Effect of Dietary Sodium Restriction on Human Urinary Metabolomic Profiles

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Abstract

Background and objectives Metabolomics is a relatively new field of "-omics" research, focusing on high-throughput identification of small molecular weight metabolites. Diet has both acute and chronic effects on metabolic profiles; however, alterations in response to dietary sodium restriction (DSR) are completely unknown. The goal of this study was to explore changes in urine metabolites in response to DSR, as well as their association with previously reported improvements in vascular function with DSR.

Design, setting, participants, & measurements Using stored urine samples from a 10-week randomized placebo-controlled crossover study of DSR in 17 middle-aged/older adults (six men and 11 women; mean age 62±8 years) who had moderately elevated systolic BP (130–159 mmHg) and were otherwise healthy, a liquid chromatography/mass spectrometry–based analysis of 289 metabolites was performed. This study identified metabolites that were significantly altered between the typical (153±29 mmol/d) and low (70±29 mmol/d) sodium conditions, as well as their baseline (typical sodium) association with responsiveness to previously reported improvements in vascular endothelial function (brachial artery flow-mediated dilation) and large elastic artery stiffness (aortic pulse wave velocity).

Results Of the 289 metabolites surveyed, 10 were significantly altered (nine were upregulated and one was downregulated) during the low sodium condition, and eight of these exceeded our prespecified clinically significant threshold of a >40% change. These metabolites were involved in biologic pathways broadly related to cardiovascular risk, nitric oxide production, oxidative stress, osmotic regulation, and metabolism. One metabolite, serine, was independently (positively) associated with previously reported improvements in the primary vascular outcome of brachial artery flow-mediated dilation.

Conclusions This proof-of-concept study provides the first evidence that DSR is a stimulus that induces significant changes in urinary metabolomic profiles. Moreover, serine was independently associated with corresponding changes in vascular endothelial function after DSR. Larger follow-up studies will be required to confirm and further elucidate the metabolic pathways that are altered in response to DSR.

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Introduction

Metabolomics is a relatively new field of "-omics" research, focusing on high-throughput identification of small molecular weight metabolites (1). Compared with other "-omics", metabolomic profiles are more characteristic of systemic phenotypes, which is particularly useful in gaining insight into changes in both physiologic and pathophysiological function (2). Metabolomics has been used to identify novel biomarkers or profiles of disease, such as CKD (3). However, various interventions, including lifestyle interventions, also influence the metabolome, and such alterations can be interpreted similarly to changes in other physiologic phenotypes. The concept of utilizing metabolomics to identify unique changes in molecular signatures in response to interventions, particularly dietary, is highly novel, and is regarded as an important new direction in nutrition-related research (1,4). Indeed, diet has both acute and chronic effects on metabolic profiles, although the chronic effects are not currently well understood (5).

Dietary sodium restriction (DSR) is a commonly recommended lifestyle intervention to reduce the risk of cardiovascular diseases, although average sodium intake remains well above recommended levels (6). Reducing sodium intake induces benefits beyond and independent of BP lowering alone (7,8), suggesting that additional physiologic mechanisms also contribute to associated cardiovascular benefits. We recently demonstrated that DSR improves vascular endothelial function in middle-aged and older adults with moderately elevated systolic BP (SBP) *via* a reduction in vascular oxidative stress (8), while also reducing large elastic artery stiffness (9). However, the physiologic mechanisms associated with DSR are incompletely understood. Thus, metabolomics may be a

novel tool for identifying new pathways mediating the beneficial effects of DSR on vascular function. In addition, because the degree of response to sodium restriction varies (10), metabolomics may allow for identification of unique predictors of responsiveness, which could be very useful clinically. Metabolomic alterations in response to DSR, as well the ability of metabolites to predict responsiveness to the intervention, are completely unknown.

Accordingly, we performed a *post hoc* analysis of a recently completed randomized placebo-controlled crossover study comparing the effect of low and typical sodium intake on vascular endothelial function and large elastic artery stiffness (8,9). Our goal was to assess urine metabolites (end products of cellular processes that can be collected noninvasively) using 24-hour urine collections and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), and to determine their association with previously reported improvements in vascular function. We utilized an untargeted approach, which allowed for the maximum likelihood of identifying metabolites that were either upregulated or downregulated in response to DSR, or related to changes in vascular function (11). This design was hypothesis-generating by nature, because there is currently no information available regarding metabolomic changes in response to DSR. Because the crossover design of the parent study allowed for isolation of dietary sodium as the only nutritional factor altered between conditions, this study allowed the ideal setting to explore these novel questions.

Materials and Methods

The details of the parent study, a randomized placebo-controlled crossover design conducted from February 2009 to January 2012, were published previously (8,9). The study was conducted at the University of Colorado Boulder Clinical and Translational Research Center (CTRC), and the metabolomics analyses were performed at the iC42 Clinical Research and Development Center at the University of Colorado Denver Anschutz Medical Campus.

Study Participants

The inclusion and exclusion criteria were described previously (8), and are summarized in the Supplemental Materials and Methods. All study participants had a resting SBP within 130–159 mmHg, which was measured in accordance with the seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (12). Participants had high normal or stage I systolic hypertension, and diastolic BP <99 mmHg, verified on a minimum of two occasions (13,14), but were otherwise free of cardiovascular disease, diabetes, kidney disease, and other clinical disorders. All procedures were approved by the University of Colorado Boulder Institutional Review Board and conformed with the Declaration of Helsinki. The nature, benefits, and risks of the study were explained to the volunteers and their written informed consent was obtained before participation.

Experimental Design and DSR

In the parent study, we used a double-blind, placebo-controlled, randomized crossover design, as described previously (8) and summarized in the schematic in Figure 1. Briefly, a low sodium intake of approximately 1500 mg/d (65 mmol/d) was compared with a typical United States sodium intake of 3600 mg/d (150 mmol/d). During the entire 10-week intervention period, participants reduced dietary sodium (target of 50 mmol/d) and were instructed to take 10 tablets spread across the day with meals. The tablets were placebo pills for 5 weeks, whereas the tablets were slow-release NaCl tablets (10 mmol [0.23 g] per tablet; HK Pharma, UK) for the other 5 weeks. The slow-release NaCl tablets aimed to return sodium intake to the approximately 150 mmol/d target. Participants were provided

