Metabolic signatures in nutrition and health : short-term diet response, sexual dimorphism and hormone chronobiology
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Chapter 2

A 48-hour vegan diet challenge in healthy women and men induces a branch-chain amino acid related, health associated, metabolic signature

Based on

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A 48-hour vegan diet challenge in healthy women and men induces a branch-chain amino acid related, health associated, metabolic signature

ABSTRACT

Research is limited on diet challenges to improve health. A short-term, vegan protein diet regimen nutritionally balanced in macronutrient composition compared to an omnivorous diet was hypothesized to improve metabolic measurements of blood sugar regulation, blood lipids, and amino acid metabolism. This randomized, cross-over, controlled vegan versus animal diet challenge was conducted on 21 (11 female,10 male) healthy participants. Fasting plasma was measured during a 3 day diet intervention for clinical biochemistry and metabonomics. Intervention diet plans met individual caloric needs. Meals were provided and supervised. Diet compliance was monitored. The vegan diet lowered triglycerides, insulin and homeostatic model assessment of insulin resistance (HOMA-IR), bile acids, elevated magnesium levels, and changed branched-chain amino acids (BCAAs) metabolism ($p < 0.05$), potentiating insulin and blood sugar control after 48 h. Cholesterol control improved significantly in the vegan versus omnivorous diets. Plasma amino acid and magnesium concentrations positively correlated with dietary concentrations. Polyunsaturated fatty acids and dietary fiber inversely correlated with insulin, HOMA-IR, and triglycerides. Nutritional biochemistries, BCAAs, insulin, and HOMA-IR were impacted by sexual dimorphism. A health-promoting, BCAA-associated metabolic signature was produced from a short-term, healthy, controlled, vegan diet challenge when compared with a healthy, controlled, omnivorous diet.

INTRODUCTION

Vegan diets are plant-based regimens that exclude meat, eggs, dairy products, and any other animal-derived foods and ingredients. In contrast, a vegetarian diet emphasizes plant-based foods but can also include dairy, eggs, honey, and fish. Populations who lack access to animal protein or cultures with historical or religious traditions have a higher percentage of vegetarians: about 35% of the Indian population eats strictly plant-based diets. Approximately 10% of all vegetarians are vegan but an increasing number of people are adopting a non-animal product diet [1].

While epidemiologic evidence published in the 1980s and 1990s supports the benefits of vegetarian diets, skepticism remains largely because of concerns about specific nutrient deficiencies of plant-based foods. Both vegan and vegetarian diets can be healthful for all life stages with appropriate selection of plant-based foods that adequately meet requirements for protein, iron, n-3 fatty acids, iodine, zinc, calcium, and vitamin B12 [2]. An intermittent vegan diet regimen that is alternated within a habitual, balanced omnivorous diet can also meet these nutritional requirements.

The growing demand for unsustainable animal-based products by an expanding and wealthier global population is negatively effecting the planet [3]. Plant-based food production requires less energy and has less of an impact on non-renewable environmental resources. Policies that promote adoption of plant-based diets may help protect the planet while improving the health of individuals [4].

The health benefits of nutrients or foods are typically analyzed after weeks or months of consuming experimental diets. Epidemiological evidence suggests habitual intake of plant-based diets (vegan and vegetarian) reduces risk of diabetes, lipid disorders, and metabolic syndrome [5]. For example, the prevalence of type 2 diabetes mellitus (T2DM) in Seventh-Day Adventists who respect a strict vegan diet is 45% of the incidence in the general population [6]. Meta-analysis of vegan diet studies show improved glycemic control compared to American Diabetes Association (ADA)'s dietary recommendations in T2DM individuals [7]. A dietary portfolio (a vegan regimen with specific amounts of plant sterols, viscous fibers, soy protein, and nuts) reduced blood lipids more than the National Cholesterol Education Program (NCEP) in individuals with hypercholesterolemia [7] using plant-based interventions that ranged from 2 to 104 weeks. Consistency in nutrient content and participant compliance are difficult to maintain in long-term studies, which may be confounded by unmeasured environmental variables that differ between participants.

Our study measured metabolic changes associated with consumption of vegan-compared to animal-based diets in the short time period of 48 hours. Diets were rigorously planned, intake compliance monitored, and clinical parameters as well as a diverse panel of plasma metabolites analyzed. The results indicated significant health-promoting benefits of a short-term, healthy vegan diet exposure.

MATERIALS AND METHODS

Study population and ethical approval

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki, approved by the Ethical Committee of Lausanne University School of Medicine (CER-VD, ref no. 222/14), and registered on ClinicalTrials.gov with the identifier NCT02223585. All participants provided written informed consent for study participation and were offered financial compensation agreed by the ethical committee (3,200 CHF) for time spent and schedule inconveniences.

A total of 56 healthy male and female volunteers were pre-screened at information sessions held at the Metabolic Unit, Nestlé Research Center (Lausanne, Switzerland). Out of the 32 participants who signed informed consent, 26 were enrolled in the study (6 screening failures), 5 dropped out and 21 healthy participants (10 men, 11 women) completed this pilot study. Two participants dropped out because of non-serious adverse events, and another 3 decided not to proceed with the study. All participants habitually ate a heterogeneous diet including animal and vegan proteins before entrance into the study.

Study inclusion criteria were age (from 18 to 55 years), regular bowel movement (at least once every 1-2 days), body mass index (BMI, from 18.5 to 27 kg.m-2). Health status was assessed by a physician during a screening visit as a standard medical visit with blood chemistry analysis. Exclusion criteria included special diets (vegetarian, high protein, and low cholesterol or weight loss program), pregnancy, food allergy, smoking, high alcohol consumption (more than 2 drinks per day), and excessive physical exercise (more than 5 moderate physical exercises per week).

Animal and vegan dietary interventions

The energy provided by vegan and animal meals was personalized for each participant according to their calculated resting energy requirements and level of physical activity. Energy requirements were calculated as a function of height, weight, age, and activity level, based on the Harris Benedict Equation [8]. Eighteen (9 animal and 9 vegan) meal

plans were designed with different caloric values, ranging from 1600 to 3000 calories (**Supplementary Figure 1**). Macronutrient composition was matched between animal and vegan-based meals, and was calculated based on 20% protein, 50% carbohydrate and 30% fat of total calories within ±5% of calculated needs of each participant.

Clinical trial design

The clinical trial was a randomized, open label, cross-over, controlled study. Study participants were randomly assigned to the animal and plant protein challenges using Medidata Balance with dynamic allocation [9].The study lasted five weeks (**Figure 1**) following a one week run-in phase (Week 1 = W1) that defines baseline of the participant's normal diet and lifestyle. Participants were then randomly assigned to either animal or vegan meals for 3 consecutive days (Tuesday, Wednesday and Thursday). Fasting blood samples were obtained after an overnight fast on each of the 3 days (days 0,1,2) of the intervention diets as indicated in **Figure 1**. The third week (W3) was a washout period during which participants resumed their usual diets. The cross-over intervention occurred during the 4th week and lasted three consecutive days also during the middle of the week. Participants were monitored during week 5 (W5) to determine if they returned to their usual health and dietary status. During each 3-day intervention, participants ate the same meals on each day, including breakfast, morning snack, lunch, afternoon snack, and dinner. All meals and snacks were prepared and provided to study participants under supervision by the Metabolic Unit staff with the exception of the dinner meal, which was packaged for home consumption. During the study period, the participants were told to avoid consumption of alcoholic beverages and limit caffeinated and sugary beverages (no more than 2 cups of coffee, black or green tea per day). Fasting blood samples were obtained after an overnight fast as indicated in **Figure 1**.

Descriptions of the materials and methods used for the diet diaries, compliance questionnaires and diet intake analysis; anthropometric, clinical data, and blood sample collection; amino acid analysis, bile acid analysis, and metabonomics analysis are found in **Chapter 2**.

