Metabolome response to glycemic load in a randomized, controlled, crossover feeding trial in humans

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ABSTRACT

Background: Observational studies show habitual low glycemic load (LGL) dietary patterns, compared with high glycemic load (HGL), to be protective against chronic conditions such as cancer, cardiovascular disease and obesity.

Objective: To investigate the metabolomes' response to glycemic load as measured in plasma and urine. We expected to differentiate between diets and see a shift in energy usage with reduced beta-oxidation and reduced inflammation after LGL.

Design: A subset of 20 participants from a previously completed controlled crossover feeding trial had metabolomics conducted on12hr fasting blood draw and 24hr urine collection from the last day of the HGL and LGL interventions. Plasma analysis was done using LC/MS, urine using LC/MS and GC/MS. Student's paired t-test, cluster and pathway analysis were conducted. Established questionnaires for mood and energy were also conducted at baseline and end of intervention

Results: 12 plasma metabolites were significantly altered between the diets; only kynurenate remained significant after FDR. 70 urine metabolites differed significantly after FDR. In both samples amino acids represented largest portion of altered metabolites. Biomarkers suggest tendency for increased depression and reduced energy after HGL, reflecting subjective measurements of mood and fatigue.

Conclusion: Clear separation was detected in plasma and urine after diets of differing GL; however no clear pathway or trend identified to suggest protective effect of LGL diet with respect to cancer or cardiovascular disease. Biomarkers and subjective measures suggest HGL results in more depressive symptoms and reduced energy while LGL reduces fatigue.

CONTENTS

INTRODUCTION

Chronic conditions, such as obesity, cancer, and cardiovascular disease (CVD), collectively account for a third of the deaths in the United States.[1,2] Numerous epidemiologic studies find low glycemic load (LGL) diets, characterized by consumption of foods that result in a relatively small blood glucose increase,[3] are associated with a reduced risk for obesity[4], some forms of cancer[4,5], diabetes[4], CVD[4] and coronary heart disease (CHD).[4,6] In contrast, the increased insulin response associated with high glycemic load (HGL) diets and oxidative stress associated with subsequent post-prandial hyperglycemia of HGL diets, are relatively well accepted and thought to contribute to the increased negative health outcomes associated with chronic HGL diets.[5,7] Less well understood are mechanisms by which metabolic changes induced by an LGL diet may result in reduced disease risk. In this study, we characterize the metabolic response to glycemic load (GL) in healthy adults. With a deeper understanding of how GL affects the metabolome, we aim to highlight areas for future research in order to elucidate mechanisms by which LGL alters disease risk, as seen in observational studies.

Metabolomics, a field initially applied to drug metabolism research, is now increasingly being applied to nutrition. As the study of a broad range of metabolites in biological samples such as plasma, urine, tissue or saliva, it can guide research related to the effects of LGL and HGL diets. Metabolomics is most commonly used to identify metabolic changes resulting from different conditions, such as a diseased or non-diseased state, or before and after a drug treatment.[8] As an emerging "omic" that describes the most transient level of an organism's biochemistry, it complements the genomics, transcriptomics, and proteomics disciplines and can illuminate consequences of environmental exposures (e.g., food), gene expression, and their combined implications.[9]

In nutritional research, metabolomics has been used to identify biomarkers as a new dietary assessment tool[10], validate food frequency questionnaires (FFQs)[11], and in dietary intervention studies to elucidate dietary effects on metabolic pathways.[12] A few studies have used metabolomics to identify biomarkers associated with glycemic index (GI) or GL[13,14] to aid dietary assessment and validate FFQs. However, no study has investigated changes in the metabolome based on dietary GL.

Several explanations have been proposed to explain the protective effects of LGL diets. In addition to avoiding the hyperglycemic and hyperinsulinemic oxidative stress of HGL diets, higher fiber content is a suggested means by which LGL mitigates chronic disease risk. While LGL diets are inherently higher in dietary fiber, human studies have shown increased fiber alone does not always result in the same degree of reduction in chronic disease risk as does reducing the GL of the diet.[5] The protective effects of LGL beyond increased fiber represents an important gap in our understanding of the metabolic effects of differing GL. Our study evaluates how HGL and LGL diets modify the metabolome using a metabolomics approach on previously collected specimens from a controlled feeding trial designed to test the effects of high and low GL.

The first aim of this study was to identify differences in plasma metabolite abundances in response to diets differing in GL. Our hypothesis is that LGL diets influence metabolic pathways differently than do HGL diets and that these effects will result in differences in metabolite abundances between the two diets.

The second aim of this study was to identify differences in plasma metabolite concentrations in energy and inflammation pathways, specifically: Krebs cycle, gluconeogenesis and glycolysis, and pathways associated with inflammation. We hypothesize that, when considered at the pathway level, metabolite concentrations evaluated as a group will show a differential response

to LGL and HGL with energy showing a shift away from beta-oxidation and towards the Krebs cycle, and inflammation pathways showing reduced activity after the LGL intervention.

The final aim of this study was to compare the altered pathways in plasma to urine, in order to further elucidate potential metabolic differences. We hypothesize that data from urine specimens will mirror differences in the same metabolic pathways, as determined in plasma.

Ultimately, our goal was to identify the mechanisms by which foods differing in GL alter metabolic pathways. This, in turn, may improve our understanding of how LGL diets reduce the risk for chronic disease as demonstrated in observational studies. Insights from this research could aid development of more detailed guidelines for healthy eating.

PARTICIPANTS AND METHODS

The metabolomics analysis was conducted in samples derived from the "Carbohydrate and Related Biomarkers" study (CARB) conducted between June 2006 and July 2009 at the Fred Hutchinson Cancer Research Center (FHCRC). CARB was a cross-over dietary intervention with two 4-week feeding periods of HGL and LGL diets given in assigned random order.[15] A four-week wash-out period took place between the two intervention periods. Metabolomics analysis was conducted on plasma and urine collected from a subset of 20 participants at the end of each intervention period.

Participants and Study Design

Non-smoking, healthy individuals between the ages of 18-45 years were recruited around the Seattle area. Exclusion criteria included impaired fasting glucose (fasting blood glucose ≥ 5.6 mmol/L), physician-diagnosed condition requiring restricted eating, food allergies, regular use of hormones or anti-inflammatory medication, pregnant or lactating, and heavy use of alcohol (>2 drinks per day). Of the 89 eligible individuals, 84 were enrolled, 2 dropped out before the study

started and 2 completed only 1 intervention period. In the parent study only the 80 participants who completed both interventions were included in the final analysis. The study population was 50% male and evenly distributed between normal weight (BMI >18.5 and ≤ 25.0) and overweight/obese (BMI ≥ 28.0 and ≤ 40.0).[16] Diet order was randomly assigned, with half the participants receiving the HGL diet first. Each intervention was four weeks long, with a four week washout period in-between.[15] A subset of 20 individuals was selected for metabolomics analysis. The group was randomly selected to represent the larger study population--they were 50% male, evenly distributed between normal and overweight, and half receiving the HGL diet first.

Nutritional Intake

The study diets were based on a repeating seven-day menu, with identical distribution of macronutrients for both the HGL and LGL diets (see Table 1 for sample menu). Only GL and fiber differed notably in the diets, with the LGL diet being half the GL of the HGL intervention (125 and 250 GL/d respectively) and twice the fiber (55 and 28 g/d respectively). Total GL for each mixed meal was calculated by multiplying the grams of carbohydrate in each food by that foods' glycemic index (GI) value. The diets were isocaloric for each individual, although minor adjustments were made as necessary to keep participants weight stable during the intervention (Table 2).

All food was provided by the FHCRC during the intervention, with dinner consumed under supervision at the Center and the next day's breakfast and lunch taken home for consumption. On Fridays, participants received all weekend meals. During the washout period individuals returned to their habitual eating patterns.[15]

Sample Collection And Analysis

At baseline, height and weight measurements were recorded for assignment to obese (BMI > 28 kg/m2) or normal weight (BMI < 25 kg/m2) category. Those with BMI 25-27.9 kg/m^2 were excluded to ensure sufficient contrast between the groups. Whole body Dual-energy X-ray absorptiometry (DEXA) scans (GE Lunar DPX-Pro) were also completed for each participant. Weight measurements were taken regularly throughout the study period, with adjustments made to food provided as necessary to keep participants weight stable.[15] 12-hour overnight fasting blood draws were taken for each participant on Day 1 and Day 28 of both treatment periods. A 24-hour urine sample was also collected, starting on Day 27 of both periods.

Metabolomics analysis

Metabolomics analysis of plasma was completed at the University of Washington's Northwest Metabolomics Research Center. Targeted metabolomics analysis on the plasma of a subset of 20 participants was carried out using liquid chromatography mass spectrometry (LC/MS) in both positive and negative ion modes against 155 known metabolite peaks.

All plasma samples were prepared at the same time. A standard protocol was used calling for 25 µL serum and 150 µL high performance liquid chromatography (HPLC) grade methanol in an Eppendorf vial to be vortexed for 2 minutes. After 20 minutes storage at -20C˚ the samples were then centrifuged at 14,000 rpm for 10 minutes. A fixed volume of 150 µL of the supernatant were collected and placed in a new Eppendorf vial with another 300 µL HPLC grade methanol, then vortexed for 10 minutes and centrifuged for 10 minutes at 14,000 rpm. A fixed volume of 250 µL was collected and combined with the original Eppendorf vial. Samples were then dried at 30C in a SpeedVac for 2 hours.