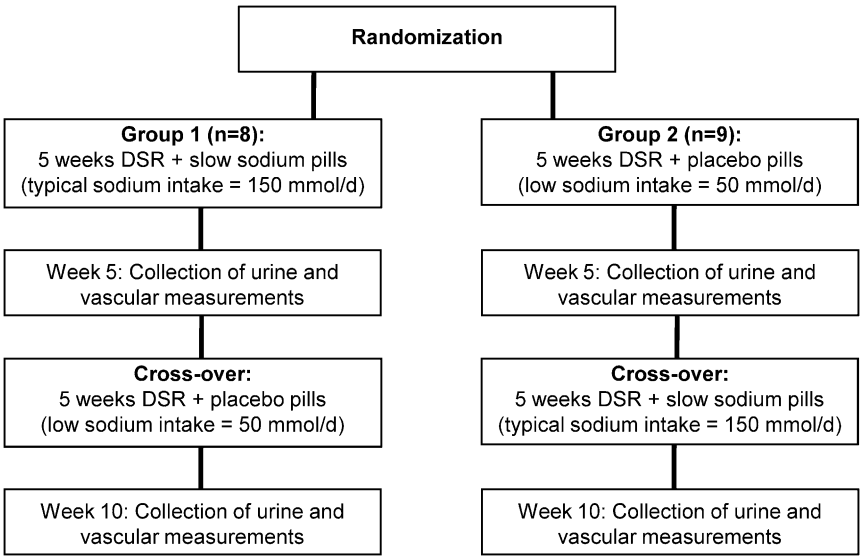

Figure 1. | **Study design.** Schematic illustrating the design of the previously published randomized placebo-controlled crossover study from which stored samples were analyzed for metabolomic profiling. DSR, dietary sodium restriction.

with comprehensive dietary education and weekly counseling by CTRC bionutritionists in order to reduce dietary sodium intake without changing caloric intake, dietary composition, or potassium intake [the results of 3-day diet record and 24-hour urinary potassium excretion analyses were all published previously (8)]. The investigators were blinded to sodium condition in the acquisition and analysis of all variables.

Vascular Function and BP

Brachial artery flow-mediated dilation (FMD_{BA}) was assessed as a measure of vascular endothelial function, and aortic pulse wave velocity (aPWV) was used as a measure of large elastic artery stiffness, as described previously (8,9). Arterial BP was assessed noninvasively from the brachial artery as described elsewhere (8,9). This *post hoc* analysis aimed to test the relations between urinary metabolites and changes in vascular outcomes with DSR, as assessed with multiple linear regression.

Urine Metabolomics

Metabolic profiling involves a comprehensive measurement of the types and concentrations of metabolites in a system at a specified time (15,16). Using stored (at −80°C) 24-hour urine collection samples from the final week (*i.e.*, week 5) of each sodium condition, we performed a LC-MS/MS–based analysis of 289 metabolites. Storage at −80°C is the recommended condition for long-term LC-MS/MS–based metabolomic analyses (17) and samples have been shown to be stable across time and freeze thaw cycles with this storage method (18). These technologies are well established in our laboratory (19–22) and utilize a positive/negative ion switching, targeted mass spectrometry–based metabolomics platform on an AB Sciex 5500 QTRAP system (AB Sciex, Concord, ON, Canada) that was established by Yuan *et al.* (23) and further extended and improved within our laboratory. This method utilizes compound-specific multiple reaction monitoring transitions. Internal standards were used for retention time correction and instrument performance monitoring. Further details are available in the Supplemental Materials and Methods. Urine samples were available from all 17 participants for the typical sodium condition, but were missing for two participants for the low sodium condition.

For those metabolites with a statistically significant ($P<0.05$) and clinically meaningful (>40%) change between sodium conditions, confirmation studies were performed by injecting pure compounds of these metabolites. This was followed by the manual integration of the peak of interest using MultiQuant 2.1 software (AB Sciex). This approach allowed for reduction of false discovery rates. Manual integration of the peak of interest was also performed for those metabolites that significantly correlated with fold change in FMD_{BA}, aPWV, and SBP, after visual inspection for outliers. Those that remained significantly associated with these outcomes after multivariate adjustment also had confirmation studies performed. All results are presented as integrated peak areas normalized to both urinary creatinine and the internal standard.

Plasma *S*-Adenosyl-L-Homocysteine

Because we identified a surprising increase in urinary *S*-adenosyl-L-homocysteine with DSR, we further explored this finding by performing a targeted analysis to also measure plasma levels of *S*-adenosyl-L-homocysteine. Using stored samples from each of the sodium conditions (available on $n=17$ and $n=16$ for the low and typical sodium conditions, respectively), plasma *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-methionine were measured by LC-MS/MS. Details of the methodology are provided in the Supplemental Materials and Methods.

Statistical Analyses

Clinical characteristics (Table 1), urinary metabolites, and plasma *S*-adenosyl-L-homocysteine/methionine were compared at the low sodium and typical sodium time points using a paired *t* test in SPSS 21 software. Principal component analysis (PCA) was performed using no weighting and Pareto scaling. For all metabolites, bivariate relations between urinary metabolites during the typical sodium condition and fold change in FMD_{BA}, aPWV, and SBP were determined using the Pearson correlation coefficient. Because there were no true baseline measurements of vascular function given the cross-sectional study design, the typical sodium condition, which consisted of dietary sodium intake equivalent to baseline screening, was used

Table 1. Select clinical characteristics

Variable	Low Sodium	Typical Sodium	*P* Value
No. of participants (women/men)	17 (6/11)	17 (6/11)	–
Age (yr)	62±8	62±8	–
Race			–
Caucasian	15 (88)	15 (88)	
Asian	2 (12)	2 (12)	
Urine sodium (mmol/24 h)	70±29	153±29	<0.001
Urine volume (ml/24 h)	2376±844	2445±1001	0.72
Modification of Diet in Renal Disease eGFR (ml/min per 1.73 m^2)	82±16	86±21	0.07
Brachial artery flow-mediated dilation (%Δ)	6.0±2.3	3.6±1.7	<0.001
Aortic pulse wave velocity (cm/s)	761±202	873±131	0.002

Data are presented as the mean±SD or *n* (%).

as the predictor condition. Step-wise multiple linear regression was then performed using SAS 9.3 software for any metabolites (integrated peak areas normalized to the internal standard and urinary creatinine) that significantly correlated with either change in $FMD_{BA,}$ aPWV, or SBP with sex, age, and SBP (except for the ΔSBP model) included in the model (model 1). All data are reported as means±SDs. Statistical significance for all analyses was set at P value <0.05.

Results

Clinical Characteristics

Detailed participant characteristics, including baseline characteristics, were published previously (8,9). Select clinical characteristics from each of the two sodium conditions are shown in Table 1. The comparison of the low versus typical sodium conditions showed that participants had lower urinary sodium excretion, indicating dietary adherence, as well as greater FMD_{BA}, and lower aPWV, with no statistically significant difference in eGFR. As a result of the crossover design and weekly counseling by CTRC bionutritionists, intake of macronutrients and micronutrients, other than sodium, were unchanged between conditions, as reported previously (8).