Statistical analysis

Statistical significance of observed differences across groups was calculated using a Wilcoxon Rank-Sum test. A Wilcoxon Signed rank test was used for paired comparison. To determine if dietary intake was correlated with plasma metabolite levels we calculated the Spearman correlation coefficients and Spearman's rho statistic to test the significance of the association. All p-values were corrected for multiple testing using false discovery rate, and values < 0.10 are reported.
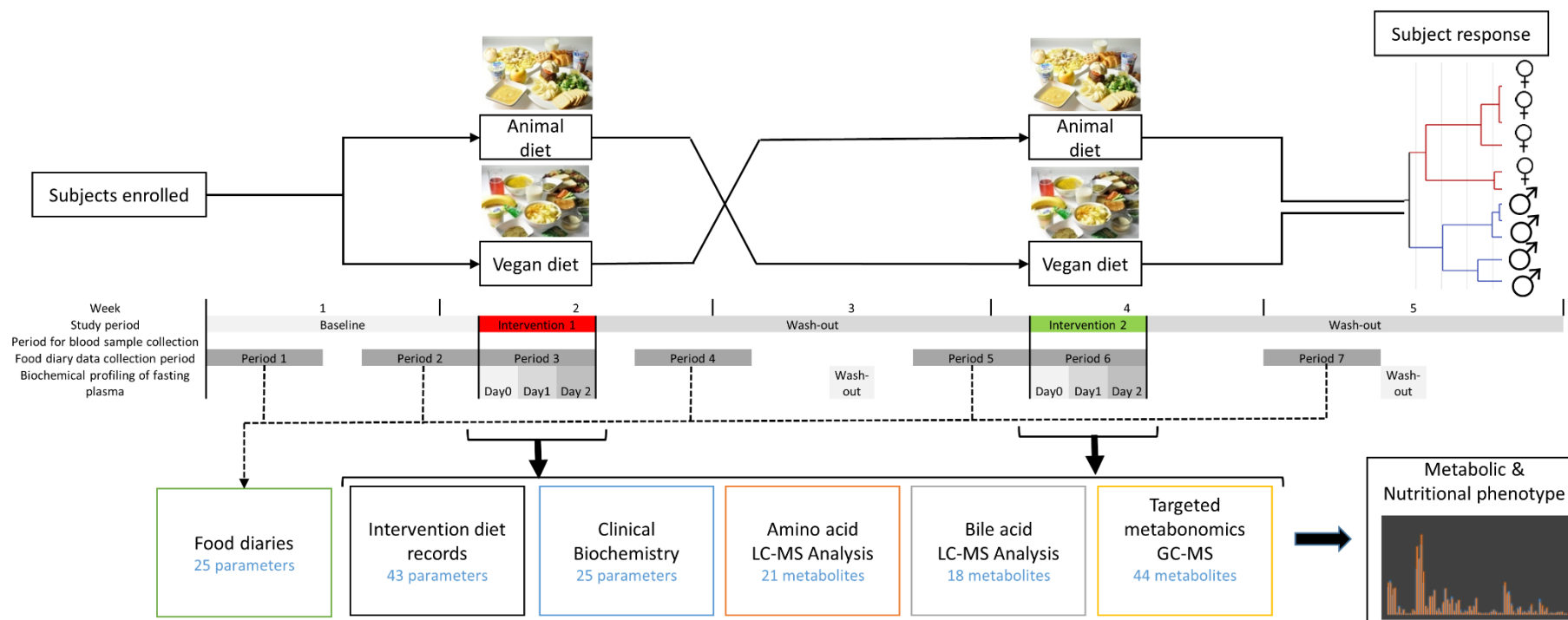
Figure 1. Overview of the experimental design and the analytical strategy. **A)** The clinical trial was a randomized, open label, cross-over, controlled study that lasted five weeks. Study participants were randomly assigned to the animal and plant protein diets to which they were challenged for three consecutive days. **B)** Dietary intake was assessed using three day food diaries each week during run-in periods, before, and after dietary interventions. During intervention periods, accurate dietary intake and metabolic status were monitored. Extensive metabolic phenotyping covered standard biochemical and nutritional measures, as well as amino acid, bile acid and targeted metabolic profiling. **C** Impact of vegan diets on individual metabolic and nutritional status was investigated in comparison to the animal dietary intervention as well as to subjects' free living dietary habits.

However, due to the small sample size, and exploratory nature of the study, the non-corrected statistical significance was used for interpretation of results

The crossover nature of this study required assessing potential carryover effects of each diet. A model was fit to assess the interaction between each metabolite and the diet sequence. A p-value below 0.05 was interpreted as a significant carry-over. To test the association between the type of diet and the changes in metabolite levels a linear mixed effect models (Model 1) was fit for each metabolite separately.
Further description of statistical methods can be found in **Chapter 2**.

Table 1. Baseline anthropometric and clinical parameters by gender

Clinical marker	Females (n=11)		Males (n=10)		p-Value
	Mean	SD	Mean	SD	
Age (years)	34.0	9.1	35.0	9.9	6.72E-01
Height (cm)	162.3	4.9	179.6	5.6	1.56E-04
Weight (kg)	58.9	8.3	75.7	10.9	3.08E-03
BMI (kg/m^2)	22.4	3.0	23.4	2.82	4.60E-01
HDL (mmol/L)	1.6	0.3	1.5	0.2	8.05E-01
Albumin (Kg/m^3)	39.7	3.8	43.7	2.9	**1.51E-02**
Alanine aminotranferase (U/L)	20.5	4.5	31.3	9.0	**1.36E-03**
Aspartate aminotransferase (U/L)	18.7	2.7	24.5	8.7	**2.19E-02**
Total cholesterol (mmol/L)	4.5	0.7	4.3	0.9	4.60E-01
Chol/HDL	2.8	0.5	3.0	1.0	6.99E-01
Creatine kinase inhibitor (mmol/L)	71.9	20.9	223.4	186.4	**6.37E-04**
Chloride (mmol/L)	105.0	1.3	104.5	2.0	7.20E-01
Creatinine (µmol/L)	67.0	11.4	93.4	10.9	**3.26E-04**
Ferritin (µg/L)	66.6	49.4	178.3	75.3	**1.06E-03**
Glucagon (pM/L)	6.2	3.1	9.3	5.4	2.18E-01
Glucose (mmol/L)	5.2	0.5	5.5	0.4	9.80E-02
Insulin (µU/mL)	17.5	6.6	13.5	5.5	2.31E-01
Iron (µmol/L)	13.8	4.2	16.9	4.3	2.18E-01
Potassium (mmol/L)	4.1	0.2	4.2	0.3	5.02E-01
LDL (mmol/L)	2.6	0.6	2.8	0.9	4.38E-01
Magnesium (mmol/L)	0.8	0.1	0.8	0.0	**2.20E-02**
Sodium (mmol/L)	141.4	1.1	142.6	1.2	**4.67E-02**
Pre-albumin (g/m^3)	288.0	39.9	369.1	69.4	**6.71E-03**
Phosphate (mmol/L)	1.1	0.2	1.2	0.2	7.51E-01
c-reactive protein (g/m^3)	1.6	1.6	1.9	3.4	2.18E-01
Total bilirubin (µmol/L)	7.2	3.2	17.0	7.0	**2.76E-03**
Triglycerides (mmol/L)	0.8	0.2	0.9	0.6	3.07E-01
Total protein (Kg/m^3)	72.7	4.9	75.2	3.7	2.91E-01
Tranferrin (Kg/m^3)	2.5	0.5	2.2	0.2	1.05E-01
Non-esterified fatty acids (µmol/L)	385.1	191.1	390.5	194.1	9.16E-01

The p-values are calculated by performing a Wilcoxon rank sum test. SD = standard deviation.

RESULTS

Population characteristics
A total of 21 (11 females, 10 males) healthy participants completed the clinical trial (**Table 1**). Individuals were similar in age and BMI . Several clinical parameters showed strong

gender specificities, including plasma clinical biochemistries (albumin, pre-albumin, ferritin, Mg, Na), hepatic functions (ALAT, ASAT, total bilirubin), and others (creatinine, creatine kinase inhibitor), as reported in **Table 1**.

Diet intervention

Forty-three nutrients were analyzed from the diet records from each 2-day intervention for the vegan and animal intervention diets (**Table 2**). Statistical analysis was performed using the average nutrient intakes from all participants over 2 days using Wilcoxon signed rank test. The 2 controlled-intervention diets were matched for macronutrient (carbohydrate, protein and fat) intakes within a pre-determined 5% variation since different food sources have unique nutrient compositions (**Supplementary Figure 1**). Menus were not matched for micronutrient intakes. Since diet intake may be altered by individual preferences, appetite, and satiety, diet intake compliance was monitored to quantify actual intake differences across the 2 intervention diets. Total carbohydrate and fat density (percentage of total calories) intakes were not significantly different between diets. Protein intake in the animal diet was 3% higher compared to vegan diet for females, and 1.3% higher for males. Intake of total calories, saturated fat, B2, B6, sodium, phosphorus, selenium, and potassium was higher on the animal diet. Intake of polyunsaturated fat, fiber, magnesium, iron, copper, vitamin A, vitamin C, vitamin B1 and folate was higher on the vegan diet. Non-heme iron, which is less easily absorbed than the heme-iron found in red meat, was also higher on the vegan diet. Although vitamin B12 was not consumed in the vegan diet, its absence is unlikely to alter the results since the half-life of this vitamin is 6 days in plasma [10]. Overall, nutrient intakes were higher in males compared to females (**Table 2**).

