Research Article

Metabolic Reprogramming: Short-term Western Diet Exposure Induces Sustained Changes in Plasma Metabolites

Biswapriya B. Misra^{1,2,*}, Ram P. Upadhayay², Vicki Mattern², John S. Parks¹, Laura A. Cox^{1,2,3}, Anthony G. Comuzzie^{2,3}, Michael Olivier^{1,2,3}

¹Center for Precision Medicine, Department of Internal Medicine, Section on Molecular Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem 27157, NC, USA.

²Department of Genetics, Texas Biomedical Research Institute, San Antonio 78227, TX, USA.

³Southwest National Primate Research Center, Texas Biomedical Research Institute, San Antonio 78227, TX, USA.

***Corresponding Author:** bmisra@wakehealth.edu (BBM)

25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

Abstract

Dietary patterns are well known to contribute to the metabolic syndrome and related disorders. We evaluated the impact of a short-term Westernized diet (WD) exposure on systemic plasma metabolomic changes in a cohort of adult male baboons. In this pilot study, five male baboons (n=5) raised and maintained on a standard monkey chow diet (high complex carbohydrates, low fat) were exposed to a challenge WD (high in saturated fats and simple carbohydrates) for 7 weeks (49 d), followed by a 57-day washout period on monkey chow (106 d). In addition to monitoring clinical measures, we used a comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (2D GC-ToF-MS) platform to assess the metabolomic changes in plasma at three time points. Twenty-three metabolites were changed in response to the WD (49 d), but the response across the animals was highly variable. All animals presented a very different metabolic profile than at baseline (0 d), and the washout period resulted in a relatively homogenous metabolic state across all animals. A short-term exposure to a WD led to long-term changes in the metabolic profile, suggesting a “reset” of the metabolic program.

Keywords: metabolomics; gas chromatography mass spectrometry; Western diet; reprogramming

1. Introduction

In 2015, an estimated 415 million adults were living with diabetes, and 5 million deaths were attributable to the disorder. The total global health expenditure due to diabetes was estimated at 673 billion USD, and predicted to rise significantly by 2040 [1]. Similar trends have been reported for obesity, with 107.7 million children and 603.7 million adults obese in 2015, and 4 million deaths globally attributed to obesity [2] . Changes in nutrition and lifestyle over the past decades have likely significantly contributed to this unprecedented rise in obesity and diabetes rates. While dietary saturated fats are recognized as a significant contributor to impaired cardiometabolic health, dietary fats alone do not seem to account for the total metabolic disruption associated with increasing risk of cardiovascular disease (CVD) and other comorbidities such as type 2 diabetes (T2D). There is emerging evidence that simple carbohydrates in the diet also negatively impact overall cardiometabolic health in conjunction with saturated fats, and that this combination of dietary elements gives rise to the full spectrum of metabolic disruption and dysregulation (elevated plasma triglyceride levels, decreased insulin sensitivity, and increasing fat accumulation). In 2009, the American Heart Association published a statement on the negative impact of a diet high in simple carbohydrates on overall cardiovascular health [3] . Unfortunately, from a public health perspective, the vast majority of individuals who typically consume a diet high in saturated fats also tend to consume higher amounts of simple carbohydrates, and this combination has been termed a Western diet (WD). A higher Western dietary pattern score is associated with an increased risk for type 2 diabetes, coronary heart disease, and colon cancer [4-8] . While dietary saturated fats and simple carbohydrates separately negatively impact cardiometabolic health, these two dietary elements consumed together likely act synergistically to increase both the rate of disease progression as well as its severity.

Long-term changes in body weight correlate with characteristic changes in serum metabolite concentrations [9]. Changes in plasma triglyceride, cholesterol, and glucose levels are often clinical

manifestations of weight gain in response to a WD. Interestingly, improved nutrition, dieting and weight loss do not invariably lead to a return to normal plasma metabolite concentrations, and these altered plasma levels of specific metabolites may be contributing to the increased cardiovascular disease risk observed in patients with repeated cycles of weight gain and loss (often termed yoyo dieting or weight cycling) in both females [10, 11] and males [12]. However, the exact pattern of metabolic changes during this weight cycling has not been characterized, and it is unknown how quickly a WD exposure (with or without weight gain and subsequent loss) causes long-term changes.

With the advent of modern metabolomics technologies, such as gas chromatography-coupled mass spectrometry, it is now possible to conduct comprehensive and unbiased analyses of serum samples to characterize the complex metabolic changes resulting from weight gain and changes in diet patterns. In a recent study, an 8% weight increase was induced within 6-8 weeks in human volunteer subjects on a WD [13]. A detailed metabolomics analysis revealed significant changes in response to the diet change and weight gain in study participants. The analysis suggested that even short-term administration of a WD, and the resulting short-term weight gain, led to metabolomic changes that persisted even after participants were returned to a normal diet, and the initial weight gain was reversed. However, the findings were highly variable among individuals, suggesting inter-individual factors (such as genetic factors or past life style) also contributing to the observed response and highlighting the challenges to investigating this phenomenon in adult human patients or volunteers.

Non-human primate (NHP) models, such as baboons, have been used extensively to study cardiometabolic disorders in response to Western and other diets [14-17]. These studies benefit from the similarity in metabolism and nutritional needs of these animals compared to humans, and a controlled environment in which diets of defined composition can be fed without having to account for variable prior exposures of animals.

For this study, we formulated a WD, mimicking the Westernized diet consumption in humans that is high in both saturated fats and simple carbohydrates. Healthy adult baboons (*Papio hamadryas*) were exposed to this challenge diet for 7 weeks, and then returned to normal monkey chow, a healthy low fat complex carbohydrate diet. We applied metabolomic analyses to evaluate the changes induced by this WD exposure, as reflected in the composition and distribution of plasma metabolites, and then assessed whether a return to a normal diet reversed the WD-induced changes. As we describe below, we observed significant changes in plasma metabolites in baboons after 7 weeks on the WD, independent of weight gain or other clinical serum measures. Interestingly, metabolite levels did not return to pre-challenge levels even after 56 d of monkey chow consumption, suggesting that even a short-term exposure to a WD leads to long-term alterations of basic metabolite levels.