Urinary Metabolomics

Table 2 displays all metabolites that significantly changed between the typical and low sodium conditions, including a brief description of the metabolite's biologic role. Ten metabolites were significantly changed with DSR, with nine increasing (from 1.3- to 2.5-fold higher) and one slightly decreasing (0.8-fold); eight of these exceeded our prespecified threshold of a clinically significant change of >40% and thus were confirmed. The results of the confirmation studies are provided in Supplemental Figure 1. Results were similar when normalized to the other internal standard (homoarginine; data not shown).

Unsupervised PCA, which considers global structure of the data, did not separate well between the groups (Supplemental Figure 2). Supervised principal component analysis–discriminant analysis (PCA-DA), which utilizes prior knowledge of sample groups to determine the variables that maximize the variation between groups and those which minimize the variation within a group did separate the two sodium conditions (Figure 2).

Plasma *S*-Adenosyl-L-Homocysteine

Because the increase in urinary *S*-adenosyl-L-homocysteine with DSR was surprising, we further explored this finding by performing a targeted analysis to measure plasma levels of *S*-adenosyl-L-homocysteine. Unlike the urinary results, the metabolite was unchanged in the plasma (typical sodium: 28.7±16.1 nM; low sodium: 25.0±14.4 nM; P=0.51). Similarly, plasma levels of *S*-adenosyl-L-methionine were also unchanged (typical sodium: 110.4±41.23 nM; low sodium: 117.4±36.0 nM; P=0.61).

Regression Analyses

Serine was the sole metabolite during the typical sodium condition that was significantly associated with change in FMD_{BA} after multivariate adjustment, as shown in Table 3. Metabolites with significant univariate correlations that did not persist after multivariate adjustment are shown in Supplemental Table 1 (these were not confirmed). In unadjusted analyses, higher levels of serine were associated with a greater increase (fold change) in FMD_{BA}, a measure of vascular endothelial function and primary outcome of the parent study, in response to DSR. This association remained significant after adjusting for age, sex, and SBP. In adjusted analyses, none of the metabolites had a significant association with change in aPWV or SBP.

Discussion

These results provide the first evidence that unique metabolomic changes occur in response to DSR. Specifically, changes were detected in 10 metabolites (nine upregulated and one downregulated) during the low sodium condition, and eight of these exceeded our prespecified threshold of a >40% change to be deemed clinically meaningful. PCA-DA analysis also showed separation between the two sodium conditions. Furthermore, one of these metabolites was independently associated with the improvements previously reported in the primary vascular outcome of FMD_{BA} (8). These findings provide proof of concept that metabolism changes in response to DSR are reflected in excreted urinary metabolites, consistent with what has been shown previously in small acute or short-term studies, including manipulation of soy intake (24), cocoa ingestion (25), and a high phytochemical diet (5).

Several metabolites were associated with biologic pathways known to be related to general cardiovascular risk. *S*-adenosyl-L-homocysteine is a precursor to homocysteine, an amino acid associated with increased cardiovascular risk (26). Because it was surprising that levels of *S*-adenosyl-L-homocysteine were increased in urine with DSR, we also evaluated levels in plasma. The lack of change in plasma levels supports that *S*-adenosyl-L-homocysteine excretion was increased, without any change in circulating levels. Similarly, the increase in methylmalonic acid during the low sodium condition was somewhat surprising. Methylmalonic acid is a vital intermediate in the metabolism of fat and protein and is also a metabolite of homocysteine (27). High levels can indicate vitamin B_{12} deficiency (28). Of note, methylmalonic acid is very efficiently excreted by the kidney, and thus urinary concentration reflects tissue depletion (29).

Serine, which was also increased with DSR, is a nonessential amino acid that is part of many biologic pathways, including methionine metabolism, sphingolipid metabolism, ammonia recycling, transcription/translation, and metabolism of serine and other small amino acids (27). It is also required to condense with homocysteine for the breakdown of the latter (27). Serine has an important role in the catalytic function of enzymes, including a residue that is phosphorylated for the activation of endothelial nitric oxide synthase and subsequent production of nitric oxide (30). Asparagine is an additional nonessential amino acid that was increased with DSR and is also involved in many biologic pathways, including roles in nervous system function and ammonia synthesis (27).

Table 2. Urinary metabolite/creatinine ratios with a significant change after DSR

Metabolite	Biologic Role/ Metabolic Pathway	Low Sodium (AU)	Typical Sodium (AU)	Fold Change with DSR	*P* Value
Methylmalonic acid	Malonic acid derivative; vital intermediate in the metabolism of fat and protein; metabolite of homocysteine	$2.23E^{-3}\pm8.79E^{-3}$	$9.46E^{-4}\pm3.87E^{-3}$	2.4	0.01
Succinate	Krebs cycle intermediate; donates electrons to the ETC	$1.74E^{-3}\pm6.74E^{-3}$	$7.67E^{-4}\pm2.9E^{-3}$	2.3	0.01
Methionine sulfoxide	Methylation; biomarker of oxidative stress	$1.78E^{-5}\pm5.27E^{-5}$	$8.07E^{-6}\pm2.19E^{-5}$	2.2	0.01
S-adenosyl-L-homocysteine	Intermediate precursor of homocysteine, which is associated with increased cardiovascular risk	$2.32E^{-5}\pm6.12E^{-5}$	$1.25E^{-5}\pm3.27E^{-5}$	1.9	0.01
Glycerophosphocholine	Choline derivative; osmolyte (counteracts urea and other macromolecules)	$2.06E^{-4}\pm5.27E^{-4}$	$1.29E^{-4}\pm3.01E^{-4}$	1.6	0.03
D-gluconate/-acid	Found naturally in fruit, honey, and wine; regulates acidity	$2.73E^{-7}\pm7.67E^{-3}$	$4.79E^{-3}\pm6.43E^{-3}$	1.6	<0.001
Serine	Nonessential amino acid; in many biologic pathways	$7.73E^{-4}\pm1.53E^{-3}$	5.16E-4±$1.03E^{-3}$	1.5	0.05
Asparagine	Nonessential amino acid; required for nervous system; role in synthesis of ammonia	$3.13E^{-4}\pm4.96E^{-4}$	$2.15E^{-4}\pm3.70E^{-4}$	1.5	<0.001
Deoxyadenosine monophosphate	Derivative of ATP; monomer in DNA	$8.19E^{-4}\pm6.97E^{-4}$	$6.19E^{-4}\pm5.15E^{-4}$	1.3	0.02
6-phospho-D-glucono-1,5-lactone	Intermediate in pentose phosphate pathway	$2.44E^{-5}\pm1.26E^{-3}$	$3.02E^{-5}\pm1.74E^{-3}$	0.8	0.04

Data are presented as means±SD. Area is normalized to urinary creatinine and the internal standard (methionine-d_3) and presented as arbitrary units. Deoxyadenosine monophosphate and 6-phospho-D-glucono-1,5-lactone were not confirmed, because the percent change (before the normalization to creatinine) did not exceed the prespecified clinically significant threshold of >40% change (these values are presented as integrated area). AU, arbitrary unit; DSR, dietary sodium restriction; ETC, electron transport chain.