Diet intervention and habitual diet intake

Three-day diet diaries were used to assess habitual diet intake for comparison with the 2 controlled diet interventions (**Supplementary Table 1**). Habitual diet intakes were significantly higher in vitamin C, fat, % calories from fat, and sodium compared to mean intakes in both the animal and vegan diets. Participants demonstrated full compliance with intake of vegan and animal meals and snacks, although there were some intake differences when satiety was reached before meal completion. Participants on the vegan diet increased their intake of vitamin A, vitamin E, iron, folate, magnesium, relative calorie intake from protein, polyunsaturated fats, and total dietary fiber; and decreased their intake of vitamin B12, carbohydrates, calories, and monounsaturated and saturated fats compared to their habitual diets. Participants on the animal diet also significantly increased intakes of vitamins B2 and B6 and decreased intakes of vitamin E, potassium, protein, relative calorie intake from protein, polyunsaturated fatty acids, and total dietary fiber compared to their habitual diets. The direction of these differences was

consistent across genders. No significant differences in intake were noted between the vegan/animal or animal/vegan washout periods (**Supplementary Table 2**).

Table 2. Significant diet intake differences between vegan and animal intervention diets

| Diet Variable | Animal diet means | | Vegan diet means | | P-value* | Gender differences** | |
	F	M	F	M		Vegan P-	Animal P-value
Kilocalories (Kcal)	1758.00	2619.75	1508.91	2255.47	**1.32E-05**	**2.86E-04**	**2.18E-04**
Alanine (mg)	4168.60	6214.95	2585.01	4098.12	**6.40E-05**	**2.86E-04**	**2.86E-04**
Cysteine (mg)	1339.82	1935.35	706.64	1194.12	**6.40E-05**	**2.17E-04**	**2.86E-04**
Glycine (mg)	3827.37	5628.07	2324.53	3706.53	**6.40E-05**	**2.86E-04**	**2.86E-04**
Histidine (mg)	2476.30	3661.50	1493.30	2368.32	**6.40E-05**	**2.86E-04**	**2.86E-04**
Isoleucine (mg)	4278.14	6398.27	2382.80	3873.30	**6.40E-05**	**2.86E-04**	**2.17E-04**
Leucine (mg)	6880.82	10366.10	3468.68	6072.80	**6.40E-05**	**1.64E-04**	**2.17E-04**
Lysine (mg)	5618.20	8634.38	3060.98	4908.35	**6.40E-05**	**2.86E-04**	**2.86E-04**
Methionine (mg)	1984.23	3029.90	793.07	1298.65	**6.40E-05**	**2.17E-04**	**2.86E-04**
Phenylalanine (mg)	3820.75	5816.07	2625.02	4257.70	**6.40E-05**	**2.17E-04**	**2.17E-04**
Proline (mg)	17998.58	24831.60	2116.40	3689.31	**6.40E-05**	**1.64E-04**	**2.86E-04**
Threonine (mg)	3418.18	5190.48	2184.59	3549.60	**6.40E-05**	**2.17E-04**	**2.86E-04**
Tryptophan (mg)	1157.75	1689.03	570.14	873.45	**6.40E-05**	**3.75E-04**	**2.86E-04**
Tyrosine (mg)	2870.95	4404.55	1773.75	2872.97	**6.40E-05**	**2.86E-04**	**2.17E-04**
Valine (mg)	4444.27	6769.45	2750.23	4454.65	**6.40E-05**	**2.17E-04**	**2.17E-04**
Polyunsaturated fat (g)	13.29	17.52	22.42	36.71	**6.41E-05**	**1.64E-04**	5.40E-03
Protein (g)	85.60	128.27	62.54	102.91	**6.41E-05**	**2.17E-04**	**2.87E-04**
Saturated fat (g)	21.89	37.44	9.48	13.92	**6.41E-05**	**5.55E-04**	**1.65E-04**
Total fiber (g)	14.67	20.32	30.52	49.02	**6.41E-05**	**2.14E-04**	**3.76E-04**
Iron (mg)	7.39	10.45	12.71	19.00	**7.41E-05**	**9.23E-04**	**2.86E-04**
Protein %	19.50	19.58	16.47	18.26	**7.42E-05**	**4.89E-04**	9.16E-01
Vitamin B12 (ug)	3.48	5.38	0.00	0.00	**1.41E-04**	NA	1.58E-03
Selenium (ug)	46.33	57.75	13.14	11.78	**1.43E-04**	4.79E-01	2.14E-03
Copper (ug)	927.11	1015.05	1872.39	2528.43	**1.43E-04**	3.43E-03	5.48E-02
Potassium (mg)	4.42	6.49	1.80	2.29	**1.43E-04**	1.65E-02	**2.76E-04**
Vitamin E (mg)	6.39	8.33	13.38	17.48	**1.43E-04**	3.75E-02	3.73E-02
Magnesium (mg)	202.25	290.25	424.75	589.12	**1.43E-04**	**1.06E-03**	**5.20E-04**
Phosporus (mg)	1331.64	2054.53	801.05	1121.75	**1.43E-04**	2.18E-03	**2.80E-04**
Vitamin B2 (mg)	1.06	1.70	0.60	0.81	**1.43E-04**	3.43E-03	**9.39E-04**
Vitamin A (mg)	0.17	0.30	4.62	3.51	**1.65E-04**	5.24E-01	**2.01E-04**
Vitamin C (mg)	40.92	49.58	61.23	80.80	**1.68E-04**	1.11E-02	1.63E-03
Vitamin B1 (mg)	0.71	1.05	0.94	1.31	**1.68E-04**	4.30E-03	**2.76E-04**
Vitamin B6 (mg)	1.60	2.27	1.20	1.42	**1.68E-04**	5.35E-03	**7.03E-04**
Folate (ug)	197.83	258.32	281.98	351.43	**1.97E-04**	2.64E-02	**3.80E-04**
Sodium (mg)	1899.36	2725.60	1423.84	2011.90	**2.31E-04**	2.18E-03	**9.44E-04**
Carbohydrate (g)	208.48	312.80	191.23	267.18	**1.23E-03**	**3.75E-04**	**2.87E-04**
FAT (g)	59.93	88.08	52.73	79.28	1.56E-03	**6.32E-04**	**1.65E-04**
FAT %	30.71	30.32	31.31	31.51	1.01E-02	9.72E-01	1.00E+00
Monounsaturated fats (g)	21.43	28.67	17.18	27.96	1.12E-02	**2.82E-04**	**2.15E-04**
Serine (mg)	3236.43	4909.48	2870.00	4586.06	1.65E-02	**2.86E-04**	**2.17E-04**
Vitamin D (ug)	1.30	1.67	1.15	0.86	3.11E-02	2.60E-01	**5.30E-04**
CHO %	47.38	47.70	50.95	47.50	7.63E-02	**1.35E-03**	1.00E+00
Arginine (mg)	4612.57	6672.82	4315.64	7102.65	8.08E-01	**2.17E-04**	**2.17E-04**

*Conventional P-values for animal vs. vegan diets calculated using Wilcoxon signed-rank test. F, female; M, male.
Significant p values for gender are in bold. A Wilcoxon rank sum test was used to calculate gender differences.

Clinical response and nutritional status

Subjects on the vegan and animal dietary interventions showed small but significant changes in several plasma biochemistry parameters related to glucose and lipid

metabolisms within 48 hours (**Table 3, Figure 2**). The most significant metabolic effect was a decrease in triglycerides and cholesterol/HDL ratio in participants consuming the vegan diet with no gender interaction. However, participants consuming the vegan diet had a significant decrease in insulin and HOMA-IR [11] that differed by gender. A marked increase in plasma magnesium, slight increase in sodium, but decreased phosphate occurred in individuals consuming the vegan diet (**Table 3**).

Effect of the dietary intervention on plasma amino acid metabolic status
Compared to the animal diet, the vegan diet intervention was associated with a decrease within 48 hours of 10 plasma amino acid levels, including total BCAAs (driven by leucine and valine), total essential amino acids (EAAs) (driven by leucine, valine, threonine, tryptophan, methionine, and lysine), and an increase in arginine and glycine (Table 3, Figure 2). The BCAA and EAA effects differed by gender with the males having significantly higher plasma levels of valine and leucine. The alpha-keto acid analogue of isoleucine (a BCAA), 3-methyl 2 oxovaleric acid, was found to be significantly increased during the vegan diet with no gender specific effects. Lysine, tryptophan, arginine, and methionine levels showed gender differences at baseline that did not carry over to the intervention response (See **Supplementary Table 3**).