The samples were run through the LC/MS in 2 sequential batches, each containing 2 quality control (QC) samples so batch variation could be assessed and adjusted for. Prior to each LC run,

samples were reconstituted with 100 uL 5 mM ammonium acetate in 95% water/5% acetonitrile + 0.5% acetic acid, and filtered through 0.45 μm PVDF filters (Phenomenex, Torrance, CA) prior to LC-MS analysis. The LC system was composed of two Agilent 1260 binary pumps, an Agilent 1260 auto-sampler and Agilent 1290 column compartment containing a column-switching valve (Agilent Technologies, Santa Clara, CA). Each sample was injected twice, 10 μL for analysis using negative ionization mode and 2 μL for analysis using positive ionization mode. Both chromatographic separations were performed in reverse phase (RP) on Thermo Accucore PFP columns (150 x 2.1 mm, 2.6 μm particle size, Thermo Fisher Scientific Inc., Waltham, MA). The flow rate was 0.250 mL/min, auto-sampler temperature was kept at 4℃, and the column compartment was set at 40℃. The mobile phase was composed of Solvents A (5 mM ammonium acetate in H2O + 0.5% acetic acid + 0.5% acetonitrile) and B (acetonitrile + 0.5% acetic acid + 0.5% water). After chromatographic separation, MS ionization and data acquisition was performed using AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) with electrospray ionization (ESI) source. The collision gas was 99.99% pure nitrogen. The data gathered through the multiple reaction monitoring (MRM) was integrated using MultiQuant 2.1 software (AB Sciex, Toronto, ON, Canada).

Targeted metabolomics analysis was performed on the urine at Metabolon, Inc. (Durham, NC) by LC/MS in both positive and negative ion modes against 278 known metabolite peaks, and gas chromatography mass spectrometry (GC/MS) in positive ion mode. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an ESI source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed

concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion.

The samples for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp is from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis.

Statistical Analysis

Each individual had samples collected at the end of both intervention periods for a total of 40 samples run through LC/MS for plasma, and 40 samples through LC/MS and GC/MS for urine. One plasma sample was compromised during sample preparation, so plasma samples from that individual were excluded and final analysis was conducted on the remaining 19 individuals.

Metabolite concentrations were naturally log-transformed for normality and statistically significant metabolite differences ($p<0.05$) in plasma were determined by performing Student's paired t-tests in Stata (v12.1, College Station, TX), then Benjamini Hochberg method was used to adjust for false discover rate (FDR)[17]. Relative concentrations of metabolites in response to the diets were calculated by dividing metabolite intensity from the LGL diet by metabolite intensity from the HGL diet. In this way the relative abundance of metabolites could be

considered in a clinical context. While the study protocol attempted to achieve weight maintenance most participants experienced a small amount of weight change. To ensure these minor fluctuations did not impact the findings we conducted a linear, mixed regression model. This adjusted for individual baseline metabolite levels as a random effect, and tested for the effects of weight change and diet on metabolite concentrations modeled as a fixed effect, using R (v3.01, with package lme4 1.0). This approach was used because each individual has intrinsic metabolite baseline levels that impact the metabolite measures after the interventions. The mixed model gives a more precise estimate for the other terms in the model (weight change, diet, sex, etc), by accounting for each individual's intrinsic baseline – the random effect. It also provides valid standard error estimates on the fixed effects in the presence of repeated measures of each individual. . The HGL metabolite response was used as the initial measurement, or random intercept, to model the repeated measurements for each participant. Metabolites were also adjusted for fat distribution as determined by the android to gynoid body fat percent ratio, using DEXA data.[18]

To consider differences occurring beyond the level of individual metabolites, pathway level analysis was done using the Global test in R (v3.0.2).[19] A priori pathways assessed were Krebs cycle, gluconeogenesis and glycolysis for energy. The tryptophan pathway was the only inflammation-related pathway with sufficient metabolites to test. We evaluated the pathways using 5 to 8 metabolites from the respective pathways, as identified in the Human Metabolome Database (HMDB)[20] and Kyoto Encyclopedia of Genes and Genomes (KEGG)[21] databases. We considered doing analysis on pathways related to adiponectin and CRP, given the findings in the parent study that these were influenced by diet;[15] however we did not have sufficient pathway data. Further to the paired t-tests, we also investigated fitting a penalized logistic regression

model in order to estimate possible associations between the diets while considering the entire set of metabolites using MatLab (R2013b, Natick, MA). Penalized regression is an extension of ordinary least-squares and logistic regression in which additional constraints are imposed in order to allow for fitting a model that has more predictor variables than subjects.[22] We specifically applied the generalized linear model Lasso model,[23] which has the effect forcing most of the regression coefficients to be zero, hence reducing the number of candidate metabolites.

To determine if the diets resulted in systemic differences in metabolic responses, principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) plots were generated using MetaboAnalyst 2.0 (http://www.metaboanalyst.ca/). Plasma data showed between-batch variability of 7%, and were therefore normalized using QC data for this analysis.

Urine concentrations were normalized by osmolality, a mathematic process whereby urine osmotic pressure is equalized across samples to ensure standard concentration for each assay. These adjusted values were naturally log-transformed to achieve a normal distribution. Student's Paired t-tests and the Benjamini Hochberg[17] FDR method determined metabolites significantly altered between interventions. PCA and PLS-DA analysis was done using MetaboAnalyst 2.0.[24]

Given that we measured both plasma and urine, albeit on different platforms, we had the opportunity to compare the results. While the targeted metabolomics platforms for plasma and urine measured different metabolites, 45 were measured in both, of which 35 were present in most urine and plasma samples for all participants. Finally, sub-pathways containing significantly altered metabolites in plasma were compared to sub-pathways with significantly altered metabolites in urine to further elucidate potential shifts in the metabolome as a result of diet.

RESULTS

Characteristics for the 20 CARB participants selected for metabolomics analysis stratified by sex are given in Table 3. As in the parent study, there are an equal number of men and women evenly distributed between normal and overweight.

Plasma metabolomics

PCA of the plasma yielded no results (data not shown). However, PLS-DA showed clear separation between the diets by the primary and secondary components (Figure 1). Components use eigenvectors to distinguish between the interventions. These mathematical models contain all detected metabolites, each with a different loading - or weighting – to identify metabolites that can be used to distinguish between the diets. The primary component's top contributors were cystathionine, trimethylamine N-oxide (TMAO), kynurenate, glycocholate, adenylosuccinate, and glucose-3-phosphate; they all had variable of importance in projection (VIP) scores >2. The secondary component's top contributors based on VIP scores of >2 were cystathionine, hippuric acid, linoleic acid, TMAO, malonic acid, kynurenate, glucose-3-phosphate, and cystamine. Of the 155 metabolites measured, 125 plasma metabolites were detected, with 12 altered significantly between the LGL and HGL interventions ($P<0.05$; Table 4). Relative abundance ratios ranged from 0.83-1.66 with a near equal number increasing as decreasing after the LGL diet relative to the HGL (Figure 4). After adjusting for FDR,[17] only kynurenate remained significant. Adjusting for weight change, sex and fat distribution did not alter these findings. Pathway analysis for the Krebs cycle, glycolysis and gluconeogenesis, and tryptophan did not yield any significant results. Penalized regression found 11 metabolites altered between diets, 5 of which were also significantly altered according to the Student's paired t-test (noted on table 4)

Urine metabolomics

PCA analysis showed clear separation between the diets (Figure 2). The top contributors to the primary component based on loadings were caffeine, 5-acetylamino-6-formylamino-3-methyluracil, and paraxanthine, none of which were significantly affected by the interventions. The secondary component's top contributors based on loadings were stachydrine, xylose, 1,6-anhydroglucose, and chiro-inositol, all of which were significantly affected by dietary GL. As expected the supervised PLS-DA also shows clear separation between the diets (data not shown). Of the 278 metabolites measured, 274 urine metabolites were detected, and 103 were statistically significantly altered between the LGL and HGL interventions. Of these, 70 remained statistically significant after adjusting for FDR (Table 5). Relative abundance ratios ranged from 0.69 to 15.18, with most metabolites increasing after LGL relative to HGL (Figure 5).

Combined plasma and urine metabolite pathway mapping

Of the 35 metabolites present in both urine and plasma for all participants, 14 had significant but weak associations between plasma and urine (Table 6); these were primarily amino acids and nucleotide metabolites (Figure 3). We looked at the sub-pathways of significant plasma metabolites and compared them to sub-pathways of significant metabolites in urine. When mapped back this way we found 50% of pathways in plasma were also represented in urine. In amino acid metabolism these were the creatine, histidine, tyrosine, and tryptophan pathways, in lipid metabolism they were the carnitine and glycerophospholipid pathways.