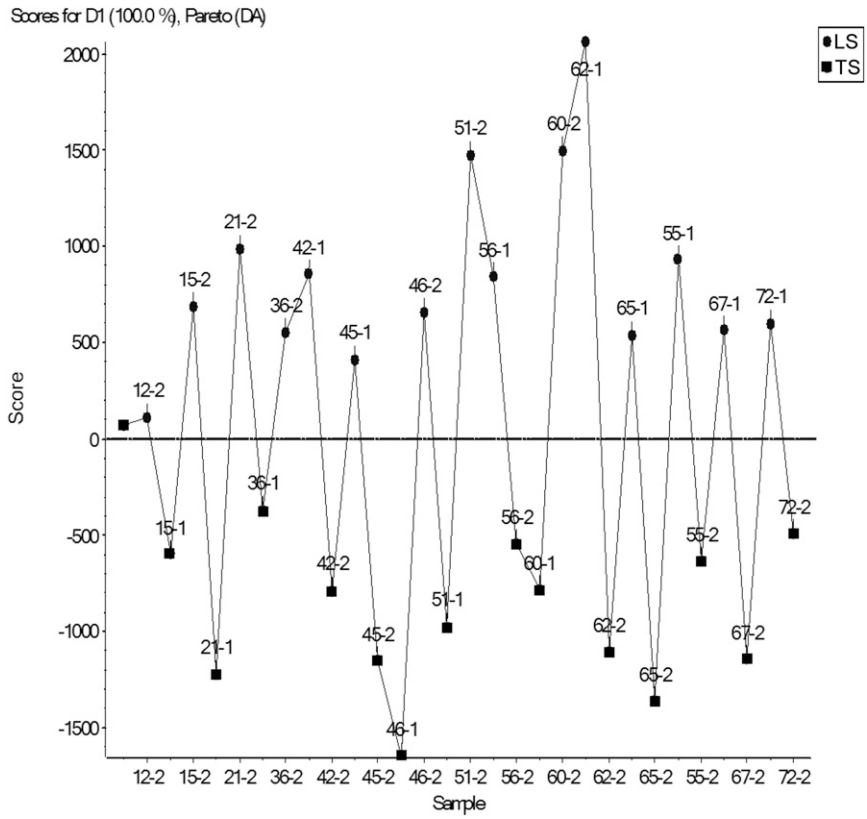

Figure 2. | **Results of supervised principal component analysis and discriminant analysis**. The results are shown by subject identification number. The Pareto algorithm was used for scaling. Ovals and squares represent the low sodium and typical sodium conditions, respectively. D1, discriminant 1; DA, discriminant analysis; LS, low sodium; TS, typical sodium.

In the parent study, we found that the improvements in vascular endothelial function with DSR were mediated in part by reduced vascular oxidative stress (8). Methionine sulfoxide is considered to be a biomarker of oxidative stress (31); thus, its increase during the low sodium condition is at odds with these previous findings. However, methionine sulfoxide reductase is a regulator of antioxidant defenses and longevity in mammals (32) and was not one of the metabolites included in this untargeted analysis. Similarly, 6-phospho-D-gluco-1,5-lactone was also lower with DSR, albeit not to our predetermined clinically significant level; thus, this peak was not confirmed. 6-Phospho-D-gluco-1,5-lactone is an intermediate in the pentose phosphatase pathway, which is considered one of the main antioxidant defense regulator systems, by providing reducing power and ribose phosphate to cells (33).

In addition, several metabolites altered with DSR have roles in osmotic regulation, sodium balance, and general metabolism. The serine protease, serpin, plays a key role in sodium handling in the kidney (34). Glycerophosphocholine is a choline derivative and osmolyte that counteracts urea and other macromolecules (35). Both were increased during the low sodium condition. Finally, D-gluconate, which is found in fruit, may reflect exogenous intake (27). However, because sodium intake was manipulated with sodium added back in pill form, a difference in fruit intake between sodium conditions would not be anticipated.

Several strengths and limitations of these analyses merit discussion. The fact that the parent study was a crossover design, with sodium intake manipulated *via* adding it back in pill form, is an important strength that allowed for

Table 3. Association between urine metabolite and change in vascular function

Metabolite	Dependent Variable	Model	Unstandardized Slope, Change (95% CI)	Standardized Slope, Change	*P* Value
Serine	ΔFMD_{BA}	Unadjusted	$1.51E^3$ ($6.06E^2$ to $2.41E^3$)	0.69	0.003
		1	$1.59E^3$ (47.64 to $3.13E^3$)	0.73	0.04

Metabolite was entered as the area normalized to urinary creatinine and the internal standard (methionine-d_3). Model 1 was adjusted for age, sex, and systolic BP. 95% CI, 95% confidence interval; FMD_{BA}, brachial artery flow-mediated dilation.

isolation of sodium as the sole manipulated dietary factor between conditions, in the exact same participants. An additional strength is the utilization of LC-MS/MS, a sensitive and quantitatively reproducible technique. The attempt to relate baseline values of metabolomic signatures with improvements in vascular function in response to DSR is a major strength of the study, providing insight into potential molecular mechanisms as well as implications for personalized medicine.

In terms of limitations, it should be noted that the population in this study comprised middle-aged and older, primarily Caucasian adults with moderately elevated SBP and the sample size was small. As such, these findings will need to be verified in a larger cohort and extended to populations other than the relatively healthy group in this study.

An additional potential limitation of this *post hoc* analysis is that we elected to only measure metabolites in urine and not also in plasma. Important advantages of urine include the fact that it is readily available, easily and noninvasively obtained, and less complex to process than blood, allowing for clinical translation. In addition, metabolites in urine are the end product of cellular processes, and are thus closely linked to phenotype (36). Future targeted analyses will be needed to examine changes in specific metabolites in plasma, in addition to urine.

Another limitation is that PCA-DA analysis, which showed separation between sodium conditions, is susceptible to overfitting because of the bias associated with prior knowledge of groups, particularly when smaller sample sizes such as ours are used. The PCA-DA results differed from the unsupervised PCA, which did not separate well between the groups. In addition, type I errors may have occurred in our analysis, because a Bonferroni correction was not feasible given the large number of screened metabolites. However, we minimized this possibility by performing confirmation studies injecting pure compounds of these metabolites, allowing for a reduction of false discovery rates by identifying a compound by its mass (mass spectrometry), fragmentation (multiple reaction monitoring transition), and chromatographic characteristics (retention time). In addition, all of the metabolites that changed with DSR were present in the urine of all analyzed samples from both conditions, which further limits false discovery rates.