2. Experimental Section

2.1. Baboon study cohort and composition of challenge diet

For this study, we selected five adult male baboons (*Papio hamadryas*) maintained as part of the baboon colony at the Southwest National Primate Research Center (Texas Biomedical Research Institute, San Antonio, TX). All animals were 7 years of age and had an average weight of 29.4 kg (26.6 – 32.5 kg). All animals were raised and maintained on a standard monkey chow diet (high complex carbohydrates, low fat) prior to the start of this dietary challenge. Each animal was exposed to the WD (high in saturated fat and simple carbohydrates) for a period of 49 d, followed by a 57 d washout period (106 d) where the baboons were returned to the baseline monkey chow diet. The caloric content of the monkey chow and WD were 3.25, and 4.59, Kcal/g respectively. The protein content of the chow and WDs were held constant between the diets while the concentrations and types of carbohydrates were modified. Further, in the WD, 40% of calories were derived from lard, and simple carbohydrates were predominantly derived from high fructose corn syrup. The detailed diet compositions are listed in **Table**

1. All animals were allowed to feed *ad libitum* throughout the study, without measuring individual food intake. All animal procedures were reviewed and approved by the Texas Biomedical Research Institute's Institutional Animal Care and Use Committee and performed by veterinary staff at the Southwest National Primate Research Center.

2.2. Clinical assays

Serum samples collected after overnight fast at baseline (day 0), after 7 weeks on the WD (day 49), and after a washout period on chow diet (day 106) were stored at -80 °C, and used for metabolomics assays as well as standard clinical chemistry measurements. Circulating biomarkers reflecting the status of lipid and glucose metabolism, as well as liver function and general systemic inflammation markers were measured using commercially available human assay kits with protocols established at the Southwest National Primate Research Center for non-human primates. Total serum cholesterol (TSC) and triglyceride (TG) concentrations were determined enzymatically using commercial reagents in a clinical chemistry analyzer. High-density lipoprotein cholesterol (HDL-C) was measured in the supernatant after heparin- Mn^{+2} precipitation. Blood glucose was measured using the Alfa Wasserman ACE clinical chemistry instrument (West Caldwell, NJ). Plasma insulin and C-peptide were measured by immunoassay using the Immulite Analyzer (Diagnostic Products Corporation, Los Angeles, CA). Interleukins, IL-6, IL-8 (pg/mL) were assayed using a sandwich-style enzyme-linked immunoassay kit, as suggested by the manufacturer (R&D Systems, Inc., Minneapolis, MN, USA). A high-sensitivity assay kit (Kamiya Biomedical, Seattle, WA, USA), with a latex particle-enhanced immunoturbidometric method, to measure C-reactive protein (CRP) concentrations. Summaries of raw data are presented as means, and standard deviations. A list of all clinical measurements used in this study is provided in **Table S1**. Numerous published studies from the institution have shown the accuracy/precision,

sensitivity/specificity of these assays over the years in baboon models and that measures for each are comparable to humans [18-20].

2.3. Metabolomic analysis using GC-MS

Gas chromatography-mass spectrometry (GC-MS) was performed as described elsewhere [21, 22] using a two-dimensional gas chromatography time-of-flight mass-spectrometer (2D GC-ToF-MS) platform. Plasma samples (30 μ L) were extracted serially, once each using mixtures of isopropanol: acetonitrile: water (3:3:2, v/v/v) and acetonitrile: water (1:1, v/v) following a standard polar metabolite extraction protocol described [23]. Pooled metabolite extracts (1500 μ L) from both phases were dried under vacuum at room temperature, and were then sequentially derivatized using methoxyamine hydrochloride (MeOX) and *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) [21]. Briefly, chemical derivatization was carried out by adding 10 μ L of MeOX (20 mgmL⁻¹ in pyridine) and shaking at 55 °C for 60 min., which was followed by trimethylsilylation reactions at 37 °C for 60 min. after addition of 90 μ L of MSTFA. Derivatized samples (1 μ L) were injected in splitless mode using an autosampler (VCTS, Gerstel™, Linthicum, MD, USA) into the 2D GC-MS system consisting of an Agilent® 7890 B gas chromatograph (GC) (Agilent Technologies, Palo Alto, CA, USA) connected to a Pegasus® 4D ToF-MS mass spectrometer (LECO Corp., St. Joseph, MI, USA) equipped with an electron impact (EI) ionization source (70 eV). Injection temperature was maintained at 250 °C, helium was used as a carrier gas with a flow rate of 1 mL min⁻¹. Separation on the GC was performed using a primary Rxi®-5Sil MS capillary column (Cat. No. 13623-6850, Restek, Bellefonte, PA, USA) (30 m $\times$ 0.25 mm $\times$ 0.25 μ m) and a secondary Rtx®-5 capillary column (Cat. No. 40201-6850, Restek, Bellefonte, PA, USA) (2 m $\times$ 0.15 mm $\times$ 0.15 μ m). The temperature program was as follows: isothermal at 70 °C for 1 min, 6 °C min⁻¹ ramp to 310 °C, and a final 11 min hold at 310 °C. The system was left at 70 °C for 5 min

before the next injection to allow temperature equilibration. Mass spectra were collected at 200 scans/s over a range of m/z 40-600. Transfer line and the ion source temperatures were both set to 280 °C. Pooled samples that served as quality control (QCs) were injected at scheduled intervals for instrumental performance, tentative identification, and monitoring of shifts in retention indices (RI).

2.4. Processing of raw GC-MS data

GC-MS data were pre-processed, cleaned, and aligned using ChromaToF version 4.71.0.0 (LECO Corp., St. Joseph, MI, USA) following settings as described elsewhere [22, 24]: viz. S/N: 25; peak width: 0.15, base line offset: 1; m/z range: 40-600. Metabolite identification included consulting the open source MSRI spectral libraries of the Golm Metabolome Database available from the Max-Planck-Institute for Plant Physiology, Golm, Germany (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>) for matching the mass spectra and retention indices (RIs) [25] and the NIST Mass Spectral Reference Library (NIST11/2011; National Institute of Standards and Technology, USA). Base peak areas for individual metabolite spectra were used for quantification. The filtered raw GC-MS data included data (base peak areas) from five biological replicates. Individual metabolite quantification data were assembled into a joint data set which included hits from both the databases.