In both plasma and urine, amino acid metabolites showed greatest response to diets and represented over a third of the alterations. In urine, another ~20% of the modifications were related to carbohydrate metabolism, none of which were significantly altered in the plasma. Of the significant amino acids in urine, a third were involved in valine, leucine and isoleucine metabolism and mostly increased modestly after the LGL diet. These pathways were not

represented in the significant plasma metabolites. Phenylalanine and tyrosine metabolites, along with threonine, glycine, and serine metabolites, made up another third of the significantly altered amino acids in urine. Tyrosine, glycine, and serine pathways were represented in the significantly altered plasma metabolites. All the significant urine nucleotides were purines and increased after the LGL diet. There were no significantly altered plasma nucleotides.

DISCUSSION

We found 12 plasma metabolites that were significantly different between the HGL and LGL diets. About half of these metabolites were more abundant after the LGL diet and half more abundant after the HGL diet; however relative abundance ratios were small, ranging from 0.83 to 1.66, such that these findings may not be clinically relevant. While these changes of 10-70% are larger than the intra-assay variation of 8%, as determined by the correlation of variance, many are still small enough that we cannot rule out they occurred due to chance or error. Alternatively these small relative abundances could be an artifact of plasma's homeostatic regulation and suggest that small changes compound over a lifetime to result in the effects associated with differing chronic dietary patterns. We only had a single end of intervention sample to test for each individual therefore intra-individual variation could not be determined. The 12 plasma metabolites were in several different pathways and, contrary to our hypothesis, did not apparently reflect any change in energy source as a result of different GL. Further analyses, focused on energy and tryptophan pathways, showed no overall pathway differences between the diets. While no singular pathway appears altered between the diets, 7 of the 12 altered metabolites are in amino acid metabolism. It is worth noting that 40% of the target metabolites for plasma were amino acid metabolites, which may influence their abundance in the significant metabolites. The PLS-DA showed clear separation between the diets with several metabolites

having VIP scores >2 in both the primary and secondary components including TMAO, and kynurenate, which were also significantly altered between the interventions. In the exploratory penalized regression, kynurenate was among those indicated as differing between diets, and was the only metabolite to maintain significance after FDR adjustment.

Amino acids involved in tryptophan and tyrosine metabolism were relatively more abundant in the plasma after the LGL diet. After the HGL diet, metabolites of histidine and taurine were higher. Taurine and histidine are excitatory amino acid with increased plasma concentrations associated with depression.[25] Reduced free plasma tryptophan, as observed on the HGL diet, has also been associated with increased depression,[26] while higher levels of free tryptophan in the blood, as found after the LGL diet, has been associated with appetite-suppression.[27] Participants of the CARB study had mood, energy and depression monitored at baseline and at the end of each intervention using the validated Profile of Mood States (POMS)26 and Center for Epidemiologic Studies Depression Scale (CES-D)27 protocols. After the HGL diet participants had reduced vigor and increased depressive symptoms while after the LGL diet participants had reduced fatigue. Depressive symptoms were also reduced after the LGL intervention, although not significantly (data not published). Similar to the parent study findings on dietary intervention effects on inflammatory cytokines, mood and energy effects were greatest in the overweight and obese groups (data not published). The other plasma metabolites differing significantly between diets included acetylcholine, a glycerophospholipid metabolite involved in neurotransmission; methyl succinate, an organic acid derivative; TMAO, a liver oxidized microbial end product; nitrosine, a reactive nitrogen species; and carnitine, a carrier protein involved in fatty acid transport .

In urine 25% of the metabolites were significantly affected by the interventions and in the unsupervised PCA analysis the 2 diets showed clear separation. The differing strength of the GL effect seen in plasma versus urine is to be expected given exogenous metabolites, such as food, are only in the blood briefly while many food metabolites end up excreted in the urine. Plasma metabolites are highly regulated to maintain homeostasis and so large changes in relative abundances would not be expected.[28] This probably explains the larger ratios in metabolite abundance seen in the urine compared with plasma.

The different targeted panels used for plasma and urine had an overlap of 35 metabolites with representation in both plasma and urine for all participants. Of these, only 3 metabolites that were statistically significantly different between diet interventions overlapped in plasma and urine: kynurenine, carnitine, and creatine. After the LGL diet, as compared to after the HGL diet, kynurenine and carnitine were higher in the plasma and lower in urine, while creatine was lower in the plasma and higher in urine. It is unclear what, if any relevance this has biologically. Insights from 3 metabolites can only be highly speculative, but a relative increase in plasma kynurenine and carnitine is generally considered beneficial, as they are associated with reduced inflammation[29] and increased antioxidant activity[30] respectively. Less creatine in the plasma and more in the urine might suggest a shift in energy metabolism away from beta-oxidation in the LGL diet; however, it is worth noting that we have no other metabolites to support this and therefore we recognize that this is only speculation.

When we performed the regression analysis to compare biological specimen without consideration for diet, a third of the metabolites had significant relationships between plasma and urine. Of the 6 nucleotides tested, 5 were purines and 1 was a pyrimidine; they all showed a significant relationship between plasma and urine. All of them were more abundant in urine after

the LGL intervention, in the plasma half increased and half decreased after the LGL intervention. Purines play a role in energy, signaling and for pyrimidine, RNA/DNA production too. These important molecules are mostly salvaged and, while they can be made de novo, are less dependent on nutrition than recycling.[31] However changes in nucleotide metabolites might indicate shifts in the salvage pathways in response to dietary availability or cellular response to the diet altering energy, signaling or RNA/DNA production. Most of the other metabolites with a significant relationship between plasma and urine were amino acids, mirroring the results for plasma and urine alone.

To our knowledge, this is the first study to use metabolomics to broadly interrogate the effects of GL on the metabolome. While not specifically evaluated in other studies of GL, metabolomics has been applied in the context of glycemic index, fiber and whole grain intake, mainly for biomarker discovery. Rasmussen et al[13] performed NMR on urine after 77 overweight participants consumed a low calorie diet for 8 weeks followed by either high or low GI diets for 6 months, with the intent of establishing biomarkers for habitual GI intake. The high GI and low GI diets differed by 15 points of GI while keeping carbohydrate intake within a similar range, although there were additional groups with higher protein and subsequently lower carbohydrate such that the total GL for the interventions had a broad range. Based on clear discrimination between the diets in PLS-DA analysis the authors concluded that formate, a one-carbon product of gut microbiome fermentation, found higher after the high GI diets, could be used to indicate dietary GI. No association was found between urinary C-peptide and GI, which the authors had hypothesized.[13] Johansson-Persson et al[14] studied the effects of high and low fiber diets in a human crossover intervention, with the intention of finding biomarkers for fiber intake using metabolomics. 25 participants consumed a daily average of 48 or 30g fiber for 5 weeks, with a 3

week washout period between interventions. Specific alteration in metabolic pathways were not assessed, however untargeted LC/MS of plasma was able to identify 2 new markers of dietary fiber intake, 2,6-dihydroxybenzoic acid and 2-aminophenol sulfate, which could be used as plasma biomarkers of dietary fiber.[14] In another study, 17 men with prostate cancer consumed 485 g/d of whole grain rye, rye bran and refined white wheat product for 6 weeks each in a crossover design.[32] A number of metabolites were more abundant in plasma after consumption of the rye bran intervention, including 3-hydroxybutyric acid, acetone, betaine, N,N-dimethylglycine and dimethyl sulfone. The authors concluded that metabolites indicated a protective shift from an anabolic to a catabolic energy state, reduced insulin load, and reduced homocysteine that could indicate mechanisms for the beneficial effects of whole grain diets.[32] Our plasma data showed betaine was increased and dimethylglycine decreased after the LGL diet, which include more whole grains, however neither reached statistical significance.

Diets higher in fruits and vegetables and most whole grains tend to be lower in GL. We previously characterized the effects of a high versus a low phytochemical diet after 10 healthy adults consumed a diet high in cruciferous vegetables, citrus fruits and soy for two weeks compared to a diet devoid of fruits and vegetables. In addition to urinary markers of dietary intervention, global metabolomics found fatty acid and niacin metabolites were more abundant after the high phytochemical diet while several acylcarnitines and amino acid metabolites were more abundant after the low phytochemical diet.[33] The current study's targeted metabolomics measuring 151 metabolites did not have similar metabolites to the 3,000+ of this untargeted approach and we therefore cannot make a direct comparison. However, this study also points to possible alterations in energy source between diets of higher and lower GL.

Metabolomics has been used in other dietary contexts as well. For example, Tulipani et al studied the effects of consuming 30 g/d mixed nuts compared to a control diet in 42 adults with metabolic syndrome. In addition to potential markers of nut intake, they found increased excretion of a number of other urinary metabolites including fatty acid, microbially-derived phenolic, and serotonin metabolites.[34] In nutrition research, metabolomics has been used to establish biomarkers for dietary intake, which commonly largely relies on self-reporting, and so could aid in reducing this source of bias and confounding. Additionally, emerging research using metabolomics to reveal response to diet has shown interesting result that require more studies with larger samples to validate.

Plasma metabolites altered between LGL and HGL

As this is an exploratory analysis we looked at all metabolites with statistically significant ($p<0.05$) changes in response to the interventions to gain insight into potential dietary effects of differing GL, even though metabolite ratios were small and only kynurenate was still significant after FDR analysis.