It is possible that the effects of DSR on metabolic profiles may be short term rather than sustained. We were unable to determine whether the changes in metabolomic profiles with DSR remained stable over time; however, the limitation of using of a single sample from each sodium condition is minimized by both the crossover design and testing a 24-hour urine collection, rather than a spot urine. Finally, the changes in urinary metabolites may have been influenced by changes in BP induced by DSR, rather than by the direct effect of changes in sodium intake, as we previously reported a reduction in BP from 140±15/82±6 to 128±10/70±6 mmHg during the low sodium condition (8).

In conclusion, this proof-of-concept study provides the first evidence that DSR is a stimulus that induces significant changes in metabolomic profiles in healthy adults. Larger follow-up studies will be required to confirm and further elucidate biologic pathways that are altered in response to DSR. Because new metabolites are continually being discovered and it is not possible to include all known metabolites in a given panel (27,37), in the future, numerous other metabolites may be found to change with DSR and may be linked with improvements in physiologic function. These findings should stimulate future research related to these questions. In addition, future research may allow the identification of metabolite biomarkers that predict physiologic responsiveness to DSR among individuals. This may have important implications for other populations, such as patients with CKD, because several of the metabolites that changed with DSR have been reported to relate to CKD progression (3,26,38).

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Disclosures

None.

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DATA SUPPLEMENT

Effect of Dietary Sodium Restriction on Human Urinary Metabolomic Profiles

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Supplemental Materials and Methods

Study Participants. The inclusion and exclusion criteria have been described previously [8]. All study participants had a resting SBP within 130-159 mmHg (measured in accordance with JNC 7 [12]), i.e., high normal or stage I systolic hypertension, and diastolic blood pressure <99 mmHg, verified on a minimum of two occasions [13, 14], but were otherwise free of CVD, diabetes, kidney disease and other clinical disorders as assessed by medical history, physical examination, ankle-brachial index ($\leq$0.9), blood chemistries and resting and exercise ECG. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula, as described previously [8]. All participants were non-smokers, had a body-mass index (BMI) <40 kg/m^2, were not taking dietary supplements known to influence vascular function, including those with antioxidant properties, and postmenopausal women (n=4) were not taking hormone replacement therapy. All procedures were approved by the Institutional Review Board of the University of Colorado Boulder and conform with the *Declaration of Helsinki*. The nature, benefits and risks of the study were explained to the volunteers and their written informed consent was obtained prior to participation.

Sample Extraction. Following centrifugation (at 14,000*g* for 10 min at 4°C), protein of a 200 µL urine aliquot was precipitated using 800 µL methanol (cooled to -80 °C). The sample was gently vortexed and incubated overnight at -80°C. Following centrifugation (at 14,000*g* for 10 min at 4°C), the supernatant was transferred into a new 1.5-ml microcentrifuge tube, and dried in a SpeedVac using no heat. The dried

metabolite sample was reconstituted in 50 µL of HPLC grade water/methanol (80/20, v/v).

Data Acquisition. The Q1 (precursor ion) and Q3 (fragment ion) transitions, the metabolite names, dwell times and the appropriate collision energies (CEs) for both positive and negative ion modes were adapted from Yuan et al [1]. In addition to this published 289 transition list, we have added 14 selective mass transitions in the positive and 4 in the negative ion mode, resulting in 307 transitions. Q1 and Q3 transitions were set to unit resolution for optimal metabolite ion isolation and selectivity. In addition, the polarity switching (settling) time was set to 50 ms; in 1.42 s using a 3-ms dwell time, we were able to obtain 6-14 scans per metabolite peak.

The source temperature was set at 500°C, curtain gas (CUR, nitrogen) at 20, collision gas (CAD, nitrogen) at high, ion source gases 1 and 2 at 33, declustering potential (DP) at +93/-93, entrance potential (EP) at +10/-10, and collision cell exit potential (CXP) at +10/-10 for positive and negative ion modes, respectively.
8 µL sample was injected onto an Amide XBridge HPLC column (3.5 µm; 4.6 mm inner diameter (i.d.) × 100 mm length; Waters). The mobile phases consisted of HPLC buffer A (pH = 9.0: 95% (vol/vol) water, 5% (vol/vol) acetonitrile, 20 mM ammonium hydroxide, 20 mM ammonium acetate) and HPLC buffer B: 100% acetonitrile.

The HPLC settings were as follows: from 0 to 3 minutes, the mobile phase was kept at 85% B; from 3 to 22 minutes, the percentage of solvent B was decreased from 85% to 2% and was kept at 2% for additional 3 minutes. At minute 26, solvent B was increased again back to 85% and the column flushed for additional 7 minutes at 85% solvent B.

Data Analysis. Once the data were acquired, MarkerView (v1.2.1, ABSciex) software was used for initial data processing. The following data extraction parameters were used: minimum peak intensity and peak width (above 1000 cps and 5 points), minimum signal-to-noise ratio of 10:1, limitation of inclusion of either three highest peaks or to one peak per chromatogram, retention time correction using the internal standards homoarginine, methionine-d3 and trimethylamine N-oxide-d9 (TMAO-d9) as well as normalization to the total area integral. Samples were grouped into LS and NS groups and a paired t-test was been used to evaluate the differences between the two patient populations for all identified metabolite peaks. In addition, principal component analysis (PCA) (unsupervised [Supplementary Figure 2] and discriminant analysis (PCA-DA) [Figure 2]) was used to visualize the differences between the LS and NS patient groups.

Since the MarkerView software identifies metabolite peaks within a small and well-defined spectral region rather that only a single peak, those metabolites that were significantly different between the LS and NS groups or predictive of fold change in FMD_{BA} and/or aPWV after multivariate adjustment were furthermore quantified using the MultiQuant (v2.1.1., ABSciex) software. Resulting metabolite peak areas were normalized to the area of the internal standard and urinary creatinine, and this ratio was used for all subsequent statistical analyses including linear regression.

Confirmation Studies. Positive identification of the metabolites of interest was performed through injection of pure compound standards onto the above described LC-MS platform (confirmation of the fragmentation pattern (MS/MS) and retention time). Representative chromatograms are presented in Supplementary Figure 1.