2.5. Statistical analyses

Standard statistical analyses (summary statistics) were performed using the statistical software R (Version 3.5.0, <http://www.R-project.org>) [2, 26] and Microsoft Excel. Normalized (internal standards), transformed ($\log_2$), imputed, and scaled peak areas representative of relative metabolite amounts were obtained using DeviumWeb [27] and are presented in tables and figures. Values reported in all tables and text are presented as means, and differences were considered significant when $P < 0.05$ (nominal P-values). Short Time series Expression Miner (STEM) [28] was used as a Java implementation with a

graphical user interface available at <http://www.cs.cmu.edu/~jernst/st/> for clustering the metabolite accumulation patterns according to three time points.

2.5.1. Univariate analysis

ANalysis Of VAriance (ANOVA) was performed using R packages. Hierarchical clustering analysis (HCA) using average linkage clustering was performed on Pearson distances using PermutMatrix [29] from the metabolite abundance data. For heat maps displaying the top 25 metabolites, data were normalized using z-scores of the abundances for individual metabolites under the peak area.

2.5.2. Multivariate analysis

Principal components analysis (PCA) was performed using the package DeviumWeb [27], where the output consisted of score plots to visualize the contrast between different samples and loading plots to explain the cluster separation. Data were scaled with unit variance without any transformation. Partial least-squares discriminant analysis (PLS-DA) was used to highlight differences between the metabolic phenotypes at three time points (0 d, 49 d, 106 d) in the study.

2.6. Pathway enrichment and clustering analysis

Pathway enrichment analysis was performed at MetaboAnalyst (www.Metaboanalyst.ca) [30], and Chemical Translation Service (CTS: <http://cts.fiehnlab.ucdavis.edu/conversion/batch>) was used to convert the common chemical names into their KEGG, HMDB, CAS, PubChem CID, LipidMAPS IDs and InChiKeys.

3. Results and Discussion

214 3.1. Changes in clinical measures in response to short-term WD exposure

215 We used baboons fed regular monkey chow and a WD as a NHP model to mimic human physiological
216 and metabolic adaptations to dietary fat and sugar overload. The WD diet constituted 7-fold greater
217 simple sugars and 2-fold lower complex carbohydrates, 4-fold higher total fat, 9- and 5-fold higher
218 saturated and monounsaturated fats than the chow diet, mimicking a typical human WD, with only a
219 modest increase in total energy content (41% higher Kcal/g in the WD).

220 Clinical assays included quantification of glucose, insulin, total cholesterol, LDLC, HDLC, Hb, HbA1c,
221 and triglyceride levels, alongside several cytokines, for the five baboons at baseline and after 49 d of
222 WD challenge. None of the clinical measures were significantly different across all animals at 49 d, but
223 insulin [0.23 fold less at 49 d (260.66 ± 88.41 pg/ml) compared to 0 d (1129.2 ± 470.82 pg/ml)], adropin
224 [4.57 fold increase at 49 d (7.52 ± 9.9 ng/ml)], and interleukin-6 (IL-6) (0.54 fold decrease at 49 d (0.75
225 ± 0.44 pg/ml)] were dysregulated after 49 d of WD administration, and showed consistent trends across
226 all animals. Adropin is a peptide hormone regulating carbohydrate and lipid metabolism, and implicated
227 in metabolic diseases, central nervous system function, endothelial function and cardiovascular disease
228 that links dietary macronutrient intake with energy homeostasis and lipid metabolism [31][32]. IL-6 is
229 known to be induced by exposure to a high fat diet [33]. Though uncommon, but in one study, when 8
230 healthy subjects were placed on a WD for 1 month, the results showed a slightly decreased IL-6. Given
231 that none of these clinical markers were significantly changed across all animals, this may suggest that
232 these measures do not represent the underlying mechanisms changing plasma parameters after a short-
233 term diet exposure. Similar to these plasma measures, the body weight of the five baboons changed
234 differentially over the 49 d diet challenge and 57 d washout period (**Fig. 1**). At 49 d of WD exposure,
235 three baboons gained weight, while two lost weight. During reversal to the monkey chow diet for 57 d,
236 two baboons gained weight, two lost weight, and one maintained the weight gain status from the WD,

suggesting high inter-individual variability in response to the short-term diet exposure. Interesting it has been shown in obese diabetic subjects with identical weight loss that the metabolite profiles are very different for gastric bypass surgery compared to a diet intervention [34]. It is likely that feeding behavior (frequency and amount) influences this, but these findings also suggest that the observed metabolic changes are likely not a result of weight gain but a primary response to WD exposure - not a secondary effect of weight gain.

3.2. Response of clinical measures to the washout period after exposure to WD

Similar to the response to the WD, none of the changes seen in clinical measures after the washout period were statistically significant across all animals. We observed a few suggestive trends, including increases in insulin [2.94 fold at 106 d (767.26 ± 329.26 pg/ml) compared to 49 d (260.66 ± 88.41 pg/ml)], and circulating adipocytokines such as INF-gamma [4.11 fold increase at 106 d (33.64 ± 48.11 pg/ml) vs 49 d (8.17 ± 12.3 pg/ml)], IL-6 [12.15 fold at 106 d (9.11 ± 7.25 pg/ml) vs 49 d (0.75 ± 0.44 pg/ml)], TNF-alpha [2.44 fold at 106 d (2.5 ± 4 pg/ml) vs 49 d (1 ± 1.85 pg/ml)], and adropin [1.72 fold at 106 d (12.95 ± 8.1 ng/ml) vs 49 d (7.51 ± 9.9 ng/ml)] (**Table S1**). Comparison of these markers at 106 d to baseline (0 d) showed that concentrations did not return to baseline measures. These increased concentrations of clinical measures indicate that the 8 week chow diet after the WD challenge did not ‘washout’ the metabolic dysregulation induced by the WD, and suggests that the short-term exposure to the WD leads to long-term alterations in clinical plasma measures, suggesting systemic changes in metabolism in these animals. While none of these increases were statistically significant, the suggestive dysregulation of adipocytokine production may contribute to changes in insulin regulation and sensitivity, similar to the systemic insulin resistance reported in mice upon short-term HFD exposure, suggesting an induction of adipose tissue dysfunction [35]. However, inter-individual genetic variation

likely influences these clinical measures, not just dietary intake, making them likely ill-suited as biomarkers to help monitor short-term responses in these animals.