Effect of the dietary intervention on plasma fatty acid profiles
Significant increases in the concentration of 3 saturated and 2 monounsaturated fatty acids, specifically dodecanoic acid (C12:0), myristic acid (C14:0), capric acid (C10:0), 5-dodecenoic acid (C12:1) and myristoleic acid (C14:1) occurred in participants consuming the vegan diets. None of these effects were gender dependent (**Table 3**).

Effect of the dietary intervention on plasma bile acids
Participants consuming the vegan diet had significantly decreased bile acids at 24hrs (GUDCA, and GCA) and at 48 hours (GUDCA, DCA, and HDCA) (**Table 3**). A trend in decreased plasma concentrations of GUDCA occurred in males consuming the vegan diet relative to the animal diet at 24 and 48 hours.

Relationships between dietary intake and plasma metabolites
Spearman's rank correlation analysis was performed to analyze changes in diet intake against blood biochemical and metabolite concentrations (**Table 4**). Strong positive correlations were identified between intakes of 11 dietary amino acids and their amino acid concentrations in the plasma. Positive correlations were also found between intakes of vitamins B2, B6, B12, and B1 and total plasma amino acids and between dietary and plasma magnesium. In addition, dietary polyunsaturated fats and total dietary fiber were negatively correlated with plasma insulin, HOMA-IR, and triglycerides.

Table 3: Vegan verses animal diet intervention response, clinical and metabonomic biomarkers

Panel / MARKER	Diet Day	Animal F* Mean	Animal M* Mean	Vegan F Mean	Vegan M Mean	Estimate DIET	P-value DIET**	P-value GENDER***
Amino Acid								
Proline (nmol/ml)	Day 3	133.69	157.05	87.65	102.67	-5.30E-01	**1.87E-11**	1.43E-01
Valine (nmol/ml)	Day 3	189.44	250.42	157.50	218.91	-3.21E+01	**2.20E-08**	**2.64E-05**
EAAs (nmol/ml)	Day 3	829.65	963.39	734.15	867.82	-9.52E+01	**1.63E-06**	**8.26E-04**
BCAAs (nmol/ml)	Day 3	332.68	454.43	294.94	413.18	-5.58E+00	**1.74E-06**	**5.80E-05**
Lysine (nmol/ml)	Day 3	154.14	175.17	136.12	150.24	-2.18E+01	**2.38E-05**	7.14E-01
Citrulline (nmol/ml)	Day 3	23.62	29.47	20.35	26.42	-2.66E+00	**3.82E-05**	7.90E-02
Threonine (nmol/ml)	Day 3	140.30	120.94	112.54	103.85	-1.14E-01	**5.92E-04**	6.48E-01
Tryptophan (nmol/ml)	Day 3	52.29	56.01	46.69	48.78	-5.44E+00	**7.91E-04**	8.90E-01
Alanine (nmol/ml)	Day 3	278.92	301.04	242.58	262.26	-3.31E+01	2.62E-03	5.15E-01
Arginine (nmol/ml)	Day 3	68.76	75.00	66.50	87.34	9.38E+00	2.88E-03	5.99E-01
Methionine (nmol/ml)	Day 3	23.18	25.29	19.93	22.96	-2.44E+00	3.57E-03	2.30E-01
3 methyl 2 oxovaleric acid (nmol/ml)	Day 3	168.88	99.96	219.16	209.77	1.45E+02	7.71E-03	4.00E-01
Leucine (nmol/ml)	Day 3	228.54	311.67	220.17	163.29	-1.63E+00	1.03E-02	**4.49E-03**
Tyrosine (nmol/ml)	Day 3	47.42	55.06	40.62	53.44	-5.21E+00	1.80E-02	1.51E-01
Glycine (nmol/ml)	Day 3	167.30	172.06	162.56	182.50	2.60E+01	3.86E-02	9.18E-01
Clinical								
Magnesium (mM.L^{-1})	Day 3	0.79	0.85	0.87	0.89	6.40E-02	**5.19E-05**	3.40E-01
Triglycerides (mM.L^{-1})	Day 3	0.83	1.22	0.72	0.58	-4.01E-01	**1.61E-04**	7.75E-01
CHOL/HDL	Day 3	2.79	3.08	2.67	2.75	-2.98E-02	**3.42E-04**	7.06E-01
Sodium (mM.L^{-1})	Day 3	140.48	141.32	140.84	142.88	1.01E+00	4.76E-03	3.79E-01
Phosphate (mM.L^{-1})	Day 3	1.11	1.17	1.02	1.05	-8.01E-02	8.56E-03	2.36E-01
Insulin (μU.mL^{-1})	Day 3	18.56	12.48	15.40	9.18	-2.98E-01	3.04E-02	**2.97E-03**
HOMA-IR	Day 3	4.24	3.04	3.49	2.16	-2.42E-01	3.95E-02	**1.07E-02**
Fatty Acids								
Dodecanoic acid (C12:0) (ng/ml)	Day 3	721.40	801.41	1338.69	1564.94	1.26E+02	**4.08E-05**	2.55E-01
5-Dodecanoic acid (C12:1) (ng/ml)	Day 3	989.79	1255.54	1806.04	2234.53	8.79E+02	9.77E-04	1.79E-01
Myristic acid (C14:0) (ng/ml)	Day 3	3067.98	3056.96	4357.64	4233.59	1.18E+03	2.27E-03	8.65E-01
Capric acid (C10:0) (ng/ml)	Day 3	686.56	603.76	1285.24	1408.98	6.94E+02	3.09E-03	9.94E-01
Myristoleic acid (C14:1) (ng/ml)	Day 3	3067.98	3056.96	4357.64	4233.59	2.14E+03	1.92E-02	2.43E-01
Bile Acids								
Glycoursodeoxycholic acid (GUDCA) (nmol/L)	Day 2	0.08	0.08	0.08	0.04	-0.4904	**6.42E-04**	3.50E-01
Glycocholate (GCA) (nmol/L)	Day 2	0.21	0.16	0.07	0.08	-0.778	**9.58E-04**	8.77E-02
Glycoursodeoxycholic acid (GUDCA) (nmol/L)	Day 3	0.09	0.12	0.09	0.06	-6.75E-01	1.46E-02	5.01E-02
Deoxycholic acid (DCA) (nmol/L)	Day 3	0.26	0.38	0.31	0.21	-3.35E-01	3.63E-02	5.82E-01
Hyodeoxycholic acid (HDCA) (nmol/L)	Day 3	0.05	0.06	0.09	0.10	1.57E-01	4.66E-02	4.87E-01