Kynurenate

Plasma kynurenate was elevated by ~ 50% in the LGL diet compared with the HGL while its precursor, tryptophan, was not higher in the LGL, therefore the increase is likely due to increased flux through the pathway. As tryptophan is metabolized it forms kynurenine that degrades to either quinolinate or kynurenate, which respectively are agonists and antagonists to the N-methyl-D-aspartate (NMDA) receptors that mediate inflammation.[35] A shift towards the kynurenate arm of the pathway suggests a reduced inflammatory effect is experienced in the LGL dietary intervention.[36] While the effect of tryptophan metabolites is most pronounced in the cerebrospinal fluid, NMDA receptors are also found in the peripheral tissue, where these plasma

metabolites elicit the same excitatory and inhibitory response.[29] When considering all plasma metabolites in the tryptophan pathway, regardless of significance, a consistent pattern is seen of a shift away from the quinolinate and increased flux through the kynurenate arm of the pathway. Although LC/MS cannot determine absolute concentrations in the plasma, animal studies suggest the ratio between kynurenate and quinolinate is what determines its protective effects; thus, increases kynurenate relative to quinolinate, as seen after the LGL diet, would be hypothesized to be beneficial.[37]

Acetylcholine

Acetylcholine was ~ 15% lower in the LGL compared to the HGL diet. Acetylcholine plays a role in neurotransmitter signaling in the central and peripheral nervous systems as part of the cholinergic anti-inflammatory response and is also an important constituent of all cell membranes.[38] In the peripheral nervous system acetylcholine has anti-inflammatory effects by inhibiting in macrophages the production of tumor necrosis factor alpha (TNF-α) and other pro-inflammatory cytokines.[39] The implications of acetylcholine concentration in plasma, however, are unclear. It is notable that pyroglutamic acid, which is associated with acetylcholine release, was also slightly lower after the LGL diet.

Methyl succinate

Methyl succinate was ~15% higher in the LGL diet compared with the HGL. Methyl esterified succinate acts on pancreatic beta-cells as an insulin secretagogue.[40] In the presence of diabetes-related drugs, such as metformin, methyl succinate has been observed to donate electrons to complex II in the electron transfer chain; this mitigates the drug activated AMP-Kinase inhibition of complex I, which ultimately reduces beta-cell death.[41] It is possible that this

endogenous metabolite plays a role in the protective effect of LGL through increased electron transfer chain efficiency.

An ancillary CARB study tested post-prandial insulin response to GL by giving a test HGL or LGL breakfast on day 28 of the interventions, with GL corresponding to the preceding diet. Insulin response was 27% lower after the LGL test breakfast compared with the HGL meal.[16] The likelihood of methyl succinate inducing insulin secretion or reducing cell death is not verifiable in these results.

Trimethylamine N-oxide

TMAO was ~70% higher at the end of the LGL intervention than the HGL. TMAO results from the gut bacterially-derived trimethylamine (TMA) being oxidized in the liver. The rate of TMA to TMAO conversion is determined by a combination of genetics, enterotype and substrate availability;[42] given the crossover nature of this study we can consider the differences to mainly be due to substrate availability although diet itself may alter the gut microbial community.[43] Precursors for TMA include carnitine, choline and betaine,[44,45] all of which are absorbed by a combination of active and passive transport in the small intestine and only reach the microbiota responsible for their conversion to TMA in the cecum and large intestine when doses are high enough to saturate these transport mechanisms.[46-48] Higher dietary content of these dietary constituents may account for the higher substrate availability. Adjusting foods to result in differing GL while keeping macronutrients constant resulted in the LGL diet having less meat than the HGL as vegetables associated with lower GL tend to have higher protein content; carnitine was therefore higher in the HGL diet. While choline was equal between the diets, the inclusion of more wholegrains and leafy greens in the LGL intervention resulted in ~80% higher betaine content due to its abundance in wheat germ and spinach.

The relationship between red meat and CVD is well accepted with carnitine derived TMAO indicated as contributing to the effect due to its interference with cholesterol clearance.[45,49] Conversely wholegrains and spinach are widely accepted as healthy and to have antioxidant properties[50] with betaine specifically identified as contributing to the effect through donation of a methyl group to homocysteine.[51] A review of studies considering dietary betaine and choline find no association with increased intake and CVD risk, while they are associated with a reduced inflammation and other risk factors.[52] While previous studies suggestion carnitine promotes atherosclerosis due to its association with TMAO, our finding that it is also increased after a diet lower in meat but higher in wholegrain and spinach needs further investigation. Solanky et al. found TMAO increased in a metabolomics analysis after a dietary intervention with soy, another food ascribed health benefits.[53] It is unclear whether TMAO derived from the carnitine in meat differs from TMAO derived from choline and betaine in plants. Perhaps more a plausible insight from these findings is that it is not an individual metabolite from a food but the combined effect of all components present that result in the effect of carnitine derived TMAO compared to betaine or choline derived TMAO.

Cystamine

Cystamine was ~20% lower in the LGL diet compared to the HGL. In healthy humans, cystamine is known to inhibit gamma-glutamylcysteine synthetase, the first enzyme in the synthesis of glutathione (GSH), a powerful antioxidant that protects against reactive oxygen species (ROS) and other toxic compounds.[54] Given the small relative change between the diets it is not possible to determine if reduced cystamine levels in the LGL diet could potentially indicate more antioxidant protection through the GSH system due to reduced inhibition.

Methylhistamine

Methylhistamine was ~10% lower after the LGL diet compared to the HGL diet. Histamine has a short half-life, and the more stable methylhistamine is considered a better measure of histamine release.[55] Histamine mediates allergic reaction and immune response through release from mast cells. However, most inflammatory cells have histamine releasing factors so it is present in any inflammatory state.[56] The reduced level of plasma methylhistamine could be an indicator of reduced inflammatory processes on the LGL.

Creatine

Creatine was ~10% lower after the LGL diet compared to the HGL diet. Its precursors, glycine, methionine and arginine had LGL to HGL ratios of 1, 0.9, and 1 respectively between the interventions. While creatine is found mostly in muscle, muscle has no ability to make it so takes it up from the blood where it comes either from dietary sources or is synthesized in the liver.[57] Once in the muscle, it is phosphorylated to phosphorylcreatine and used as a source of quick-release energy, such as in fast twitch muscle.[57] In healthy adults, it is not clear if a small change in response to diet has clinical implications.

Proline

Proline was ~10% higher after the LGL diet compared to the HGL diet. The food consumed on the diets had similar proline content, so it is unlikely changes are due to substrate availability. Proline plays an important role in saliva due to its multivalent function and ability to neutralize tannins. It is a significant component of collagen, and protects proteins in general against nonspecific proteolytic degradation. Proline also mediates multiple aspects in the immune system, including potent antimicrobial and cytotoxic effects on bacteria, enhancing macrophage phagocytosis, reducing ROS damage in the epithelium, and generally enhancing immune

response.[58,59] As a non-essential amino acid with many roles in the body it is hard to interpret exactly what increased plasma levels indicate in terms of any potential reduction in disease risk.

Homovanillate

Homovanillate was ~20% higher after the LGL diet compared to the HGL diet. Homovanillate is a catecholamine metabolite and plasma concentrations have been associated with dopaminergic activity in the central nervous system.[60] In healthy individuals, mental stress[60] and tobacco use[61] are associated with decreased levels of homovanillate while moderate exercise can marginally increase it for a brief amount of time.[62] Given the cross-over design of the study, it is unlikely that differences in tobacco use or physical activity are the reasons for the changes. Diets high in monoamines have also been shown to increase plasma homovanillate to a greater degree and for a longer duration than exercise.[62] Examples of foods containing monoamines are bananas, unsweetened orange juice, certain cheeses, and tomato.[62] Both intervention menus included cheese and tomato, neither had bananas; only the LGL diet included orange juice. It is possible the increased levels of homovanillate are due to substrate availability in the diets. It is not clear whether this alone indicates a protective effect as a result of the LGL diet.

Pyroglutamic acid

Pyroglutamic acid was ~10% lower after the LGL diet compared to the HGL diet. As a glutamate derivative, plasma concentrations of pyroglutamic acid have an inverse relationship with glutathione production, where increases indicate reduced detoxification activity via glutathione.[63] In animal models, injecting pyroglutamic acid resulted in increased acetylcholine and GABA production.[64,65] In the LGL intervention, acetylcholine production was also reduced. It is unclear if this small reduction in pyroglutamic acid infers increased antioxidant activity, or what implications there are for chronic disease risk.

Nitrotyrosine

Nitrotyrosine was ~15% higher after the LGL diet compared to the HGL diet. As a metabolite of myeloperoxidase, nitrotyrosine indicates an inflammatory state mediated by neutrophils and monocytes and causes oxidative stress that has been associated with CVD.[66] Several animal studies have shown an association between nitrotyrosine and endothelial nitric oxide synthase (eNOS) activity.[67,68] The nitric oxide produced by eNOS has been found to play a role in energy metabolism, mitochondrial biogenesis, and to trigger adiponectin synthesis in adipocytes.[69] Increased adiponectin among overweight and obese participants after the LGL diet was one of findings of the parent CARB Study .[15] Generally, increased nitrotyrosine is considered a health risk.[66] It is interesting to see that carnitine, which mitigates damage from nitrotyrosine is also increased in the LGL diet.