3.3. *Changes in baboon plasma metabolome in response to short-term WD exposure and upon return to chow diet*

Metabolomics-based studies are rapidly maturing to allow the use of small molecule chemical profiling in complex biosystems research related to diet and nutrition [36]. Advances towards a clearer understanding of the role of specific dietary constituents in conferring cardioprotective and/or antidiabetic benefits require carefully controlled dietary intervention studies. Using a comprehensive 2D GC-MS platform, we obtained a snapshot of circulating metabolites in plasma reflecting the diet-induced physiological changes in baboons. We identified and quantified the relative amounts of 115 metabolites in the study at all-time points (**Table S2 and S3**). These metabolites covered 56 different KEGG-based metabolic pathways of which 21 were over-represented ($P < 0.1$), such as aminoacyl-tRNA biosynthesis, nitrogen metabolism, alanine, aspartate and glutamate metabolism, cyanoamino acid metabolism, glycine, serine and threonine metabolism, fatty acid biosynthesis, and D-Glutamine and D-glutamate metabolism among others (**Table S5**).

Upon comparison of metabolite profiles between 0 and 49 d when WD was administered, we observed no statistically significant ($P < 0.05$) changes in individual metabolites. While we found trends for changes across the time course, similar to the clinical measures described above, due to the high variability (possibly from genotypic differences and thereby differential metabolic rates, and the small number of animals examined as part of our pilot study), there was no statistically consistent and significant difference between samples collected at 0 d and 49 d. However, we observed suggestive changes ($P < 0.1$) in 69 metabolites. Interestingly, all of these metabolites were ‘lower’ compared to the baseline (49 vs. 0 d; **Table S4**). These changes included saturated fatty acids (caproic acid, capric acid,

arachidic acid, lauric acid, butyric acid, pentadecanoic acid) and other lipid compounds such as alpha-linoleic acid and glycerol (**Fig. 2**). Obesity and insulin resistance (IR) are associated with impaired elongation and desaturation of fatty acids, reflected in the serum by, e.g., higher proportions of myristic and palmitic acid and lower proportions of longer-chain n-6 and n-3 fatty acids [37, 38]. Decreased circulation of saturated fatty acids can also be considered beneficial because increased flux of saturated fatty acids into peripheral tissues can lead to accumulation of toxic lipid molecular species in these tissues [39, 40]. Plasma long-chain fatty acid concentrations have also been reported to be associated with the degree of obesity in humans [41]. Increased plasma glycerol concentrations are observed during the early phase of human obesity [42]. A human WD frequently contains excessive saturated and trans fatty acids [43].

In addition to the lipid-related molecules, we observed changes in amino acids (1-methylhistidine, isoleucine, phenylalanine, asparagine, tryptophan, **Fig. 2**). Metabolic roles for amino acids are diverse, from providing substrates for mRNA translation, initiators of signal transduction and neurotransmission, biosynthesis of other nitrogen-containing compounds, to serving as substrates for gluconeogenesis, one-carbon methyl reactions, and tricarboxylic acid cycle [44]. The key role of amino acid metabolism early in the pathogenesis of diabetes and assessment of diabetes risk-management is well-established [45].

3.4. Clustering of diet-groups based on metabolite abundance

A major goal of this study was to compare the effects of the WD challenge and the washout period on quantity and quality of circulating plasma metabolites, as a function of systemic metabolic dysregulation. We observed that for time points 0 d, 49 d, and 106 d, two models explained the direction of changes for 105 of the 115 total metabolites we measured (**Fig. 3, Table S6**). The first model (P-value, 3.0×10^{-27} , 85 metabolites) demonstrated a pattern of 0, 0, -1, indicating no changes to metabolites from time point 0 d to 49 d, followed by a decline in the levels of metabolites (-1) at 106 d.

305 The 85 metabolites in the first model belonged mostly to amino acid metabolism (asparagine, glycine,
306 aspartic acid, valine, isoleucine, leucine, threonine, tryptophan, tyrosine, proline, cysteine, and glutamic
307 acid), nitrogen metabolism (glycine, hydroxylamine), butanoate metabolism (butanal, butyric acid,
308 succinic acid), fatty acid biosynthesis (myristic acid, dodecanoic acid, and capric acid), cholesterol,
309 maltose, myo-inositol, myo-inositol-phosphate, uridine, creatinine, canavanine among others. The
310 second model (0, 1,-1) was based on 20 metabolites (P-value, 1.3×10^{-7}) that belonged to amino acid
311 metabolism (glutamine, serine, lysine, ornithine, 1-methyl histidine) ethanol, and glycerol.

312 It is striking that all metabolites in these two groups had lower circulating plasma levels at 106 d, when
313 compared to the start of the study (0 d). Contrary to expectation, the levels did not simply return to
314 baseline after washout, but settled on a different, lower “normal set point”, suggesting that the short-
315 term WD challenge, regardless of the immediate impact during the diet administration, had long-term
316 consequences, a process we have called ‘metabolic reprogramming’ [46]. The most evident
317 visualization of this phenomenon is provided in the plots in **Fig. 2** where levels are lower and variation
318 among animals decreases at 106 d when compared to 0 d and 49 d.

319 To understand the contribution of the metabolite abundances in clustering and separation of the three
320 time points of this diet challenge, hierarchical clustering analysis (HCA) was performed to classify the
321 metabolites into clusters based on top 25 metabolites that were different for the group means (ANOVA).
322 This clustering also suggests a tighter relatedness of 0 d with 49 d compared to metabolite levels after
323 the washout period at 106 d (**Figure S1**). Moreover, the upper clusters were formed mostly by amino
324 acids. Again, this analysis points to the clear metabolic differences in the 0 d and 106 d samples,
325 confirming a “reprogramming” of the metabolic state due to the short-term exposure to the WD. This
326 strongly suggests that the alterations in plasma metabolites after 106 d are truly reflective of a changes

in underlying systemic metabolic regulation, and not simply a result of physiological changes such as weight gain induced during the WD exposure.