*F=female; M=male. The model is a mixed effect model for each day and marker separately. A random effect per subject was used. A t test (null hypothesis: coeff = 0) was used. **The p-values correspond to the coefficient of interest in the regression model. ***P-values correspond to gender main term effect. P-values bolded if False Discovery Rate <0.10.
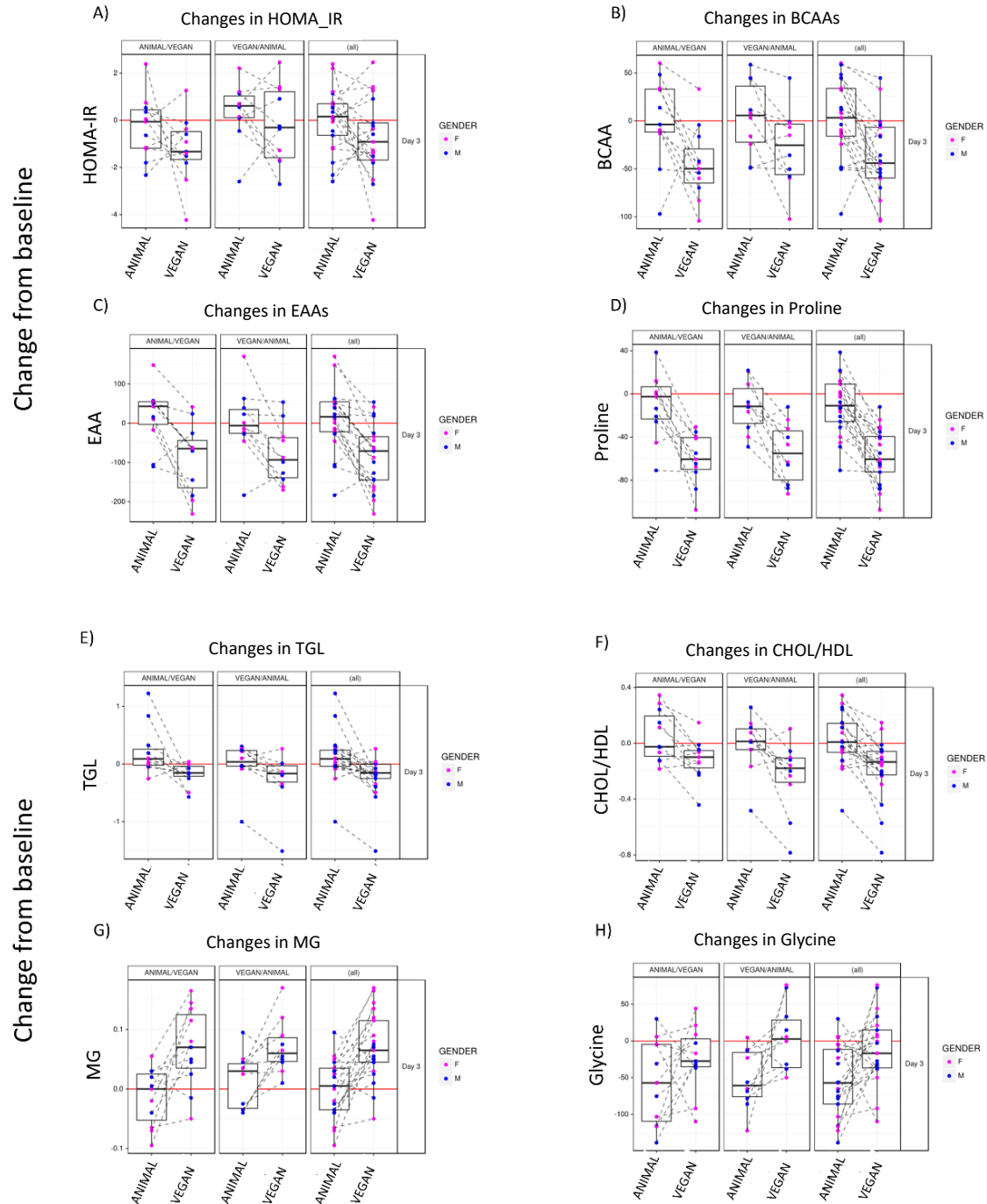
Figure 2A-H. Vegan vs. animal diet metabolic response signature by diet day and diet sequence. Key metabolite differences are graphically depicted showing day-3 changes from baseline for both diets, both directions (animal, washout, then vegan and vice versa). Gender differences shown with pink (female) and blue (male) dots. A mixed linear model was used to evaluate significant differences. A) HOMA-IR p=3.95E-02 (diet) p=1.07E-02 gender; B) BCAAs p (diet)=1.74E-06 p (gender)=5.80E-05; C) EAAs p(diet)=1.63E-06 p(gender)=8.26E-04; D) Proline p(diet)=1.87E-11; E) TGL p(diet)=1.61E-04; Chol/HDL p(diet)=3.42E-04; F) Mg p(diet)=5.19E-05; G) Glycine p(diet)=3.86E-02. BCAAs=branched chain amino acids; EAA=essential amino acids; TGL=triglycerides; Chol/HDL=cholesterol to high density lipoprotein ratio; MG=magnesium; HOMA-IR is calculated by glucose*insulin/22.5.
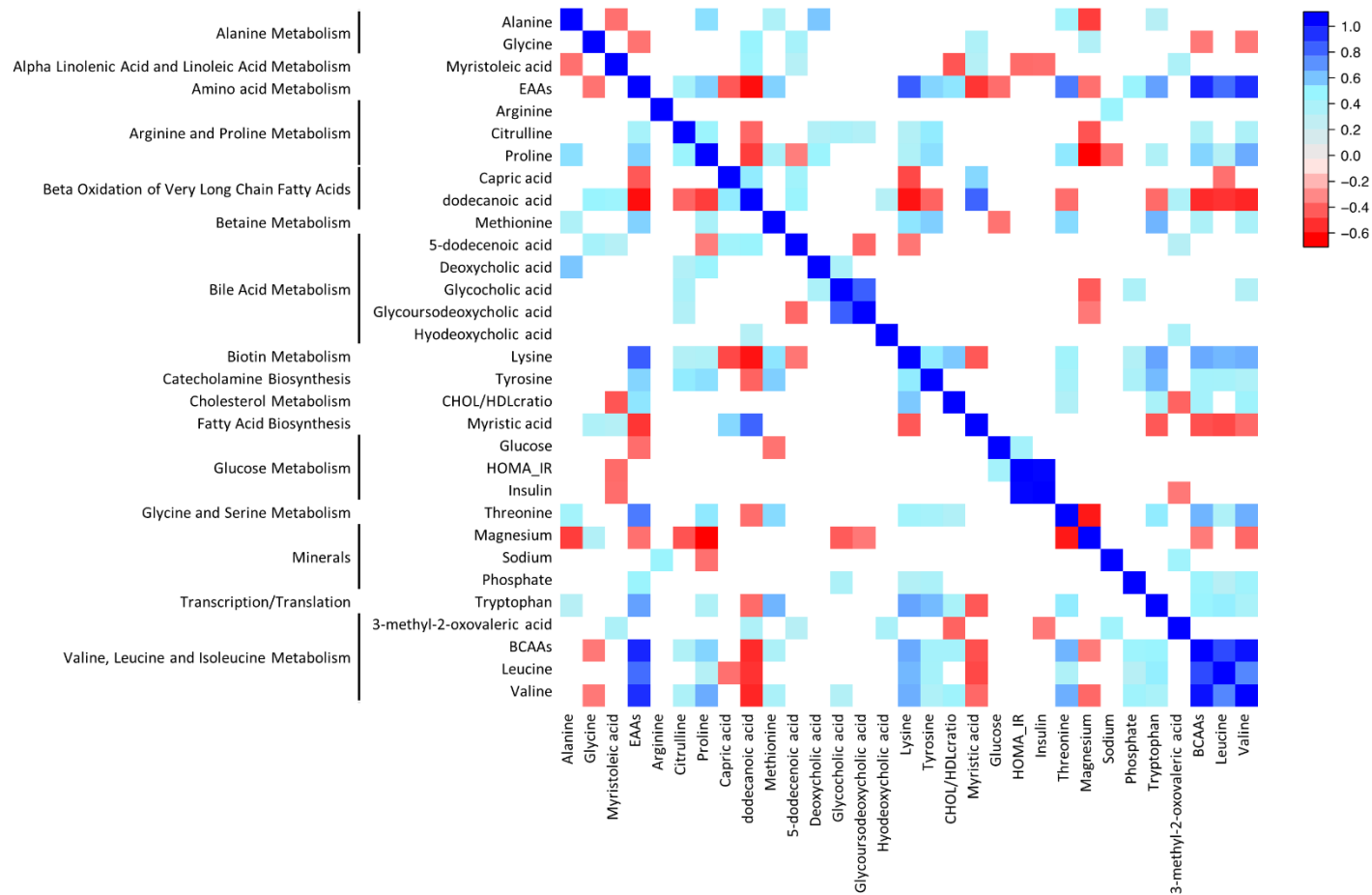
Figure 3. Heatmap with color gradients related to the similarity between blood biochemical variables based on spearman correlation coefficients, using a statistical significant treshold at 95% confidence interval. A blue cell represents a positive correlation between the corresponding biochemical species with a value ranging from 0.3 to 1; red represents an anti-correlation between the variables, with a value ranging from -0.3 to 0.60; a white cell indicates no correlation. Variables were ordered with biochemical pathways or classes.

Correlation analyses between blood plasma biochemical species

In addition to the correlations identified between biochemical metabolite classes (e.g., correlations of an amino acid with another amino acid, or of one bile acid with another), strong correlations between metabolites of different molecular classes were found: individual bile acids, fatty acids, and lipids correlated with individual amino acids (Figure 3). The branched-chain amino acids were negatively correlated with myristic acid and magnesium but positively correlated with the Chol/HDL ratio and phosphorus. The essential amino acids were negatively correlated with dodecanoic acid, myristic acid, capric acid, HDL, glucose, and glycine and positively correlated with Chol/HDL ratio, phosphorus, and triglycerides. The metabolite 3-methyl-2-oxovaleric acid had an inverse correlation with insulin, Chol/HDL ratio, and TGs and a positive correlation with the myristoleic and dodecaonic acids and hyodeoxycholic acid (HDCA).

DISCUSSION

Vegan diet patterns have been historically associated with diabetes prevention, promotion of blood sugar control, improved insulin sensitivity, decreased total and LDL cholesterol, and higher levels of HDL [5, 12], To our knowledge, this is the first short-term, plant-based (vegan) diet study focused on metabolic health that identified improvement in clinical and metabolic parameters associated with insulin resistance in healthy subjects.