Carnitine

Carnitine was ~10% higher after the LGL diet compared to the HGL diet. Carnitine is found in animal products such as meat and dairy. While both diets had an average daily protein intake of 90 g, the source of protein differed. The HGL diet had a higher animal protein intake than plant protein at 57 and 33 g respectively, while the LGL diet was more balanced at 47 g animal and 44 g plant protein. While the ratio between the diets is close to 1, it is interesting to note then that the LGL diet had less carnitine in it yet resulted in higher plasma carnitine. The body can also make carnitine from lysine and methionine, which were also both slightly lower in the LGL intervention. This suggests increased carnitine after the LGL diet is not due to substrate availability but changes in metabolism. In healthy subjects, increased carnitine has been associated with increased total antioxidant capacity by enhancing activity in antioxidant enzymes including superoxide dismutase, glutathione peroxidase, and catalase.[70] Specifically, it has been seen to reduce the effects of nitrotyrosine, which was also increased in the LGL diet.[30]

Urine

In the urine 70 of the 274 metabolites were significantly altered in the diets after adjusting for FDR. Of the 11 metabolites measured in the Krebs cycle 8 of them were increased in the LGL diet, 4 of them significantly. Only α-ketoglutarate, isocitrate and succinylcarnitine decreased in LGL, and this decrease was not significant. Conversely, the two fatty acid metabolites measured, propionylcarnitine and hexanoylglycine, were down after LGL, the former significantly. There were no beta-oxidation metabolites were in the urine panel so we were unable to investigate a potential shift away from fat to carbohydrate as an energy source in LGL due to a more constant level of plasma glucose. Additionally secretion of bile acid metabolites were increased in LGL, potentially indicating a mechanism for this diet being protective of CVD. However, it is not possible to know with these data if this is due to GL overall or only the fiber inherent in the diet.

Study strengths and limitations

This study had several strengths including the crossover design where individuals act as their own control. The 4-week duration is also relatively long for a controlled nutritional intervention where all foods and most beverages are provided. The participants largely maintained their weight on a standardized diet with all food supplied that held macronutrients constant while only differing in GL, and had good compliance (97% consumed > 90% of provided food with no difference in compliance between interventions). Targeted metabolomics, unlike global metabolomics, also enabled us to be more certain about the identities of the metabolites; however, we were only able to measure 125 metabolites whereas global metabolomics can measure thousands of metabolites. To our knowledge this is the only metabolomics analysis of the effects of GL diets, tested under controlled dietary conditions.

A limitation of the study is that we only used LC/MS for the plasma metabolomics and some compounds, such as sugars, are not reliably detected with this method. Runs with GC/MS and

NMR could provide more detailed insight into metabolic changes between diets differing in GL in plasma. Additionally, LC/MS cannot determine absolute concentrations of metabolites, which is possible using NMR and would be useful given relatively small change in concentration to determine their biologically significance. Conducting the metabolomics for plasma and urine in different labs reduced our ability to do more robust data analysis as the labs had established different metabolite panels for their target metabolomics assays. Our study can also not separate the effects of GL and fiber, so it is not clear if the changes seen are a result of lower GL in general or differences in fiber specifically. Fitness and activity level impact the metabolome[71] and while participants were asked to keep this constant detailed data on daily physical activity was not collected so could not be adjusted for. Similar to other metabomomic studies, we were able to adjust for several factors but age, gender, and fasting status account for only a small proportion of total metabolome variation and a larger sample would be needed to reduce the role chance played in these findings.[72] Our sample size was small, although comparable to other crossover metabolomic studies, and as with any pilot study our power is limited. However, information from this pilot study will be used to support a future R01 submission for a larger RCT, including information on: methods of recruitment and compliance, estimated effect size from a randomized trial and sample size needed, and new metabolic pathways that may serve as additional biomarker endpoints.

CONCLUSION

In comparing LGL and HGL diets we found significantly altered metabolites that demonstrated a metabolic response to differing GL but indicated no shift in specific pathways to explain the protective effect of an LGL diet observed in the literature. One finding of interest was that biochemical and subjective self-reporting measures both showed lower energy and increased depressive symptoms after the HGL diet, while LGL showed reduced fatigue. Of the significantly altered plasma metabolites, most prominently represented pathways were in protein metabolism, which were consistent with the results from urine. Despite measurable separation between the diets, the magnitude of the ratios between the diets were small and could be due to chance, or indicate that small effects accumulate over long-term dietary exposure to result in the observed protective effect of LGL.

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TABLES AND FIGURES

Table 1:

Sample daily meal plans for high glycemic load (HGL) and low glycemic load (LGL) intervention diets in the CARB study. Menus were designed to contain similar food with specific items swapped out to alter glycemic load (GL) of overall meal. In this way minimal change to the diet outside of GL was achieved.

HGL Breakfast		**LGL Breakfast**	
Grape-nut cereal	Dates	All Bran	Blueberries
2% milk	Dried cranberries	2% milk	Nut crunch
Sweetener		Sweetener	Tomato juice
		Strawberries	
HGL Lunch		**LGL Lunch**	
White bread	Cauliflower	Pumpernickel	Carrots
Roast beef	Potato salad	Roast beef	Tabouli
Mayo	Ranch dressing	Mayo	Hummus
Mustard		Mustard	
Tomatoes	*Desert*	Tomato	*Desert*
Lettuce	Fruit roll ups	Lettuce	M&Ms
Onions	Jellybeans	Onions	Pears
Pickles	Apricots	Pickles	
HGL Dinner		**LGL Dinner**	
Chicken breast	Sour cream	Chicken breast	Sour cream
Green pepper	White rice	Green pepper	Tortilla
Red pepper	Taco shell (hard)	Red pepper	
Onions	*Desert*	Onions	*Desert*
Mexican sauce	Rice pudding	Mexican sauce	Chocolate mousse
Salsa	Cranberry juice	Salsa	Apple juice
HGL Snack		**LGL Snack**	
Energy bar		Dried apple	Chocolate power bar

Table 2:

Summary of macronutrient and glycemic load (GL) in high glycemic load (HGL) and low glycemic load (LGL) intervention diets. Differences in diet were minimal outside of GL (and therefore fiber) to ensure it was primarily responsible for dietary effects.

Mean daily intake	**HGL diet**	**LGL diet**
Energy, kcal (SD)	2583 (451)	2569 (478)
Carbohydrate, g (SD), % (SD)	368 (63) 57% (0.8%)	387 (71) 60% (0.7%)
Protein, g (SD), % (SD)	97 (17) 15% (0.2%)	98 (18) 15% (0.4%)
Fat, g (SD), % (SD)	86 (16) 30% (0.8%)	87 (17) 31% (0.6%)
GL (SD)	262 (46)	126 (23)
Fiber, g (SD)	28 (5)	55 (10)

Table 3:

Baseline characteristics for subset of 20 participants whose samples underwent metabolomic analysis, from the original 80 participants in the CARB study.

Demographics	**Men**	**Women**
N	10	10
Age, y (SD)	31.3 (9.2)	31.9 (9.5)
BMI group		
Normal (BMI < 25 kg/m^2)	5	4
Overweight (BMI > 28 kg/m^2)	5	6
Mean body fat %[1]		
BMI < 25 kg/m2 (SD)	28.74 (5.59)	20.57 (4.37)
BMI > 28 kg/m2 (SD)	45.58 (4.64)	36.19 (6.02)
Mean hip/waist ratio[2]		
BMI < 25 (SD)	0.74 (0.17)	1.08 (0.21)
BMI > 28 (SD)	1.03 (0.11)	1.21 (0.11)
Fasting blood glucose, mmol/L (SD)		
BMI < 25 (SD)	4.9 (0.28)	4.9 (0.41)
BMI > 28 (SD)	4.8 (0.78)	4.8 (0.65)

[1] Body fat % measured by DEXA

[2] Hip to waist ratio calculated using android body fat % divided by gynoid body fat %, from DEXA scan data and used as indication of fat distribution

Table 4:

Plasma metabolites detected as significantly altered between high glycemic load (HGL) and low glycemic load (LGL) dietary intervention using liquid chromatography mass spectrometry. Sorted by p-value, from most to least significant.

Super Pathway	Sub Pathway	Plasma Metabolite	△	Ratio[1]	P-value	
Amino acid metabolism	Tryptophan Metabolism	Kynurenate *	↑	1.46	0.0002	
Lipid metabolism	Glycerophospholipid metabolism	Acetylcholine	↓	0.86	0.0026	$p < 0.01$
Organic Acids and Derivatives	Dicarboxylic Acids and Derivatives	Methyl succinate	↑	1.15	0.0038	
Gut Bacteria Metabolite		Trimethylamine N-oxide	↑	1.66	0.0038	
Metabolism of other amino acids	Taurine and Hypotaurine Metabolism	Cystamine *	↓	0.83	0.0084	
Amino acid metabolism	Histidine Metabolism	Methylhistamine *	↓	0.88	0.033	
Amino acid metabolism	Creatine metabolism Glycine and Serine Metabolism Arginine and Proline Metab.	Creatine	↓	0.89	0.035	$p > 0.01$ and $p < 0.05$
Amino acid metabolism	Arginine and Proline Metabolism	Proline *	↑	1.11	0.036	
Amino acid metabolism	Tyrosine Metabolism	Homovanillate	↑	1.24	0.036	
Metabolism of other amino acids	Glutathione Metabolism	Pyroglutamic acid	↓	0.92	0.037	
Product of RNS		Nitrotyrosine *	↑	1.15	0.042	
Biosynthesis of unsaturated fatty acids	Fatty acid metabolism	Carnitine	↑	1.12	0.045	

[1] Ratio is mean ratio change calculated LGL/HGL, so >1 indicates metabolite higher after LGL intervention and <1 indicates metabolite lower after LGL intervention

* Indicated in penalized regression as significantly different between diets.