3.5. Metabolic dysregulation induced by WD prevented a return to baseline

We further looked into the metabolic changes in the baboons at 106 d as compared to the 0 d baseline levels. The dimension reduction strategy of unsupervised PCA was used to reduce all metabolites measured in plasma into a smaller number of orthogonal variables that revealed grouped and differential responses of the baboons to diet changes with an interpretable visualization. Upon PCA evaluation, the resulting plots explained ~77 % of the variation by two PCs (PC1- 61.8% and PC2-15.4%) attributed to the variation from the time (and diet) components (**Fig. 4A**). A supervised approach of classification, PLS-DA analysis, was conducted to indicate grouped responses of baboons to diet where the first two PCs explained 80% of the variations in the grouped responses (**Fig. 4B**). The clustering of 106 d samples reflects the reduced variability of the baboon metabolite abundances, and clearly separated from the 0 d and 49 d plasma samples.

Further analysis of the metabolites driving the separation to identify the discriminatory metabolites using biplots derived from PLS-DA analysis revealed that sugars (glucose, galactose, mannose), urea, and fatty acids (palmitic and stearic acids) show higher discriminatory potential than others (**Fig. 5A**). Peining et al. [13] attributed this change to decreased catabolism due to increased calorie intake to explain changing metabolites at the time points in their weight loss study [13] .

Peining et al. also showed trends in metabolites such as isoleucine, myo-inositol, tryptophan, serine and glutamic acid [13] that changed in the same direction as the changes observed in our study. They reported a cluster of biomolecules that was increased upon weight gain but failed to return to baseline at

the end of the weight loss period, including folate, phenylalanine and molecules related to branched chain amino acid (BCAA) degradation [13].

Even though several fatty acids did not show statistically significant changes, likely due to the small sample size (n=5) in this pilot study, the direction of the changes were consistent and qualitatively similar across these classes. In fact, different classes of lipids (for example, n-3 versus n-6) may have opposing net effects on specific processes, such as inflammation, and their relative proportions determine the net biological effect [47]. Most notably, measurements from this method are semi-quantitative, and reflect ion abundances for only a small subset of metabolites that are amenable to GC analysis, which may not always correlate with targeted methods that provide absolute concentration values. In addition, the statistical analysis of high-dimensional data is subject to overfitting and over interpretation because of the “small n/large p” paradigm. Despite these limitations of this pilot analysis, it is striking that the study clearly demonstrated that even after a relatively long washout period (diet with regular chow of 57 days), the metabolomic profiles do not return to pre-challenge levels, suggesting that the diet exposure has long-term metabolic consequences on the animals, independent of weight gain (the study design reported elsewhere [13]) or any other consistent clinical or pathological changes.

While the changes in plasma metabolites observed in our analysis suggest an interesting long-term physiological response to the 7 week WD challenge in our animals, further studies will be needed to elucidate mechanisms and address weaknesses in our existing pilot. Most notably, the findings will need to be confirmed in a larger number of animals. Finally, future studies may require collection of food consumption rates for individual animals, a factor that may have influenced our pilot analysis, and may allow a more detailed analysis of genetic factors contributing to the variability in individual responses. Therefore, we consider the modeling results presented here as hypothesis generating until confirmed

with more targeted approaches that allow a more comprehensive and accurate correlation of genetic predisposition, systemic physiology, and plasma metabolite changes. Nonetheless, despite the underlying inter-individual variability and the small sample size, the consistent finding of altered plasma metabolite concentrations even after a return to a normal diet, and the reduced variability observed across the animals suggests a systemic regulatory mechanism that alters these metabolites in response to a WD diet in a tightly controlled way. This metabolic reprogramming may provide a first insight into potential pathophysiological mechanisms contributing to the negative health consequences of weight cycling and short-term diet changes in humans.

4. Concluding remarks

In our baboon study, short-term exposure to a WD did not induce significant metabolomic changes after 7 weeks (49 d). However, samples collected after the washout period of 57 d showed large-scale metabolic changes in these animals, resulting in metabolite levels that were lower relative to the 0 d time point, and less variable across all animals. The post-washout period metabolite levels did not revert back to the 0 d baseline abundance levels, suggesting metabolic reprogramming with the establishment of a tightly regulated and controlled new “normal set point” in response to the short-term WD challenge. These results provide new insights into potential targets and related pathways that reflect the metabolic health status in baboons. Some of these metabolites and metabolic signatures may be indicative of long-term physiological changes and chronic disease susceptibility. Additional studies will be required to further elucidate the metabolic consequences of these changes in plasma metabolites, and how this alters the metabolic regulation in these animals.

Author Contributions

AC, LAC, MO designed the research; VM, RPU, BBM conducted the research; MO, LAC, AC provided essential reagents and materials; BBM analyzed and interpreted data, BBM, MO, LAC, JSP wrote the manuscript; and have primary responsibility for the final content and edits. All authors read and approved the final manuscript.

Disclosures

None.

Acknowledgements

Research reported in this manuscript was supported by a grant in aid from the National Institutes of Health (NIH): P01 HL028972. This investigation used resources, which were supported by the Southwest National Primate Research Center grant P51 RR013986 from the National Center for Research Resources, NIH, currently supported by the Office of Research Infrastructure Programs (ORIP), NIH through grant P51 OD011133. This investigation was conducted in facilities constructed with support from ORIP through grant numbers C06 RR14578, C06 RR15456, C06 RR013556, and C06 RR017515.