Plasma S-Adenosyl-L-Homocysteine. Using stored samples from each of the sodium conditions (available on n=17 for low sodium and n=16 for typical sodium conditions), plasma S-adenosyl-L-homocysteine and S-adenosyl-L-methionine were measured by LC-MS/MS. The API5000 mass spectrometer (AB Sciex) was run in the positive electrospray ionization mode (ESI) using MRM. The following ion transitions were used: SAM: 399.0→250.1; d3-SAM (internal standard): 402.0→136.2; SAH: 385.0→136.2 and d5-SAH (internal standard): 391.0→137.2. For additional details on the sample preparation and analysis conditions, please refer to Klepacki et al. [2].

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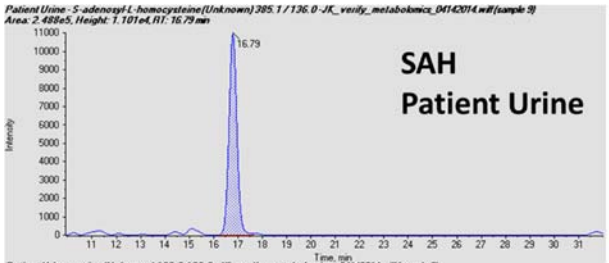
Supplemental Figure Legend

Supplementary Figure 1. Representative chromatograms from confirmation studies. Representative chromatograms from conformation studies showcasing the matching retention times and fragmentation patterns (MS/MS) between the pure compound standards and identified urinary metabolites.

Abbreviations: GPC: Glycerophosphocholine, SAH: S-Adenosyl-L-homocysteine.


Patient Urine - S-adenosyl-L-homocysteine (Unknown) 385.1 / 136.0 - JK_verify_metabolomics_04142014.wiff (sample 9)
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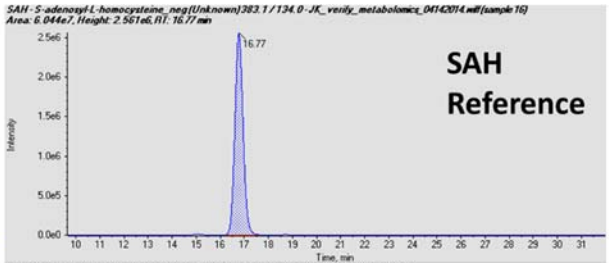
16.79
Intensity
Time, min
SAH
Patient Urine


SAH - S-adenosyl-L-homocysteine, neg (Unknown) 383.1 / 134.0 - JK_verify_metabolomics_04142014.wiff (sample 16)
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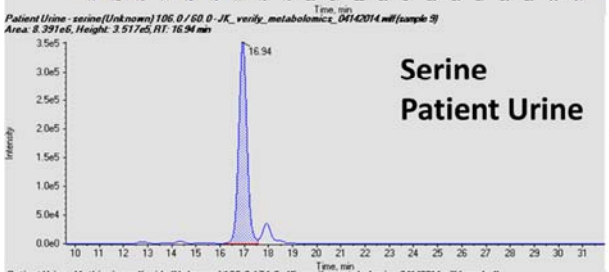
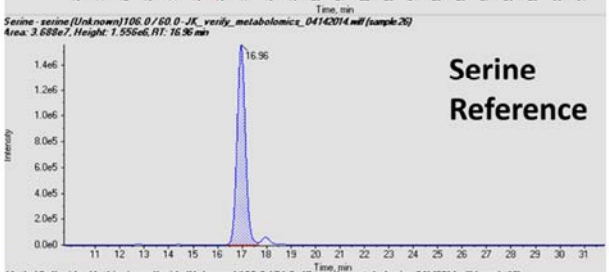
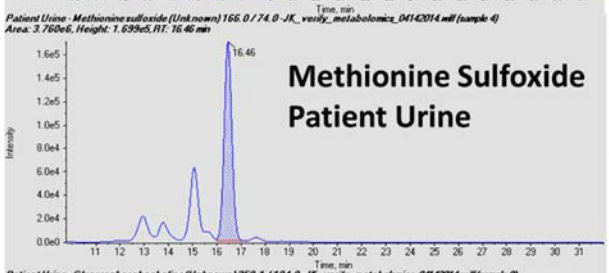
16.77
Intensity
Time, min
SAH
Reference


Patient Urine - serine (Unknown) 106.0 / 60.0 - JK_verify_metabolomics_04142014.wiff (sample 9)
Area: 8.391e6, Height: 3.517e5, RT: 16.94 min
16.94
Intensity
Time, min
Serine
Patient Urine


Serine - serine (Unknown) 106.0 / 60.0 - JK_verify_metabolomics_04142014.wiff (sample 26)
Area: 3.688e7, Height: 1.556e6, RT: 16.96 min
16.96
Intensity
Time, min
Serine
Reference


Patient Urine - Methionine sulfoxide (Unknown) 166.0 / 74.0 - JK_verify_metabolomics_04142014.wiff (sample 4)
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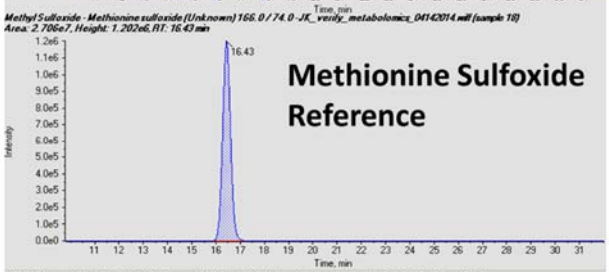
16.46
Intensity
Time, min
Methionine Sulfoxide
Patient Urine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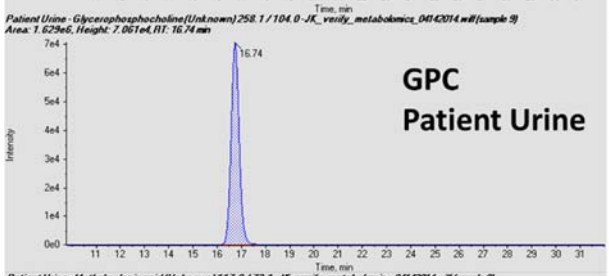
16.43
Intensity
Time, min
Methionine Sulfoxide
Reference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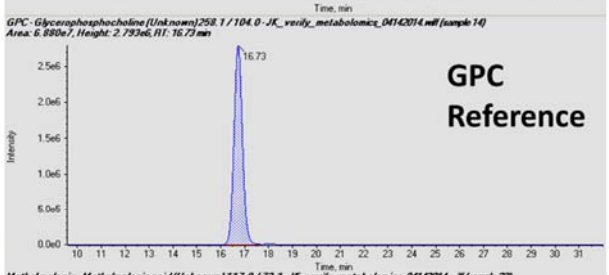
16.74
Intensity
Time, min
GPC
Patient Urine


