confirmed in future studies. In addition, we excluded 35 metabolites from analysis that were not stable with delays in processing up to 24 hours, as demonstrated in our previous pilot testing (11), in order to focus on metabolites likely to be informative in our study population. Thus, some of the metabolite categories in our analysis contained small numbers of metabolites (e.g., purines and pyrimidines, vitamins) and certain categories were excluded entirely (e.g., carbohydrates); this limits our ability to make conclusions about the influence of blood collection characteristics on certain metabolite types. Finally, several factors may limit the generalizability of our results. Our primary analyses summarized results within metabolite categories. Correlations between metabolites within categories will influence the amount of variability in percentage differences observed (Supplemental Table S2). Therefore, our results may not be generalizable to other metabolomics platforms in which correlations between metabolites differ from those in our study or platforms that measured different sets of metabolites. Also, our results may not apply to study populations that differ significantly from the generally healthy, middle- and older-aged adults in our study.

In conclusion, the results of our analysis suggest that fasting time, season of blood draw, and time of day of blood draw are not important sources of variability for several major classes of metabolites measured by an LC-MS metabolomics platform, including amines, amino

acid derivatives, amino acids, and lipids and lipid metabolites. Thus, epidemiologic studies with archived blood specimens may be useful settings for future metabolomics research, even if strict blood collection protocols were not instituted or data on blood collection characteristics are not available. However, our results also suggest that sample collection factors may be important to consider if certain metabolite classes are of particular scientific interest. Investigation of water-soluble vitamins should be restricted to individuals fasting at least 8 hours. In addition, evaluating a potential influence of season of blood draw on vitamin levels may be prudent (e.g., in sensitivity analyses). Due to variability by season and time of day of blood draw, analyses of bile acids may be difficult in epidemiologic studies. If bile acids are of particular interest, analyses should be limited to participants fasting at least 8 hours at blood draw. Overall, our results may be useful for epidemiologic studies to *a priori* exclude metabolites subject to substantial measurement error from analyses, and thus reduce penalties for multiple testing, and when considering variables related to blood collection to include in analysis plans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Age-standardized characteristics of the study population[a]

Characteristic	Health Professionals Follow-up Study (n=171)	Nurses' Health Study (n=252)
Age at blood draw, years, mean (SD)	65.2 (7.8)	60.2 (6.2)
White, %	94.4	99.4
Height, inches, mean (SD)	70.1 (2.9)	64.4 (2.4)
Body mass index, kg/m^2, mean (SD)	26.0 (3.2)	25.9 (5.0)
Physical activity, MET-hrs/wk, mean (SD)	38.0 (35.1)	17.7 (20.3)
Season of blood draw, %		
Feb-Apr	10.0	36.2
May-Jul	27.7	31.4
Aug-Oct	45.9	15.1
Nov-Jan	16.4	17.3
Fasting time at blood draw, %		
≤4 h	31.9	18.5
5-8 h	11.6	5.0
9-12 h	36.8	45.7
≥13 h	19.7	30.8
Time of day of blood draw, %		
6-8am	29.9	37.7
9-10am	37.0	45.7
11am-noon	9.9	7.6
1-2pm	11.6	5.2
3pm-midnight	11.6	3.8

MET, metabolic equivalent; SD, standard deviation.

[a]All values (except age at blood draw) are standardized to the age distribution of the study population.

Table 2

Distribution of inter-assay coefficients of variation for metabolites in each category[a]

Metabolite category	No. of metabolites	Median (minimum – maximum) inter-assay coefficient of variation (%)
Amines	5	8.9 (8.2 - 22.5)
Amino acid derivatives	13	13.0 (9.6 - 23.9)
Amino acids	28	10.4 (6.9 - 18.0)
Bile acids	5	17.6 (12.1 - 20.5)
Lipids and lipid metabolites	88	9.6 (4.0 -25.0)
Organic acids	11	13.3 (5.2 - 24.8)
Purines, pyrimidines, and derivatives	3	9.0 (6.8 - 20.5)
Vitamins	3	14.8 (11.5 - 16.1)
Other[b]	10	10.5 (6.6 - 16.3)

[a]Metabolites with an inter-assay coefficient of variation >25% were excluded from analysis.

[b]Other metabolites include: allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine , salicylurate, sorbitol, and uracil.

Table 3

Percentage difference in geometric mean metabolite LC-MS peak areas, by fasting time[a]

Metabolite category	N	Fasting ≥ 13 h	Fasting 9-12 vs ≥13 h		Fasting 5-8 vs ≥13 h		Fasting ≤4 vs ≥13 h	
			Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent
Amines	5	Referent	−2.7 (−10.6, 2.4)	100%	−4.6 (−21.0, 6.5)	80%	−2.9 (−16.3, 6.7)	80%
Amino acid derivatives	13	Referent	1.5 (−5.5, 8.2)	100%	−2.7 (−11.4, 3.7)	92%	4.8 (−3.8, 24.6)	77%
Amino acids	28	Referent	2.0 (−3.8, 5.3)	96%	−2.4 (−9.4, 10.8)	96%	2.8 (−5.5, 20.7)	86%
Bile acids	5	Referent	0.4 (−8.4, 17.4)	80%	−11.3 (−29.1, 18.7)	60%	53.1 (3.0, 129.9)	20%
Lipids and lipid metabolites	88	Referent	0.2 (−4.6, 8.6)	98%	0.5 (−3.8, 10.7)	92%	0.9 (−4.5, 26.4)	83%
Organic acids	11	Referent	−5.1 (−13.7, 3.5)	91%	0.6 (−4.3, 10.2)	91%	−0.6 (−18.1, 10.7)	73%
Purines, pyrimidines, and derivatives	3	Referent	−1.2 (−2.9, 1.8)	100%	−3.9 (−7.4, −1.7)	100%	−2.6 (−26.6, 0.7)	67%
Vitamins	3	Referent	7.6 (0.8, 10.4)	100%	9.6 (9.2, 16.0)	67%	58.6 (24.1, 77.3)	0%
Other[b]	10	Referent	−0.4 (−16.4, 10.7)	90%	−2.8 (−24.0, 18.6)	50%	12.4 (−24.2, 84.5)	30%

N, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hrs/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), and total caloric intake (kcal, continuous).