Table 5:

Urine metabolites detected as significantly altered between high glycemic load (HGL) and in low glycemic load (LGL) dietary interventions in a crossover feeding study, using liquid and gas chromatography mass spectrometry. After adjusting for false discoveries using the Benjamini Hochberg method, all significantly altered metabolites were increased after the LGL intervention, other than where indicated (*)

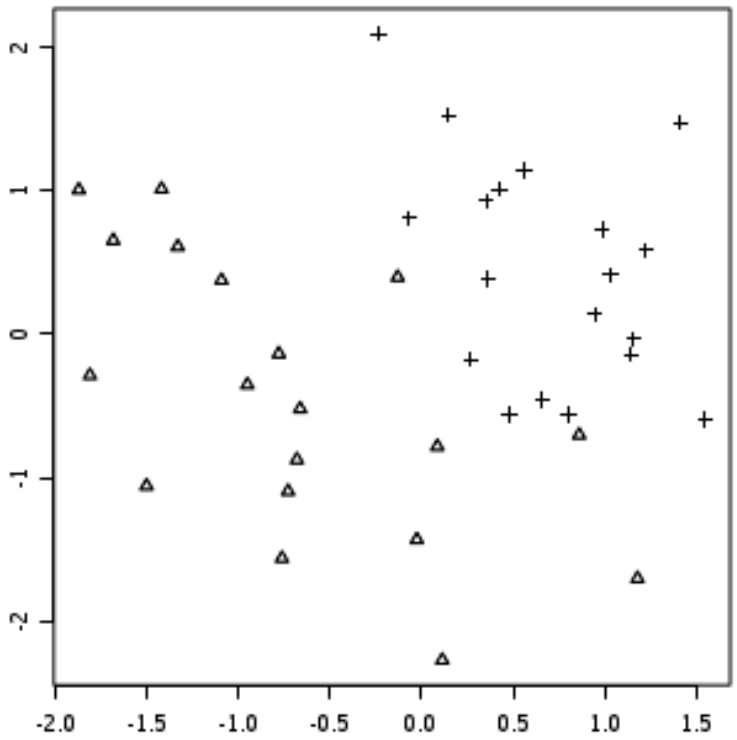
Group	Sub Pathway	Metabolite
Amino Acid	Glycine, serine and threonine	3-Methylcrotonylglycine*, Dimethylglycine, N-Acetylthreonine, Sarcosine
	Guanidino and acetamido	4-Guanidinobutanoate
	Histidine	1-Methylhistidine, 1-Methylimidazoleacetate*
	Phenylalanine & tyrosine	3-Hydroxyphenylpropionate, 3,4-Dihydroxyphenylacetate, 3,4-Hydroxyphenyllactate, 4-Hydroxyphenylacetate, Gentisate, Phenol Sulfate, Tyramine*
	Tryptophan	Kynurenine
	Urea cycle; arginine-, proline-,	Dimethylarginine, Homocitrulline*, Stachydrine
	Valine, leucine and isoleucine	2-Methylbutyroylcarnitine*, 3-Methyl-2-Oxovalerate, Isobutyrylcarnitine
CHO	Aminosugars	Erythronate
	Fructose, mannose, galactose, starch, and sucrose	Fructose, Mannitol, Sorbitol, Sorbose
	Glycolysis, gluconeogenesis, pyruvate	1,6-Anhydroglucose, 2-Isopropylmalate
	Nucleotide sugars, pentose	Arabinose, Arabitol, Glucuronate, Lyxose, Ribitol, Threitol, Xylonate, Xylose, Xylulose
Vitamins	Ascorbate and aldarate	Arabonate, Ascorbate, Threonate
	Thiamine	Thiamin
Energy	Krebs cycle	2-Methylcitrate, Itaconate, Succinate
Lipid	Bile acid	Glycolithocholate Sulfate, Taurolithocholate 3-Sulfate
	Carnitine	Carnitine*
	Fatty acid (also BCAA)	Propionylcarnitine*
	Inositol	Chiro-Inositol, Myo-Inositol, Scyllo-Inositol
	Mevalonate	3-Hydroxy-3-Methylglutarate
	Sterol/Steroid	5alpha-pregnan-3beta,20alpha-diol disulfate*, Andro Steroid Monosulfate*
Nucleotide	Purine , (hypo)xanthine/inosine containing	Xanthosine
	Purine , guanine containing	7,8-Dihydroneopterin, Guanidine
	Purine , urate	Allantoin
Peptide	Dipeptide derivative	Anserine
Xenobiotics	Benzoate	4-Hydroxybenzoate, 4-Hydroxyhippurate, 4-Hydroxymandelate, Catechol Sulfate, Hippurate
	Drug	4-Acetylphenol Sulfate, Citramalate
	Sugar, sugar substitute, starch	Erythritol
	Xanthine	3-Methylxanthine, 3,7-Dimethylurate, 7-Methylxanthine

Table 6:

Urine and plasma metabolites with statistically significant associations ($p<0.05$) after regression to compare biological specimen without consideration for diet.

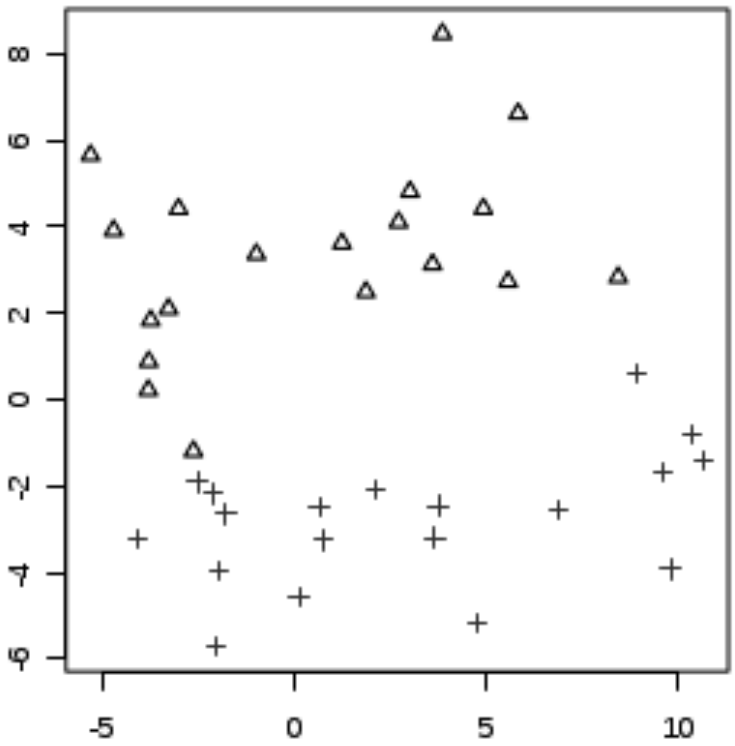
Super pathway	Sub-pathway	Metabolite	P>|t|	Plasma Coef.
Amino acid	Lysine metabolism	2-Aminoadipate	0.000	-0.22
Nucleotide	Purine metabolism, adenine containing	Adenosine	0.000	0.19
Nucleotide	Purine metabolism, urate metabolism	Allantoin	0.000	-0.64
Amino acid	Glycine, serine and threonine metabolism	Glycine	0.000	-1.07
Amino acid	Phenylalanine & tyrosine metabolism	Homovanillate	0.000	-0.06
Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism	Pyruvate	0.000	0.04
Nucleotide	Pyrimidine metabolism, uracil containing	Uracil	0.000	-.012
Nucleotide	Purine metabolism, (hypo)xanthine/inosine)	Xanthine	0.000	0.02
Nucleotide	Purine metabolism, (hypo)xanthine/inosine)	Xanthosine	0.000	-0.06
Amino acid	Histidine metabolism	Methylhistidine	0.009	-0.03
Lipid	Glycerolipid metabolism	Choline	0.014	1.74
Lipid	Carnitine metabolism	Carnitine	0.016	-0.14
Amino acid	Tryptophan metabolism	Xanthurenate	0.024	0.08
Nucleotide	Purine metabolism, urate metabolism	Urate	0.044	0.21

Figure 1:



PLS-DA plot of plasma after a high glycemic load (HGL, **Δ**) and low glycemic load (LGL, +) randomized crossover-feeding study. Pairwise score plots between the selected components show a clear separation can be seen in the plasma. The first component accounts for 10.7% and the second for 7.8% of the variance. While the first 2 components account for less than 20% of the variance, and only 12 metabolites significantly altered between the diets, our ability to distinguish between the diets in this discriminate analysis suggests the glycemic load of a diet affects plasma metabolites broadly.