GPC - Glycerophosphocholine (Unknown) 258.1 / 104.0 - JK_verify_metabolomics_04142014.wiff (sample 14)
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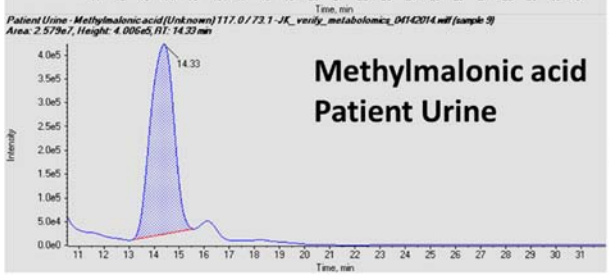
16.73
Intensity
Time, min
GPC
Reference


Patient Urine - Methylmalonic acid (Unknown) 117.0 / 73.1 - JK_verify_metabolomics_04142014.wiff (sample 9)
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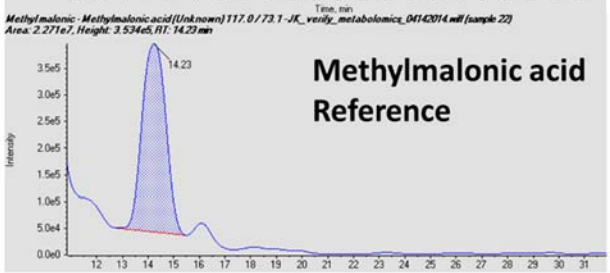
14.33
Intensity
Time, min
Methylmalonic acid
Patient Urine


Methylmalonic - Methylmalonic acid (Unknown) 117.0 / 73.1 - JK_verify_metabolomics_04142014.wiff (sample 22)
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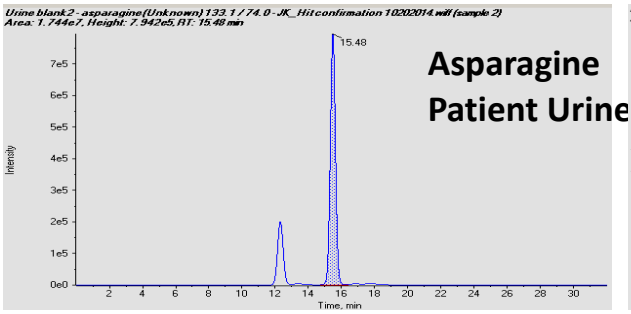
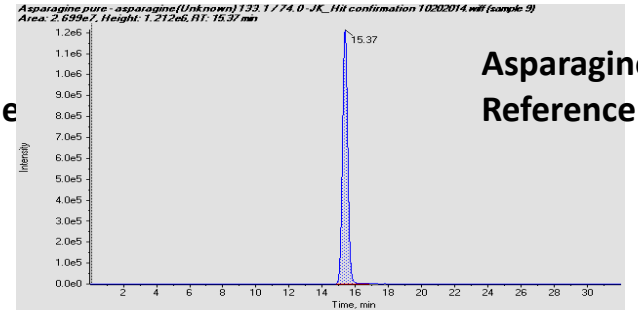
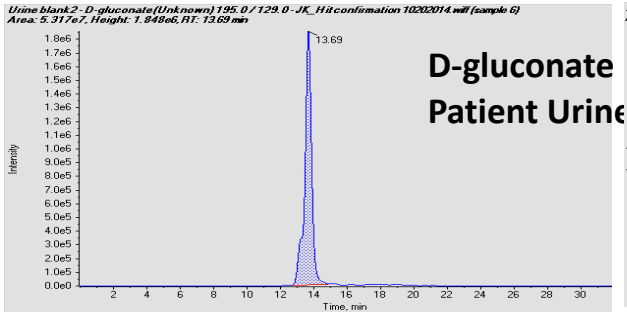
14.23
Intensity
Time, min
Methylmalonic acid
Reference