[b]Other metabolites include: allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine , salicylurate, sorbitol, and uracil.

Table 4

Percentage difference in geometric mean metabolite LC-MS peak areas, by season of blood draw[a]

Metabolite category	N	Feb-Apr	May-Jul vs Feb-Apr		Aug-Oct vs Feb-Apr		Nov-Jan vs Feb-Apr	
			Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent
Amines	5	Referent	−1.2 (−13.4, 9.5)	100%	3.7 (−13.9, 12.1)	100%	−2.0 (−15.8, 3.0)	80%
Amino acid derivatives	13	Referent	2.5 (−2.4, 6.0)	100%	6.2 (1.6, 14.0)	100%	−4.4 (−9.9, 3.1)	100%
Amino acids	28	Referent	1.7 (−2.7, 19.0)	86%	5.3 (−0.8, 23.9)	82%	−3.2 (−6.4, 2.6)	100%
Bile acids	5	Referent	13.7 (0.2, 27.2)	80%	45.7 (21.2, 53.7)	0%	2.5 (−6.6, 30.2)	80%
Lipids and lipid metabolites	88	Referent	−1.4 (−4.7, 3.4)	100%	−2.8 (−9.9, 3.6)	98%	−1.6 (−7.6, 2.1)	100%
Organic acids	11	Referent	5.4 (−0.6, 37.3)	64%	6.2 (0.1, 35.0)	55%	−0.1 (−1.9, 9.5)	91%
Purines, pyrimidines, and derivatives	3	Referent	5.9 (0.5, 8.4)	100%	10.9 (0.5, 20.4)	67%	3.1 (−0.4, 8.7)	100%
Vitamins	3	Referent	15.4 (3.9, 16.9)	33%	7.6 (−5.0, 12.3)	100%	2.7 (−1.1, 27.3)	67%
Other[b]	10	Referent	4.3 (−26.8, 21.3)	50%	4.9 (−4.4, 19.6)	90%	−2.0 (−17.2, 4.0)	90%

N, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hrs/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), and total caloric intake (kcal, continuous).

[b]Other metabolites include: allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine , salicylurate, sorbitol, and uracil.

Table 5

Percentage difference in geometric mean metabolite LC-MS peak areas, by time of day of blood draw[a]

Metabolite category	N	6-8am	9-10am vs 6-8am		11am-noon vs 6-8am		1-2pm vs 6-8am		3pm-midnight vs 6-8am	
			Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent	Percentage difference, median (10th-90th percentile)	Percentage of metabolites within 15% of Referent
Amines	5	Ref	2.5 (1.8, 8.5)	100%	2.2 (−10.1, 19.0)	80%	0.4 (−30.4, 28.1)	60%	0.6 (−6.0, 11.3)	100%
Amino acid derivatives	13	Ref	5.4 (0.3, 8.4)	100%	5.9 (−4.4, 10.5)	100%	0.8 (−9.7, 6.3)	92%	2.7 (−7.5, 17.8)	85%
Amino acids	28	Ref	−3.5 (−6.3, 2.0)	100%	−8.2 (−15.4, −0.1)	89%	−7.4 (−14.4, 6.7)	89%	−6.3 (−15.5, 7.1)	82%
Bile acids	5	Ref	−21.7 (−29.6, −9.8)	20%	−28 (−39.2, −19.4)	0%	3.1 (−41.5, 9.1)	80%	−10.7 (−39.2, −1.0)	60%
Lipids and lipid metabolites	88	Ref	0.4 (−2.9, 2.6)	100%	2.7 (−5.1, 12.5)	94%	0.7 (−7.5, 10.4)	93%	−1.6 (−10.5, 4.8)	92%
Organic acids	11	Ref	1.4 (−0.3, 9.1)	100%	−0.4 (−10.3, 10.8)	91%	1.3 (−2.9, 30.2)	64%	5.2 (0.9, 27.0)	83%
Purines and pyrimidines	3	Ref	−0.9 (−3.7, 22.4)	67%	7.3 (5.1, 8.3)	100%	−1.7 (−3.5, −1.4)	100%	−4.7 (−9.7, 2.2)	100%
Vitamins	3	Ref	17.6 (12.4, 24.8)	33%	−13.0 (−16.9, 3.0)	67%	17.0 (14.3, 36)	33%	13.7 (−8.9, 47.7)	67%
Other[b]	10	Ref	−2.4 (−17.8, 23.7)	80%	−2.0 (−23.4, 61.7)	60%	2.2 (−22.3, 56.5)	60%	9.1 (−17.1, 187.2)	60%

N, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hrs/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), total caloric intake (kcal, continuous), and fasting time (≤4, 5-8, 9-12, ≥13 hours).

[b]Other metabolites include: allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine , salicylurate, sorbitol, and uracil.

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