List of abbreviations

2D GC-ToF-MS: Two dimensional gas chromatography time-of-flight mass spectrometry,
ANOVA: analysis of variance,
BCAA: branched chain amino acid,
CVD: cardiovascular diseases,

418 HCA: hierarchical clustering analysis,
 419 HDL: high density lipoprotein,
 420 KEGG: Kyoto Encyclopedia of Genes and Genomes,
 421 LDL: low density lipoprotein,
 422 *m/z*: mass-to-charge ratio,
 423 MeOX: methoxyamine hydrochloride,
 424 MSTFA: N-methyl-N-trimethylsilyl-trifluoroacetamide,
 425 NHP: non-human primate,
 426 PCA: principal component analysis,
 427 PUFA: polyunsaturated fatty acid,
 428 QC: quality control,
 429 WD: Western diet,

430

431 **References**

- 432 1. Ogurtsova K, Fernandes JDDR, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. IDF
 433 Diabetes Atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes Res Clin Pr*.
 434 2017;128:40-50. doi: 10.1016/j.diabres.2017.03.024. PubMed PMID: WOS:000402469500006.
- 435 2. Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, Lee A, et al. Health Effects of
 436 Overweight and Obesity in 195 Countries over 25 Years. *New Engl J Med*. 2017;377(1):13-27. doi:
 437 10.1056/NEJMoa1614362. PubMed PMID: WOS:000404730000005.
- 438 3. Johnson RK, Appel LJ, Brands M, Howard BV, Lefevre M, Lustig RH, et al. Dietary Sugars
 439 Intake and Cardiovascular Health A Scientific Statement From the American Heart Association.
 440 *Circulation*. 2009;120(11):1011-20. doi: 10.1161/Circulationaha.109.192627. PubMed PMID:
 441 WOS:000269772800013.
- 442 4. van Dam RM, Rimm EB, Willett WC, Stampfer MJ, Hu FB. Dietary patterns and risk for type 2
 443 diabetes mellitus in US men. *Ann Intern Med*. 2002;136(3):201-9. doi: 10.7326/0003-4819-136-3-
 444 200202050-00008. PubMed PMID: WOS:000173637400003.
- 445 5. Fung T, Hu FB, Fuchs C, Giovannucci E, Hunter DJ, Stampfer MJ, et al. Major dietary patterns
 446 and the risk of colorectal cancer in women. *Arch Intern Med*. 2003;163(3):309-14. doi: DOI
 447 10.1001/archinte.163.3.309. PubMed PMID: WOS:000180928100006.
- 448 6. Fung TT, Schulze M, Manson JE, Willett WC, Hu FB. Dietary patterns, meat intake, and the risk
 449 of type 2 diabetes in women. *Arch Intern Med*. 2004;164(20):2235-40. doi: DOI
 450 10.1001/archinte.164.20.2235. PubMed PMID: WOS:000224965600008.

- 451 7. Fung TT, Willett WC, Stampfer MJ, Manson JE, Hu FB. Dietary patterns and the risk of
452 coronary heart disease in women. *Arch Intern Med.* 2001;161(15):1857-62. doi: DOI
453 10.1001/archinte.161.15.1857. PubMed PMID: WOS:000170236900008.
- 454 8. Hu FB, Rimm EB, Stampfer MJ, Ascherio A, Spiegelman D, Willett WC. Prospective study of
455 major dietary patterns and risk of coronary heart disease in men. *Am J Clin Nutr.* 2000;72(4):912-21.
456 PubMed PMID: WOS:000089494200005.
- 457 9. Wahl S, Vogt S, Stuckler F, Krumsiek J, Bartel J, Kacprowski T, et al. Multi-omic signature of
458 body weight change: results from a population-based cohort study. *Bmc Med.* 2015;13. doi: ARTN 48
459 10.1186/s12916-015-0282-y. PubMed PMID: WOS:000351724800001.
- 460 10. Montani JP, Schutz Y, Dulloo AG. Dieting and weight cycling as risk factors for
461 cardiometabolic diseases: who is really at risk? *Obes Rev.* 2015;16:7-18. doi: 10.1111/obr.12251.
462 PubMed PMID: WOS:000350285800002.
- 463 11. Merz CNB, Olson MB, Kelsey SF, Bittner V, Reis SE, Reichek N, et al. Weight cycling and
464 cardiovascular outcome in women with suspected ischemia: A report from the NHLBI-sponsored WISE
465 Study. *Plos One.* 2018;13(12). doi: ARTN e0207223
466 10.1371/journal.pone.0207223. PubMed PMID: WOS:000451886500005.
- 467 12. Schofield S, Parkinson J, Henley A, Sahuri-Arisoylu M, Sanchez-Canon G, Bell J. Metabolic
468 dysfunction following weight cycling in male mice. *Int J Obesity.* 2017;41(3):402-11. doi:
469 10.1038/ijo.2016.193. PubMed PMID: WOS:000395816000006.
- 470 13. Piening BD, Zhou WY, Contrepois K, Rost H, Urban GJG, Mishra T, et al. Integrative Personal
471 Omics Profiles during Periods of Weight Gain and Loss. *Cell Systems.* 2018;6(2):157-+. doi:
472 10.1016/j.cels.2017.12.013. PubMed PMID: WOS:000427421500006.
- 473 14. Bishop AC, Libardoni M, Choudary A, Misra B, Lange K, Bernal J, et al. Nonhuman primate
474 breath volatile organic compounds associate with developmental programming and cardio-metabolic
475 status. *Journal of breath research.* 2018;12(3):036016.
- 476 15. Higgins PB, Bastarrachea RA, Lopez-Alvarenga JC, Garcia-Forey M, Proffitt JM, Voruganti VS,
477 et al. Eight week exposure to a high sugar high fat diet results in adiposity gain and alterations in
478 metabolic biomarkers in baboons (*Papio hamadryas* sp.). *Cardiovasc Diabetol.* 2010;9. doi: ArtN 71
479 10.1186/1475-2840-9-71. PubMed PMID: WOS:000284466600001.
- 480 16. Mahaney MC, Karere GM, Rainwater DL, Voruganti VS, Dick EJ, Jr., Owston MA, et al. Diet-
481 induced early-stage atherosclerosis in baboons: Lipoproteins, atherogenesis, and arterial compliance. *J*
482 *Med Primatol.* 2018;47(1):3-17. Epub 2017/06/18. doi: 10.1111/jmp.12283. PubMed PMID: 28620920;
483 PubMed Central PMCID: PMCPMC5839476.
- 484 17. Cox LA, Comuzzie AG, Havill LM, Karere GM, Spradling KD, Mahaney MC, et al. Baboons as
485 a model to study genetics and epigenetics of human disease. *ILAR J.* 2013;54(2):106-21. Epub
486 2013/11/01. doi: 10.1093/ilar/ilt038. PubMed PMID: 24174436; PubMed Central PMCID:
487 PMCPMC3924757.
- 488 18. Kochunov P, Wey HY, Fox PT, Lancaster JL, Davis MD, Wang DJJ, et al. Changes in Cerebral
489 Blood Flow during an Alteration in Glycemic State in a Large Non-human Primate (*Papio hamadryas*
490 sp.). *Front Neurosci-Switz.* 2017;11. doi: ARTN 49
491 10.3389/fnins.2017.00049. PubMed PMID: WOS:000393914000001.
- 492 19. Chen AQ, Akinyemi RO, Hase Y, Firbank MJ, Ndung'u MN, Foster V, et al. Frontal white
493 matter hyperintensities, clasmotodendrosis and gliovascular abnormalities in ageing and post-stroke
494 dementia. *Brain.* 2016;139:242-58. doi: 10.1093/brain/awv328. PubMed PMID:
495 WOS:000370205000032.