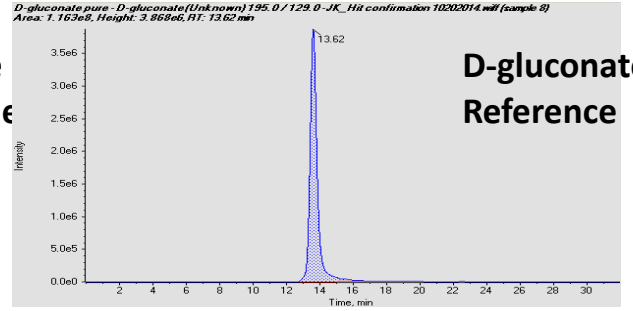
Asparagine Patient Urine



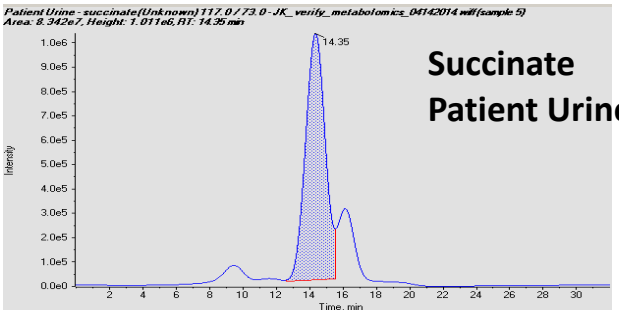
Asparagine Reference



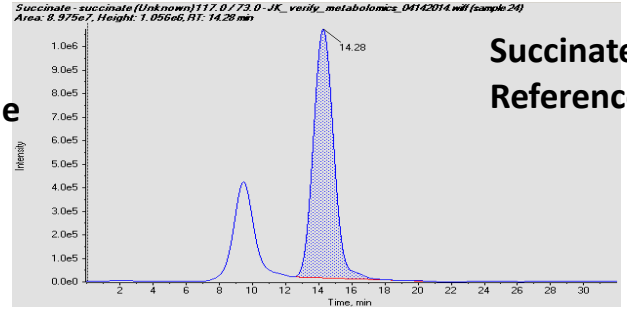
D-gluconate Patient Urine



D-gluconate Reference

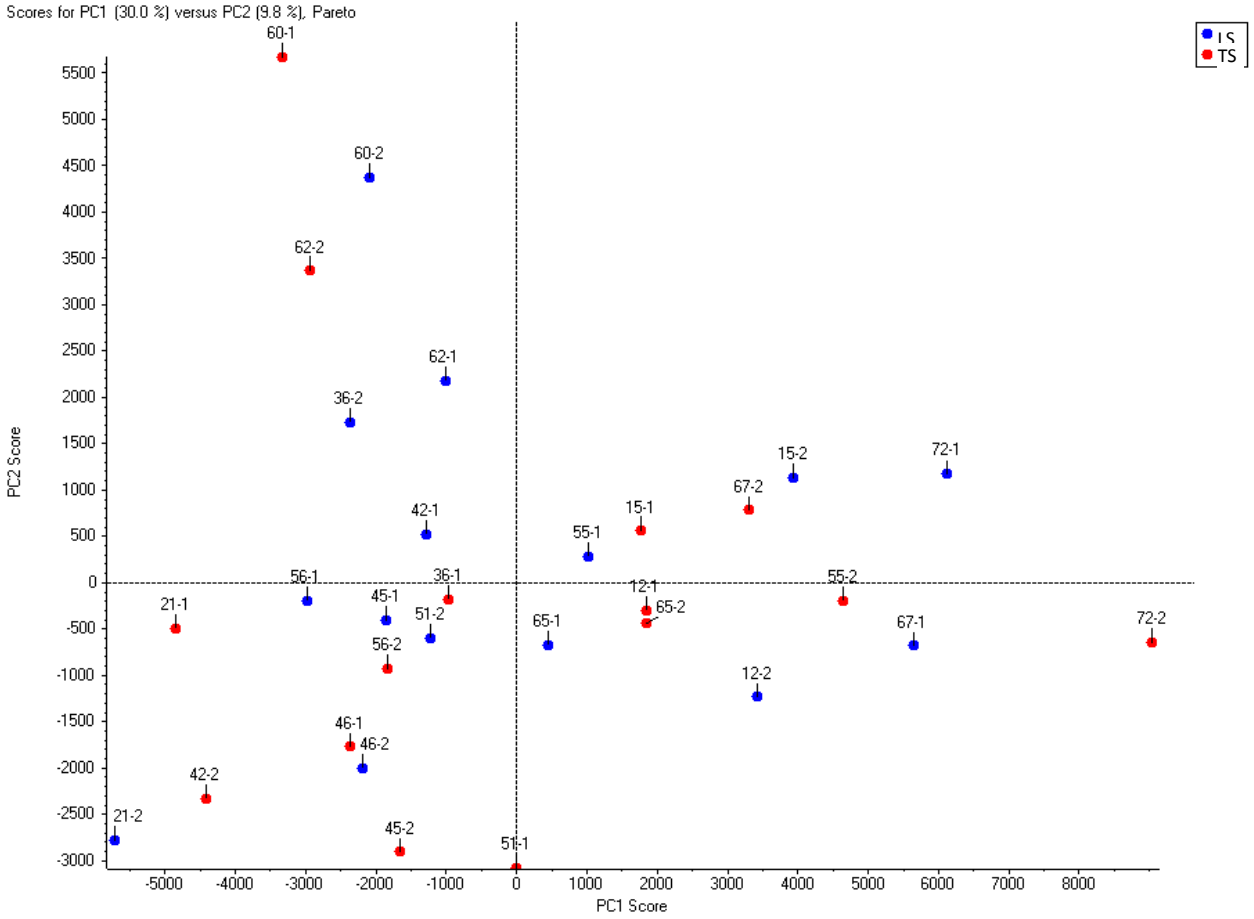


Succinate Patient Urine



Succinate Reference

Supplementary Figure 2. Results of unsupervised principal component analysis (PCA) (low sodium: LS and typical sodium: TS). The Pareto Algorithm was used for scaling.



Supplemental Table 1

Table 3. All Statistically Significant Univariate Associations Between Urine Metabolites and Change in Vascular Function and SBP (Unconfirmed)

Metabolite	Dependent Variable	Model	Unstandardized Slope [change] (95% CI)	Standardized Slope [change]	p-value
Serine	ΔFMD_{BA}	unadjusted	$1.51E^3$ ($6.06E^2$, $2.41E^3$)	0.69	0.003 *
		1	$1.59E^3$ (47.64, $3.13E^3$)	0.73	0.044 *
N-acetyl-L-alanine	ΔFMD_{BA}	unadjusted	$2.63E^2$ (73.57 , $4.52E^2$)	0.62	0.010 *
		1	$2.82E^2$ ($-1.08\ E^2$, $6.71E^2$)	0.67	0.14
N-acetyl-L-alanine	ΔSBP	unadjusted	-8.08 (-14.31, -1.86)	-0.58	0.014 *
		1	-1.27 (-11.92, 9.38)	0.09	0.80
Oxaloacetate	ΔFMD_{BA}	unadjusted	$1.49E^2$ (39.51, $2.58E^2$)	0.62	0.011 *
		1	$1.27E^2$ (-52.29, $3.07E^2$)	0.53	0.15
Orotate	ΔFMD_{BA}	unadjusted	$8.08E^2$ ($1.28E^2$, $1.49E^3$)	0.56	0.023 *
		1	$7.50E^2$ ($-3.67E^2$, $1.87E^3$)	0.52	0.17
Guanine	ΔFMD_{BA}	unadjusted	$1.02E^3$ ($3.65E^2$, $1.67E^3$)	0.67	0.005 *
		1	$8.80E^2$ (-25.70, $1.79E^3$)	0.58	0.056

D-gluconate	ΔFMD_{BA}	unadjusted	$2.85E^2$ ($28.07, 5.41E^2$)	0.54	0.032 *
		1	$1.94E^2$ ($-2.12E^2$, $5.99E^2$)	0.37	0.32
D-gluconate	ΔSBP	unadjusted	-12.45 (-24.64, -0.27)	-0.49	0.046 *
		1	2.33 (-15.34, 19.99)	0.09	0.80
Adenosine	ΔFMD_{BA}	unadjusted	$8.95E^2$ ($1.62E^2$, $1.63E^3$)	0.57	0.020 *
		1	$7.91E^2$ ($-5.80E^2$, $2.16E^3$)	0.51	0.23
Adenosine	ΔSBP	unadjusted	-47.60 (-75.83, -19.37)	-0.68	0.003 *
		1	-29.27 (-81.90, 23.37)	-0.42	0.25
Pyruvate	ΔFMD_{BA}	unadjusted	$3.64E^3$ $5.76E^2$, $6.71E^3$)	0.56	0.023 *
		1	$2.63E^3$ ($-2.05E^3$, $7.31E^3$)	0.41	0.24
Pyruvate	ΔSBP	unadjusted	-95.36 ($-1.89E^2$, -1.27)	-0.49	0.047 *
		1	-0.20 ($-1.25E^2$, $1.24E^2$)	-0.001	0.99
Quinolinate	ΔFMD_{BA}	unadjusted	$1.17E^3$ (91.37, $2.26E^3$)	0.53	0.036 *
		1	$1.04E^3$ ($-4.35E^2$, $2.52E^3$)	0.47	0.15

Metabolite was entered as area normalized to urinary creatinine and the internal standard (methionine-d3). Model 1 was adjusted for age, gender, and baseline systolic blood pressure. CI, confidence interval; FMD_{BA}, brachial artery flow-mediated dilation; SBP, systolic blood pressure * $p<0.05$