A well-balanced vegan diet improves lipid and insulin metabolic status

Participants enrolled in this study were not vegans and habitually consumed animal protein-based diets typical of the Swiss culture. The animal- and vegan-protein foods provided in this intervention were intentionally designed to be more nutritious than typical diets and were balanced in macronutrient content. The foods were consumed in a semi-controlled, supervised environment and intake measured to reduce uncertainty in nutrient consumption. Participants in our study showed statistically significant improvements in insulin and HOMA-IR (33% reduction vegan vs. animal), TGs, and the Chol/HDL ratio after only 48 hours of consuming the vegan diet. Elevated serum lipids, including total CHOL and TGs and a high cholesterol/HDL ratio have been associated with insulin resistance [13-15]. Improvements in these metabolic syndrome-associated risk factors were likely related to higher fiber content (39.8 g and 17.5 g from the vegan and animal based protein diets consecutively), micronutrient density, and non-oxidized polyunsaturated fats (PUFAs), and lower glycemic index food choices found in a healthful vegan diet pattern. Inverse correlations were also found between dietary PUFA intake and total dietary fiber with insulin, HOMA-IR, and TGs, consistent with higher PUFA and fiber intakes (**Table 2, Table 4**). Major contributors of PUFAs from the vegan

diet include cashew butter, rapeseed oil, sunflower oil, hummus, hemp protein powder, and soy yoghurt (**Supplementary Figure 1**).

A significant increase in plasma magnesium from the vegan diet was found and a highly significant positive correlation between dietary magnesium intake and plasma magnesium, insulin, and HOMA-IR was detected (**Tables 3 and 4**), cashew butter, hummus, lentils, and kidney beans eaten as part of the vegan diet regimen in this study are rich sources of magnesium. Increased intake of magnesium has been shown to reduce the risk of impaired glucose tolerance and insulin metabolism [16, 17] consistent with the improved insulin control and decreased triglyceride levels observed in this study (**Table 2, Figure 4**).

In addition to the changes in blood lipid profile, the saturated fatty acids dodecanoic, capric, and myristic acids were significantly elevated after the vegan diet. These saturated fatty acids (SFAs) likely reflect the fatty acid composition of the coconut milk provided at dinner each evening over the 2 days. The monounsaturated species of these fatty acids, 5-dodecanoic and myristoleic acids, were also significantly elevated after the vegan diet and reflect the digestion and metabolism of their dietary precursors. Intake of dodecanoic acid may decrease the ratio of total to HDL cholesterol ratio by raising HDL [18], [19]. Although only a trend between dodecanoic acid HDL was seen in the present study, a positive correlation between capric acid and HDL and an inverse correlation between Chol/HDL ratio and myristoleic acid was found, suggesting that these fatty acids may improve the lipoprotein profile (**Figure 3**).

In relation to observed lipid changes, some bile acid species were higher after consuming the animal protein relative to the vegan diets, consistent with their role in the digestion and absorption of dietary fat and cholesterol; and their synthesis in the liver from cholesterol. This effect may be explained by the higher saturated fat and cholesterol content in the animal diet (red meat) and the higher fiber in the vegan diet may have resulted in a relative decrease in bile acid absorption. Furthermore, bile acids are also essential metabolic integrators and signalling factors, far beyond their role as lipid solubilizers and simple regulators of bile-acid homeostasis[20]. In particular, it is now well established how glucose and insulin can enlarge bile acid pool size and their blood circulating levels, by modulating Cholesterol 7 alpha-hydroxylase, a rate limiting enzyme in bile acid synthesis[21]. Blood bile acid concentrations have been associated with fasting and postprandial insulin and glucose, with further implication in diabetes and obesity research [22, 23].The higher circulating levels of certain bile acids (and secondary bile acid DCA) and insulin observed in individuals on the animal diet compared to the vegan diet may therefore be directly related. It may be envisioned that

a vegan diet strategy may offer protective benefits against insulin resistance, as noted here with improved HOMA-IR, and lowered circulating insulin and specific bile acid species [24]. Future evaluation of the alternating plant – animal protein diet in insulin resistant individuals is warranted to determine if this strategy is beneficial for improving metabolic control.

Table 4: Spearman's rank correlations between plasma concentrations and dietary nutrient intakes

Diet	Plasma	r	P-value*
Amino acids (AAs)			
PRO	PRO	0.74	**2.42E-08**
VAL	VAL	0.73	**5.35E-08**
LYS	LYS	0.62	**1.05E-05**
LEU	LEU	0.59	**3.94E-05**
TYR	TYR	0.52	**4.04E-04**
MET	MET	0.52	**4.82E-04**
PHE	PHE	0.47	**1.74E-03**
ARG	ARG	0.46	**2.27E-03**
ILE	ILE	0.42	**5.61E-03**
TRP	TRP	0.42	**6.01E-03**
ALA	ALA	0.36	**1.77E-02**
B2	Total AAs	0.71	**3.28E-07**
B6	Total AAs	0.71	**3.54E-07**
B12	Total AAs	0.60	**4.22E-05**
B1	Total AAs	0.34	3.23E-02
Mg	Mg	0.54	**3.48E-04**
PUFA	Insulin	-0.55	**1.56E-04**
PUFA	HOMA-IR	-0.54	**2.07E-04**
PUFA	TG	-0.49	**8.76E-04**
Saturated Fat	TG	0.30	**4.80E-02**
TDF	Insulin	-0.51	**5.55E-04**
TDF	HOMA-IR	-0.50	**7.38E-04**
TDF	TG	-0.49	**1.10E-03**

*Conventional p-values are shown and those marked in bold were significant after False Discovery

A vegan diet may improve insulin sensitivity by modulating AA bioavailability
Elevated fasting BCAA and aromatic amino acids have been associated with higher metabolic risk of insulin resistance and obesity (21-29). The vegan diet induced a strong modulatory effect within 48 hours on the circulating levels of EAAs and BCAAs (Figure 2). In addition, blood concentrations of EAAs were directly correlated with intake of these amino acids suggesting this improved metabolic health status was associated with dietary protein (**Table 4, Figure 4**). Several studies captured specific AA signatures related to HOMA-IR conditions in lean and obese subjects [25], Chinese and Asian-Indian males [26], and in a weight loss cohort study of 500 men and women [27]. These reports showed a similar elevated AA signature in high versus low HOMA-IR status

including BCAAs (valine, leucine, isoleucine), aromatic (phenylalanine, tyrosine), alanine, glutamine, asparagine, and arginine which were positively associated with insulin resistance. The same AA differences from the animal vs vegan diets were found in this study with the exception of phenylalanine and asparagine. In a 6-month weight loss intervention study of 500 men and women, change in HOMA-IR was not strongly associated with amount of weight lost but rather with an AAs signature that included alanine, proline, BCAAs (valine leucine/isoleucine), methionine, aromatic amino acids (ArAAs) (phenylalanine, tyrosine), glutamine and ornithine [27]. The association of this AAs profile and HOMA-IR in different populations is consistent with the associations found in our participants consuming the vegan diet. Increased concentrations of the circulating essential BCAAs resulted from diet intake and protein catabolism. However, BCAA may increase insulin resistance when consumed with high fat diets, at least in rats [25]. The animal protein diet in the study reported here was slightly higher in overall fat content and significantly higher in saturated fat and BCAAs, which may potentiate an increase in HOMA-IR (**Table 3**). The vegan diet produced reductions in, but not strong statistical correlations between HOMA-IR, BCAAs, and ArAAs, which may be related to the short duration of our study (**Table 3, Figure 3**). Longer term (1 and 6 week) vegetarian plus fish diets significantly reduced and showed strong correlations between HOMA-IR, BCAAs, and ArAAs [28]. Plasma concentrations of 3-methyl-2-oxovaleric acid, a keto-acid product of isoleucine metabolism, decreased on the animal protein diet which may be due to increased activity of branched-chain keto acid dehydrogenase (BCKDH) [29].

Gender dimorphism may influence the impact of dietary modulation of glucose and insulin metabolism

Twelve amino acids, including the BCAAs, showed significant gender differences at baseline (**Supplementary Table 3**). The BCAAs, insulin and HOMA-IR showed gender differences post intervention (**Table 3**). Recent studies found that males had significantly higher BCAAs, phenylalanine, tyrosine, alanine, proline, methionine, glutamine and ornithine [30]. In contrast, insulin and HOMA-IR showed gender differences in intervention response with plasma concentrations slightly higher in females in the study presented here (**Table 3**). These results were inconsistent with published literature on correlations between gender dimorphism and insulin resistance [31, 32]. However, gender differences in insulin concentrations and calculated HOMA-IR may be augmented by consuming defined short-term or long-term diets or by differences in estrogen levels between studies based on age or unknown menstrual cycle status, or any other difference in study conditions. Males were also found to have slightly higher magnesium levels (**Table 3**), consistent with published literature [33].

Flexitarian dieting for metabolic health improvement?