20. Higgins PB, Shade RE, Rodriguez-Sanchez IP, Garcia-Forey M, Tejero ME, Voruganti VS, et al. Central GIP signaling stimulates peripheral GIP release and promotes insulin and pancreatic polypeptide secretion in nonhuman primates. *Am J Physiol-Endoc M*. 2016;311(4):E661-E70. doi: 10.1152/ajpendo.00166.2016. PubMed PMID: WOS:000388457000001.
 21. Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR. Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature protocols*. 2006;1(1):387-96.
 22. Misra BB, Bassey E, Bishop AC, Kusel DT, Cox LA, Olivier M. High Resolution GC/MS Metabolomics of Non-Human Primate Serum. *Rapid Commun Mass Spectrom*. 2018. Epub 2018/06/07. doi: 10.1002/rcm.8197. PubMed PMID: 29874398.
 23. Fiehn O, Wohlgemuth G, Scholz M, Kind T, Lee DY, Lu Y, et al. Quality control for plant metabolomics: reporting MSI-compliant studies. *The Plant journal : for cell and molecular biology*. 2008;53(4):691-704.
 24. Winnike JH, Wei X, Knagge KJ, Colman SD, Gregory SG, Zhang X. Comparison of GC-MS and GC× GC-MS in the analysis of human serum samples for biomarker discovery. *Journal of proteome research*. 2015;14(4):1810-7.
 25. Kopka J, Schauer N, Krueger S, Birkemeyer C, Usadel B, Weckwerth W, et al. Bergmüller, E., Dörmann, P., and . GMD@ CSB. DB: the Golm metabolome database. *Bioinformatics* pp. 2004;21(8 SRC - BaiduScholar):1635-8.
 26. Sokal RR, Rohlf FJ. *The principles and practice of statistics in biological research*: WH Freeman and company San Francisco; 1969.
 27. Grapov D. DeviumWeb: Dynamic Multivariate Data Analysis and Visualization Platform. 2014.
 28. Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. *Bmc Bioinformatics*. 2006;7. doi: Artn 191
- 10.1186/1471-2105-7-191. PubMed PMID: WOS:000237263300001.
29. Caraux G, Pinloche S. PermutMatrix: a graphical environment to arrange gene expression profiles in optimal linear order. *Bioinformatics*. 2005;21(7):1280-1. Epub 2004/11/18. doi: 10.1093/bioinformatics/bti141. PubMed PMID: 15546938.
 30. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic acids research*. 2015;43(W1):W251-W7.
 31. Pendyala S, Walker JM, Holt PR. A High-Fat Diet Is Associated With Endotoxemia That Originates From the Gut. *Gastroenterology*. 2012;142(5):1100-+. doi: 10.1053/j.gastro.2012.01.034. PubMed PMID: WOS:000303113600028.
 32. Kumar KG, Trevaskis JL, Lam DD, Sutton GM, Koza RA, Chouljenko VN, et al. Identification of Adropin as a Secreted Factor Linking Dietary Macronutrient Intake with Energy Homeostasis and Lipid Metabolism. *Cell Metabolism*. 2008;8(6):468-81. doi: 10.1016/j.cmet.2008.10.011. PubMed PMID: WOS:000261379600006.
 33. Cortez M, Carmo LS, Rogero MM, Borelli P, Fock RA. A High-Fat Diet Increases IL-1, IL-6, and TNF-alpha Production by Increasing NF-kappa B and Attenuating PPAR-gamma Expression in Bone Marrow Mesenchymal Stem Cells. *Inflammation*. 2013;36(2):379-86. doi: 10.1007/s10753-012-9557-z. PubMed PMID: WOS:000316020100015.
 34. Laferrère B, Reilly D, Arias S, Swerdlow N, Gorroochurn P, Bawa B, et al. Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss. 2011;3(80):80re2-re2.
 35. Lee YS, Li PP, Huh JY, Hwang IJ, Lu M, Kim JI, et al. Inflammation Is Necessary for Long-Term but Not Short-Term High-Fat Diet Induced Insulin Resistance. *Diabetes*. 2011;60(10):2474-83. doi: 10.2337/db11-0194. PubMed PMID: WOS:000295998700006.