Figure 2:

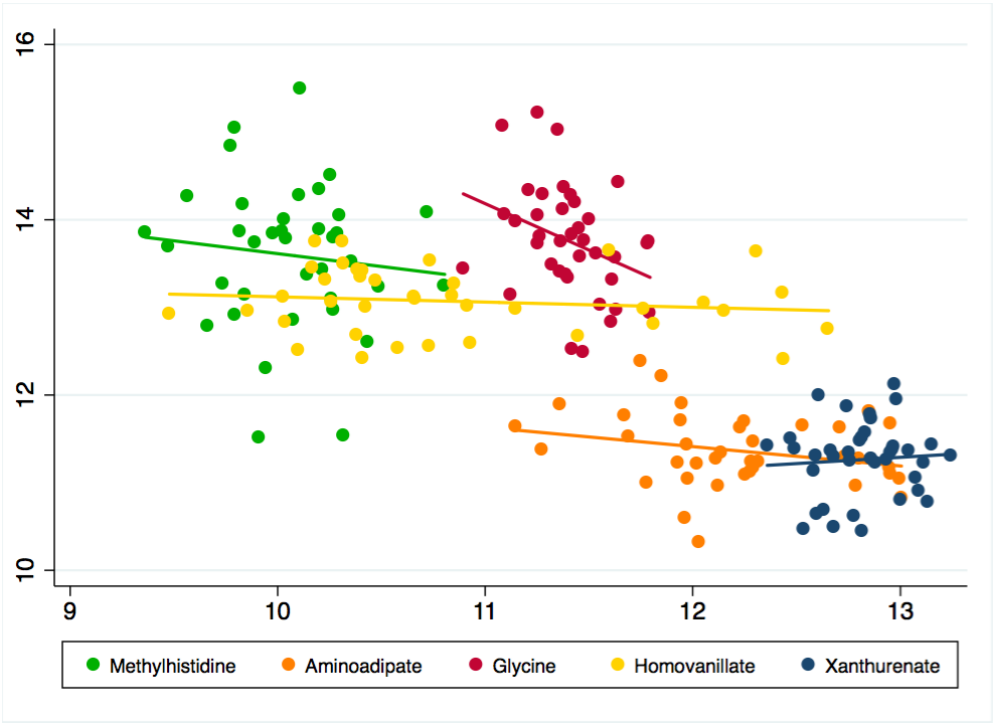


PCA plot of urine after a high glycemic load (HGL, **Δ**) and low glycemic load (LGL, +) randomized crossover-feeding study. Pairwise score plots between the selected components show a clear separation can be seen in the urine between HGL and LGL diets, even in an unsupervised test.

Figure 3:

Associations in CARB study metabolomic data. A) Amino acids with significant association between plasma and urine metabolites. B) Nucleotides with significant association between plasma and urine metabolites.

A)



B)

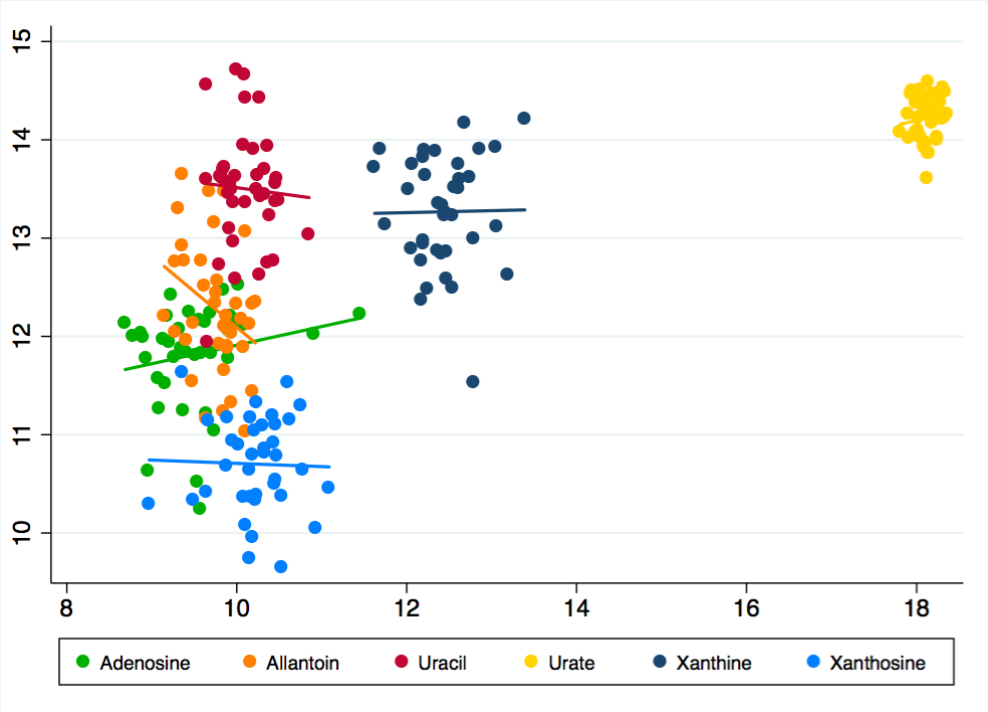


Figure 4

Radar plot showing relative abundance of significantly affected plasma metabolites after low glycemic load diet (**black**) relative to high glycemic load diet (**grey**). Values are log-transformed LC/MS metabolite intensity values, center point is 9.

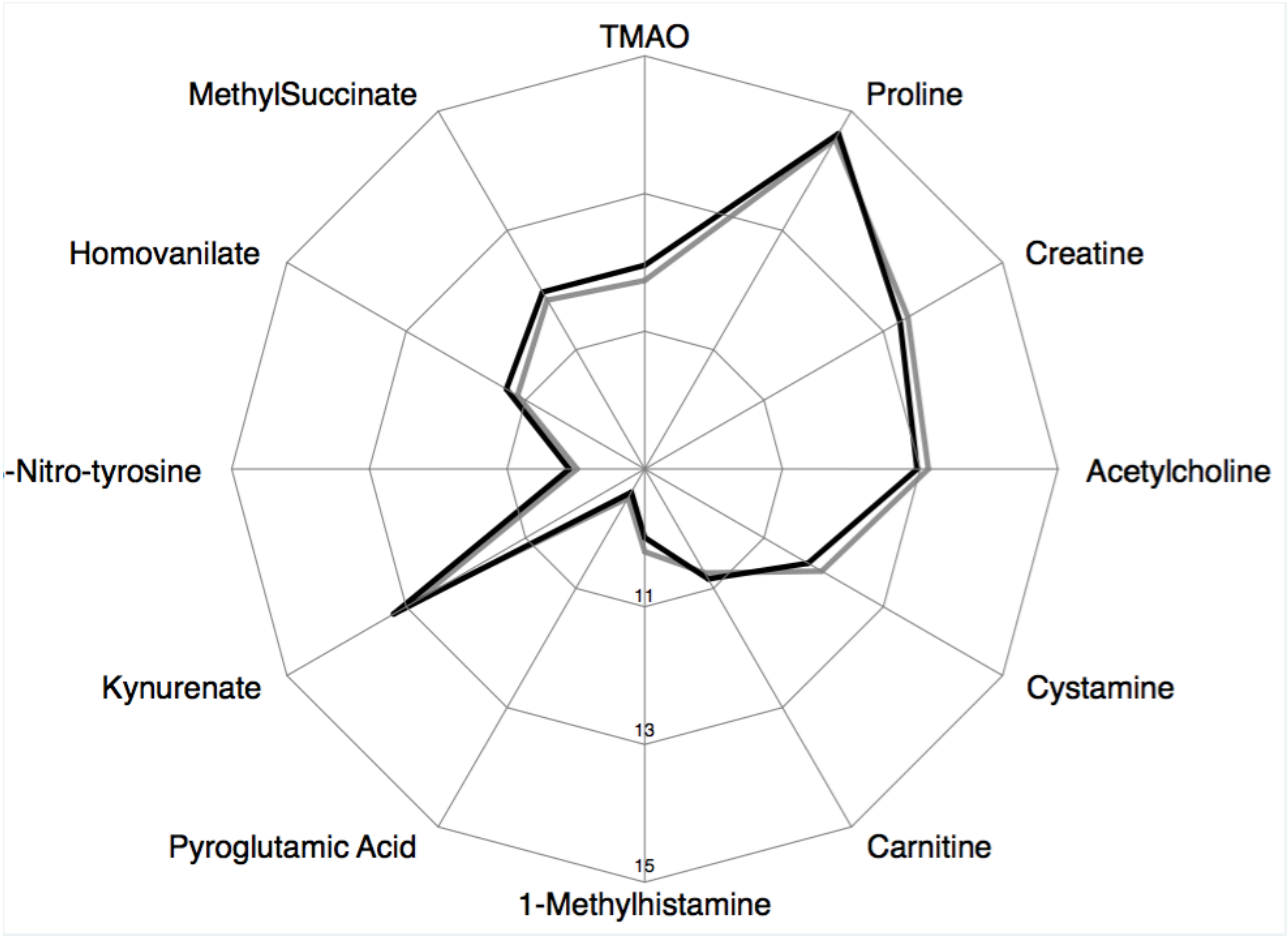
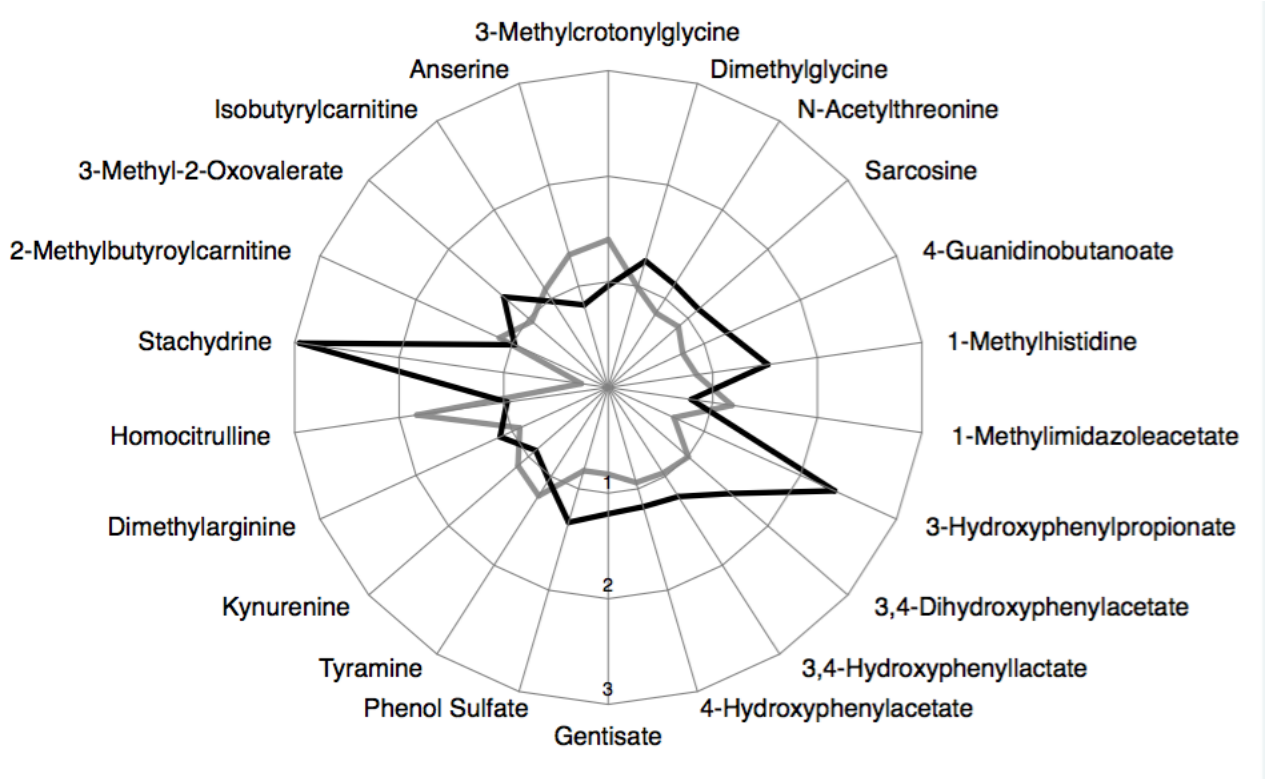