Intermittent or periodic fasting has been proposed as a novel approach to weight management and modulation of markers of metabolic syndrome. A similar flexitarian approach of alternating between animal and vegan protein choices requires only periodic alteration of an individual's dietary habits. Reducing the amount of habitual change on a frequent basis may increase adherence to dietary lifestyle change which has been shown to be the most important factor for diet success [34]. Additionally, a flexitarian diet optimizes nutrient intake from all protein sources and prevents potential micronutrient deficiencies from an exclusively vegan diet. Based on the results shown here, an intermittent vegan diet with healthful macronutrient and micronutrient balance may beneficially modulate blood insulin, lipids, and amino acids (**Figure 4A**).

Study limitations & opportunities

This was a small, pilot study of 21 participants with concomitant limitations of sample size and risk of false positives. Even though vegan meals appeared to have a higher volume of food, caloric intake was less on this diet compared to animal protein diet due to the satiating effect of the high fiber containing plant foods and despite efforts to match calories and macronutrient contents (**Table 2**). We did not distinguish between the different types of fiber (soluble, insoluble, inulin) and phytosterols such as plant stanols and sterols that are known to have an effect on lipids/cholesterol, bile acids, and glucose. However, the complexity of metabolite-metabolite and varying genotype-metabolite interactions challenge simplistic single metabolite interpretations of the phenotypic response to the diet interventions. Even though many nutrient parameters were recorded, this study relied on food diaries with manual database entry for analysis which highlights the lack of availability of high quality, reliable nutrition data capture and measurement technologies. This was a study of non-obese healthy participants without insulin resistance. However, the results presented here suggest that this diet strategy be tested in a non-healthy population to examine its beneficial effects on insulin and AA metabolism.

The strength of the study was the development and testing of a specific diet strategy that more closely resembled habitual animal protein-based diets compared to a vegan diet comprised of easily accessible food choices in a controlled, cross-over design. These results can be translated more easily to a diet strategy to promote health improvement compared to single food ingredient interventions. Moreover, the small size of the study permitted a focused, quality controlled measurement and analysis of dietary intake.An intermittent, high protein vegan diet may not only impact weight control but may be more environmentally sustainable than current animal-based protein diets.
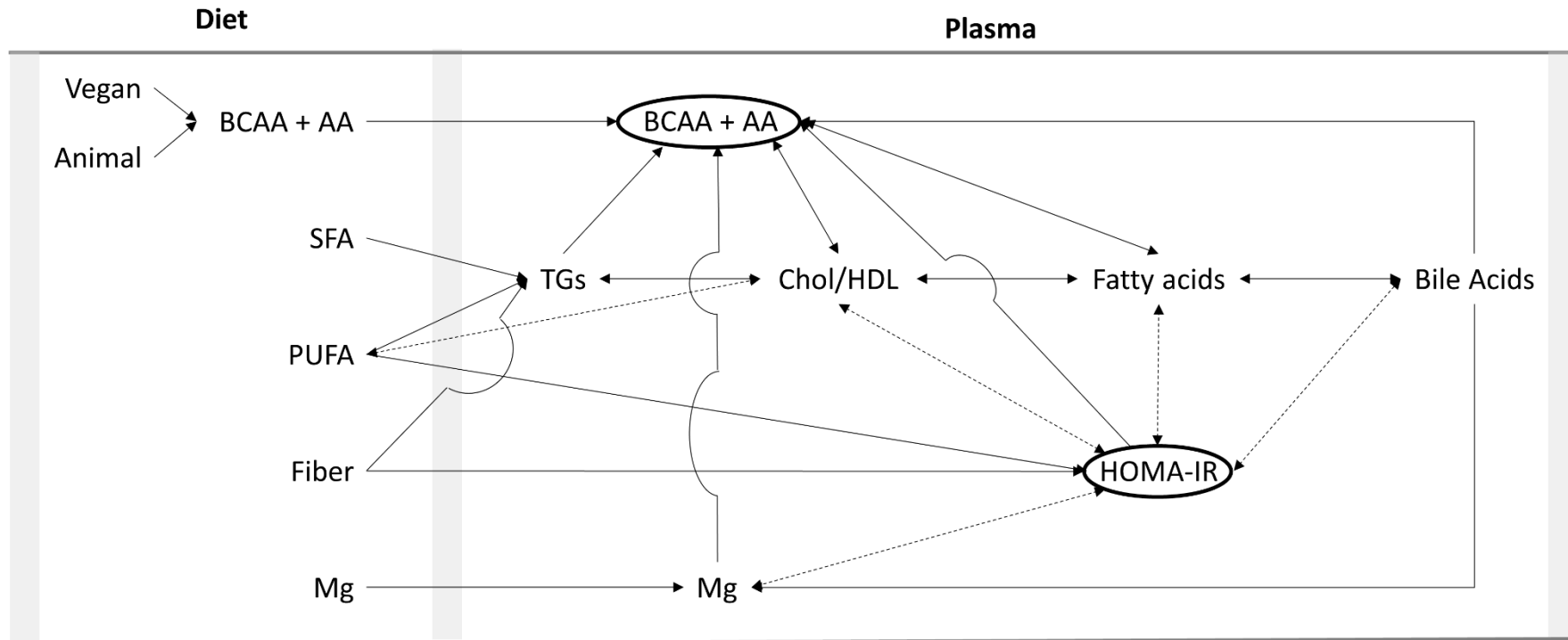
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Figure 4A - The vegan diet decreased plasma AA ,HOMA-IR, Chol/HDL, and bile acids while increasing Mg; with the animal diet having opposing effects. Dietary SFAs increased TGs in the animal diet; high dietary PUFA and fiber intakes from the vegan diet reduced HOMA-IR and TGs. Dietary AA increased 11 plasma AA. Solid lines and arrows depict significant Spearman's correlations between diet and plasma variables and between plasma metabolites; dotted lines depict known associations. Biomarkers that showed significant gender dimorphic responses to the diet interventions are circled. AA=amino acid; BCAA=branched chain amino acids; TGs=triglycerides; Chol/HDL=cholesterol to high density lipoprotein ratio; Mg=magnesium; HOMA-IR is calculated by glucose*insulin/22.
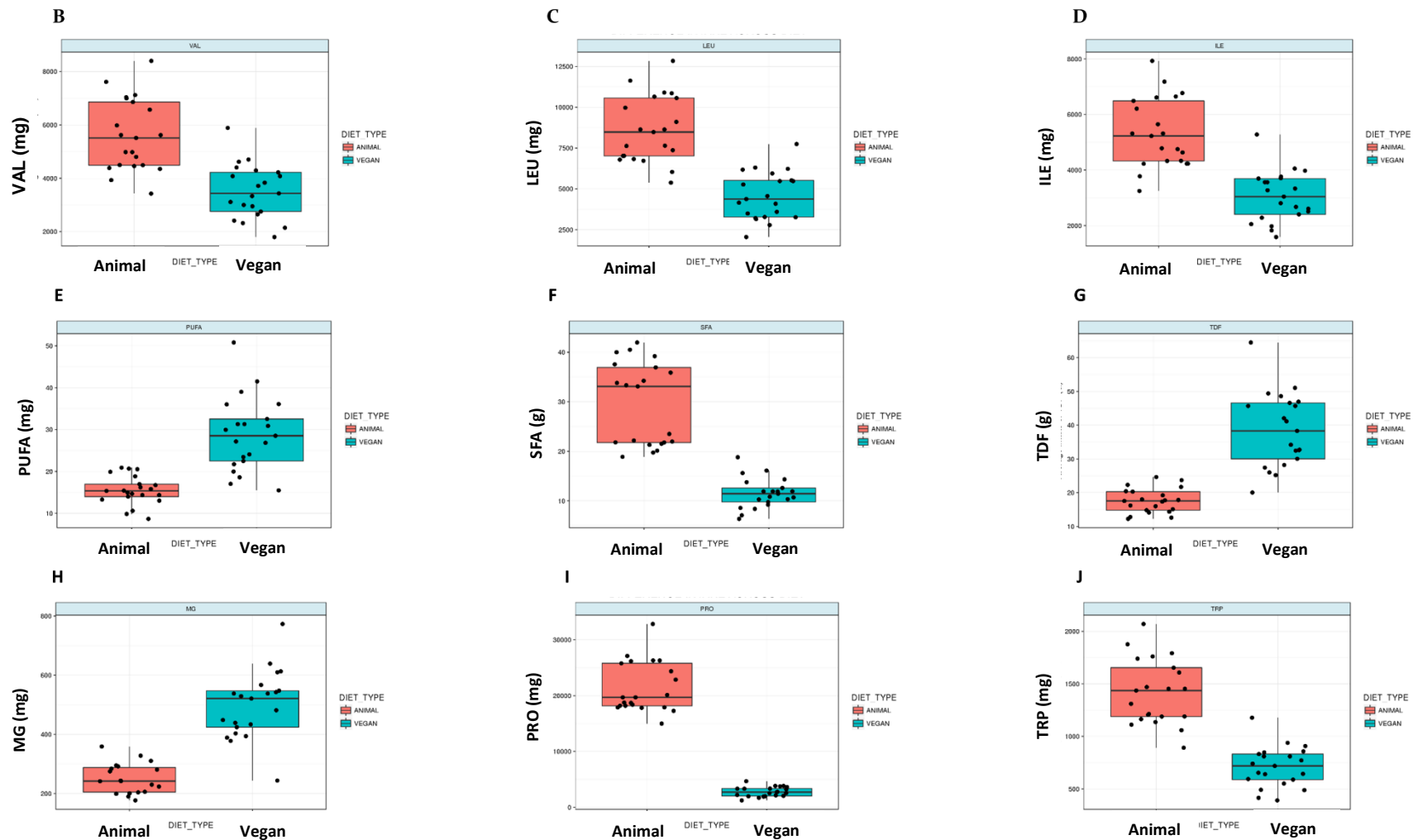
Figure 4B-J - Dietary intake differences across diet interventions for correlated nutrients. Nutrient intake differences in animal vs. vegan diets using a Wilcoxon signed-rank test. A subset of nutrients also found to be correlated with significant metabolites is shown here. B) VAL (valine) p=6.40E-05; C) LEU (leucine) p=6.40E-05; D) ILE (Isoleucine) p=6.40E-05; E) PUFA (polyunsaturated fatty acids) p=6.41E-05; F) SFA (saturated fatty acids) p=6.41E-05; G) TDF (total dietary fiber) p=6.41E-05; H) MG (magnesium) p=1.43E-04; I) PRO (protein) p=6.41E-05; J) TRP (tryptophan) p=6.40E-05. All nutrient intakes were significantly higher in males vs. females (p<0.05).