36. Jones DP, Park Y, Ziegler TR. Nutritional Metabolomics: Progress in Addressing Complexity in Diet and Health. *Annu Rev Nutr.* 2012;32:183-+. doi: 10.1146/annurev-nutr-072610-145159. PubMed PMID: WOS:000307963100010.
37. Vessby B. Dietary fat, fatty acid composition in plasma and the metabolic syndrome. *Current Opinion in Lipidology.* 2003;14(1):15-9. doi: 10.1097/01.mol.0000052859.26236.5f. PubMed PMID: WOS:000180877500004.
38. Storlien LH, Baur LA, Kriketos AD, Pan DA, Cooney GJ, Jenkins AB, et al. Dietary fats and insulin action. *Diabetologia.* 1996;39(6):621-31. doi: Doi 10.1007/Bf00418533. PubMed PMID: WOS:A1996UQ00200001.
39. Shimabukuro M, Zhou YT, Levi M, Unger RH. Fatty acid-induced beta cell apoptosis: A link between obesity and diabetes. *P Natl Acad Sci USA.* 1998;95(5):2498-502. doi: DOI 10.1073/pnas.95.5.2498. PubMed PMID: WOS:000072366600100.
40. Wei YR, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol-Endoc M.* 2006;291(2):E275-E81. doi: 10.1152/ajpendo.00644.2005. PubMed PMID: WOS:000238841200010.
41. Scaglioni S, Verduci E, Salvioni M, Bruzzese MG, Radaelli G, Zetterstrom R, et al. Plasma long-chain fatty acids and the degree of obesity in Italian children. *Acta Paediatr.* 2006;95(8):964-9. doi: 10.1080/08035250600764834. PubMed PMID: WOS:000239953000013.
42. Lestunff C, Bougneres PF. Glycerol Production and Utilization during the Early Phase of Human Obesity. *Diabetes.* 1992;41(4):444-50. doi: DOI 10.2337/diabetes.41.4.444. PubMed PMID: WOS:A1992HK69200007.
43. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr.* 2005;81(2):341-54. PubMed PMID: WOS:000226943100003.
44. Layman DK, Anthony TG, Rasmussen BB, Adams SH, Lynch CJ, Brinkworth GD, et al. Defining meal requirements for protein to optimize metabolic roles of amino acids. *Am J Clin Nutr.* 2015;101(6):1330s-8s. doi: 10.3945/ajcn.114.084053. PubMed PMID: WOS:000355641900032.
45. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. *Nature Medicine.* 2011;17(4):448-U83. doi: 10.1038/nm.2307. PubMed PMID: WOS:000289245100035.
46. Anderson RM, Weindruch R. Metabolic reprogramming in dietary restriction. *Mechanisms of Dietary Restriction in Aging and Disease.* 35: Karger Publishers; 2007. p. 18-38.
47. Calder PC. n- 3 Polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids.* 2003;38(4):343-52.

Figure Captions

Fig 1. Variability in body weight reflecting individual response to diet challenge. Line chart showing change in body weight from 0 to 49 d and to 106 d for the five baboons in the study.

Fig 2. Relative abundances of different metabolites obtained from the metabolomics analysis. Box whisker plots (displaying the lower (Q1) and upper (Q3) quartiles and the median) showing relative metabolite abundance of (A) amino acids, (B) fatty acids (* are saturated fatty acids), and (C) carbohydrates, at each time point 0 d, 49 d, and 106 d. Only metabolites with $P < 0.1$ are shown. P-values were obtained from within-subject ANOVA (for three time points 0, 49, and 106 d).

Fig 3. Patterns in changes of metabolite abundance at time points 0 d, 49 d, and 106 d. For metabolites, the two models were significant with 85 and 20 metabolites showing patterns of 0 (unchanged), 0 (unchanged), -1 (lower) (A) and 0 (unchanged), 1 (higher), -1(lower) (B), respectively.

Fig 4. Multivariate analysis displaying score plots for supervised and unsupervised multivariate analysis. (A) PCA and (B) PLS-DA analysis. PCA was performed using five replicate data of relative metabolite abundances, and the generated PC1 and PC2 were plotted. PCA score plots display a clear separation of the sample groups (three time points) based on the abundance of 115 metabolites.

Fig 5. Metabolites contributing to the clustering of samples at the three time points. (A) A biplot from PLS-DA analysis displaying the metabolites contributing to the clusters of time points. For simplicity only the metabolites with higher discriminatory potential are shown. (B) A line chart displaying the trends of metabolites (amino acids and myo-inositol) over the study period.

Supporting Information

S1 Table. Clinical measures (raw data) obtained from the 5 animals fed WD diet for 49 d and brought back to chow diet.

603 **S2 Table. List of 115 metabolites identified and their raw relative abundances obtaining using the**
 604 **2D GC-ToF-MS platform alongside their identifiers.** Identifiers are KEGG: Kyoto Encyclopedia of
 605 Genes and Genomes, HMDB: Human Metabolome Database, PubChem ID, ChEBI, METLIN IDs.

606

607 **S3 Table. Processed, normalized and transformed relative metabolite abundance data for the**
 608 **three time points, 0, 49, and 106 d as well as the mean, SD, RSD, and fold changes (FC).**

609

610 **S4 Table. ANOVA and post-hoc (Fisher's LSD) results for the metabolite abundance data**
 611 **conducted on the three time points.**

612

613 **S5 Table. Enrichment analysis for KEGG-based pathways represented by the metabolites**
 614 **quantified in this study.**

615

616 **S6 Table. Pattern analysis displaying the list and values of 85 and 20 metabolites fitting into two**
 617 **models, (0, 0, -1) and (0, 1, -1), respectively.**

618

619 **S1 Fig. Hierarchical cluster analysis (HCA) of mean and normalized abundances of metabolites**
 620 **obtained from five biological replicates across the time points 0, 39, and 106 d. Red and green**
 621 **indicate increasing and decreasing concentrations of metabolites, respectively. Values were**
 622 **subjected to average linkage clustering (Pearson distance). Scale indicates high (3, red) to green (-3,**
 623 **low) abundances.**

624

Fig1

Fig2

Fig3

Fig4

Fig5

Table 1. Comparison of study diets.

	Monkey Chow	Western Diet (WD)
Energy (Kcal/g)		
	3.25	4.3
Carbohydrates (g/100g)		
Total	62	51
Simple	3	22
Complex	59	29
Protein (g/100g)		
Total	28	10
Fat (g/100g)		
Total	10	39
Saturated	2	18
Monounsaturated	3	16
Polyunsaturated	5	5
Trans	0	0

bioRxiv preprint doi: <https://doi.org/10.1101/534980>; this version posted February 20, 2019. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.