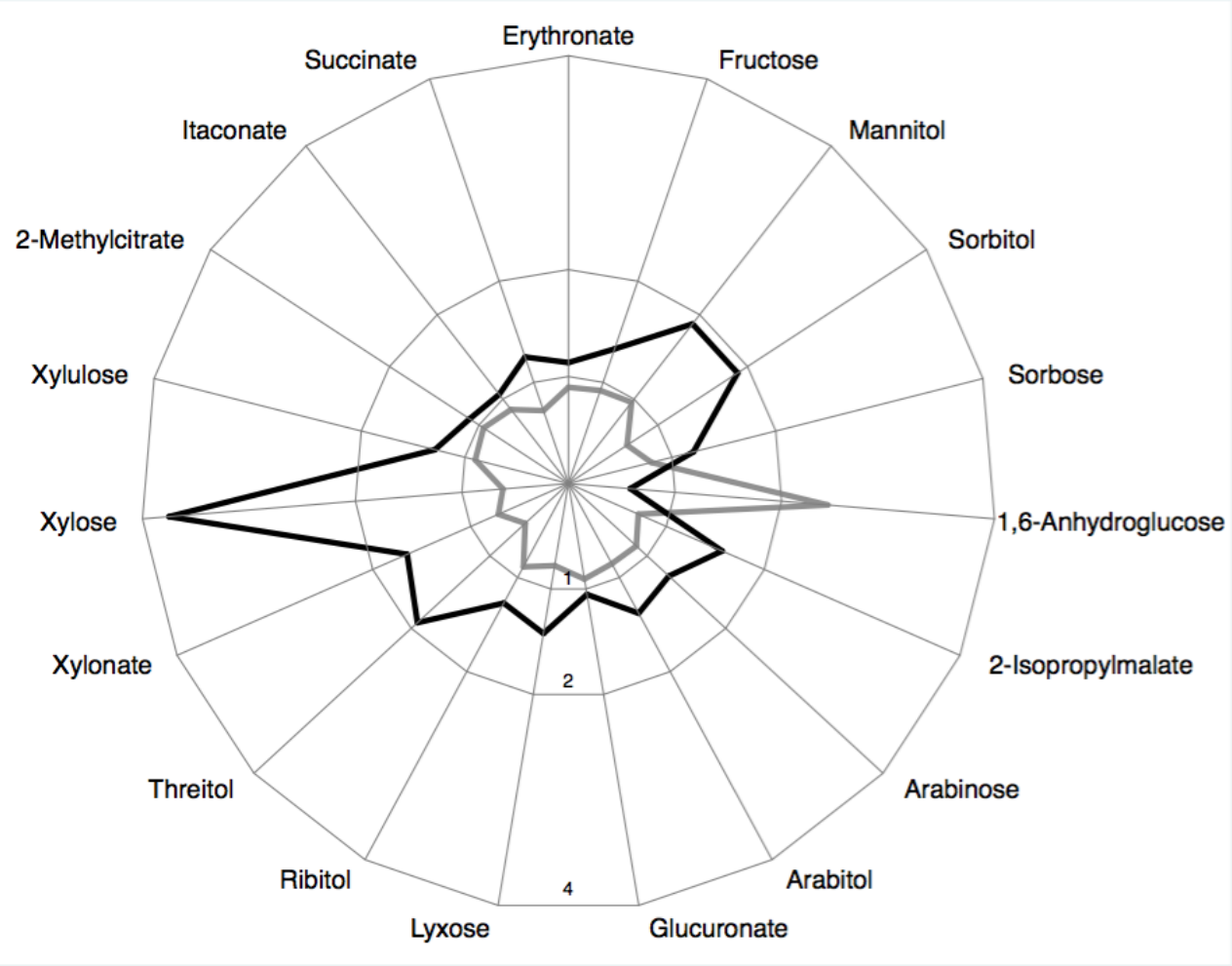
Figure 5

Radar plot showing relative abundance of significantly affected urine metabolites after low glycemic load diet (**black**) relative to high glycemic load diet (**grey**) in A) amino acids and peptides, B) carbohydrate and energy metabolites, C) lipids, nucleotides, vitamins and xenobiotic. Values are osmolality normalized LC and GC/MS metabolites intensity readings, center point is zero.



A) A**mino acids and peptides:** Stachydrine, also known as Proline Betaine, and considered to be protective in the kidneys due to its osmoprotective properties[73] and is a biomarker for citrus in the diet[33]. 3-hydroxyphenylpropionate is a major metabolite of caffeic acid and the metabolite associated with the antioxidant properties of coffee.[74] It is also a microflora degradation product of polyphenols found abundant in chocolate.[75] The LGL diet was higher in chocolate.

B) **Carbohydrate and energy metabolites:** Most CHO and energy metabolites were higher in urine after LGL, notably all the sugar alcohols: xylose, threitol, mannitol and sorbitol. These were higher in the LGL diet due to being added to items such as the sugar free maple syrup and naturally occurring in the canned pears. The one metabolite notably higher after the HGL diet, 1.6-anhydroglucose, is derived from starch.



C) **Lipids, nucleotides, vitamins and xenobiotic:** While most metabolites were more abundant after LGL, the few that were reduced showed no pattern apart from all steroid nucleotides. After FDR only andro steroid monosulfate and 5alpha-pregnan-3beta-,20alpha-diol sulfate were still significant. This potentially indicates that in the presence of higher fiber, more of this was excreted bound to stool in LGL. Chiro-inositol is present in many food sources, most notably buckwheat. The last 2 days of both intervention diets included buckwheat pancakes for breakfast. For the HGL intervention these were reconstituted from a highly processed boxed package, where the LGL included wholegrain and were made from scratch. The different quality of the buckwheat in the diets most likely accounts for the large difference in abundance. Urinary chiro-inositol is associated with increased insulin sensitivity and decreased blood pressure in some populations.[76]