CONCLUSION

We analyzed the effects of vegan- versus animal protein-based diets on metabolic health parameters. A slightly higher than average protein intake was chosen for both diets to optimize metabolic health impact. We produced a branched-chain amino acid-associated metabolic signature from a short term, healthy, vegan diet challenge (Figure 4). These results suggest an improvement in the ability to adapt to changes in intake of different nutrients or levels of nutrients while maintaining a healthy metabolism [35]. Intermittently substituting vegan meals in otherwise animal-based diets may decrease the unsustainable environmental impact of animal-based diets. Future research should be conducted to evaluate the benefits of this diet strategy in an obese, insulin-resistant population.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

ANIMAL DIET Menu

Breakfast
Croissant
Strawberry jam
Coffee with milk and sugar

Snack
Yogurt Nature
Fresh apple

Lunch
Beef Hamburger with mustard and fresh tomato slices
Chips
Cucumber salad
Applesauce

Snack
Crackers

Dinner
Dijon chicken with pasta
Mixed lettuce with balsamic vinaigrette
Apricot tart

Drinks
Water

"Emergency Snack"
Yogurt, skimmed with fruit
10 Raw plain almonds

Kcals	Kcals
1600	1600
1800	1800
2000	2000
2200	2200
2400	2400
2600	2600
2800	2800
3000	3000

VEGAN DIET Menu

Breakfast
Healthy quinoa rice milk müesli with cashew butter
Hemp protein powder
Black coffee

Snack
Homemade hummus + Hemp protein powder
Carrots, cucumber
Gluten-free bread, Kombucha drink

Lunch
Brown rice and red beans salad with baby spinach, white balsamic vinaigrette
Gluten Free Bread
Green Tea

Snack
Fresh banana with soy yogurt

Dinner
Asian lentil coconut curry +Hemp protein powder
Gluten-free bread
Ginseng and green tea

Drinks
Water

"Emergency Snack"
Soy Yogurt Nature (Coop Naturaplan)

Supplementary Figure S1. The animal and vegan diet menu plans were the same each day for 2 days. Food portions were provided based on 8 personalized calorie plans in accordance with the caloric needs of the individual participants. Hemp protein powder was used for the vegan diet to boost total protein.

Supplementary Table S1. Mean diet intake differences between baseline and intervention diets

Diet Variable	Baseline vs. vegan P-value*	Baseline vs. animal P-value*
Vitamin B12 (ug)	**6.41E-05**	NS
Sodium (mg)	**6.41E-05**	**1.68E-04**
Polyunsaturated fat (g)	**6.41E-05**	**2.29E-04**
Saturated fat (g)	**6.41E-05**	NS
Magnesium (mg)	**7.42E-05**	NS
Fat (g)	**8.56E-05**	**3.01E-04**
Folate (ug)	**8.57E-05**	NS
Kilocalories (Kcal)	**8.58E-05**	NS
Protein (%)	**1.14E-04**	**6.41E-05**
Vitamin C (mg)	**2.29E-04**	**1.68E-04**
Vitamin A (mg)	**3.01E-04**	NS
Carbohydrate (g)	**4.77E-04**	NS
Vitamin B2 (mg)	**6.58E-04**	**1.38E-03**
Fat%	**1.56E-03**	**6.59E-04**
Vitamin E (mg)	**1.76E-03**	**6.73E-04**
Monounsaturated fats (g)	**3.92E-03**	NS
Total fiber (g)	**3.92E-03**	**8.48E-04**
Iron (mg)	**4.87E-03**	NS
Protein (%)	NS	NS
Phosporus (mg)	NS	**1.43E-04**
Protein (g)	NS	**1.52E-04**
Vitamin C (mg)	NS	NS
Sodium (mg)	NS	NS
Polyunsaturated fat (g)	NS	NS
Fat (g)	NS	NS
Vitamin B6 (mg)	NS	**5.00E-04**
Vitamin E (mg)	NS	NS
Fat%	NS	NS
Total fiber (g)	NS	NS
Vitamin B2 (mg)	NS	NS

*Wilcoxon signed-rank test comparing paired average animal and vegan diet intakes with corresponding baseline and all comparisons are bolded as they also met a false discovery rate of $P<0.10$.

Supplementary Table S2. Comparison of baseline and washout diet intakes

Diet Variable	P-value*
Carbohydrate	**6.36E-03**
Total fiber	**1.23E-02**
Magnesium	1.65E-02
Kilocalories	1.99E-02
Sodium	2.85E-02

*Wilcoxon signed-rank test comparing paired average washout food diaries
with corresponding baseline average and those marked in bold were
after False Discovery Rate ($P<0.10$).

Supplementary Table S3. Baseline plasma amino acid status by gender

Amino Acid (nmol/ml)	Female	SD	Male	SD	P-value
BCAAs	322.879	43.869	457.996	42.248	**1.65E-04**
Leucine	94.528	12.853	141.503	11.265	**1.65E-04**
Valine	174.545	25.471	244.347	30.226	**2.18E-04**
Isoleucine*	53.806	9.205	72.146	7.75	**4.91E-04**
EAAs	786.518	80.857	962.85	88.423	**1.36E-03**
Ornithine*	32.559	10.591	45.087	6.989	**4.35E-03**
Phenylalanine*	45.7	6.158	55.726	5.765	**4.35E-03**
Methionine	22.111	3.387	26.377	4.035	**5.41E-03**
Lysine	137.13	30.114	174.895	29.075	**1.24E-02**
Glutamic acid*	16.452	8.07	33.702	19.751	**1.83E-02**
Glutamine*	471.3	59.72	562.596	81.189	**1.83E-02**
Tyrosine	47.073	11.555	59.167	8.034	**2.65E-02**
Arginine	67.603	19.984	82.202	14.837	**4.48E-02**
Tryptophan	52.314	7.933	59.029	7.642	**4.48E-02**
Alanine	302.367	37.867	337.712	65.431	5.28E-02
Proline	137.206	28.536	168.398	42.841	8.45E-02
Citrulline	23.213	5.957	29.073	6.732	9.80E-02
Glycine	187.707	100.015	213.71	68.119	1.30E-01
Asparagine	41.154	6.475	46.753	10.575	1.93E-01
Threonine	128.34	41.449	110.838	23.28	5.50E-01
Serine	91.633	15.776	95.653	19.314	7.51E-01
Histidine	78.044	8.474	77.989	7.283	9.16E-01
Cystine	49.772	21.201	53.412	11.122	1.00E+00

Conventional P-values calculated using Wilcoxon signed-rank test.
*Amino acids with gender difference at baseline but no significant post-intervention
BCAAs=branched chain amino acids, EAAs=essential amino acids
Significant P-values <0.05 in bold