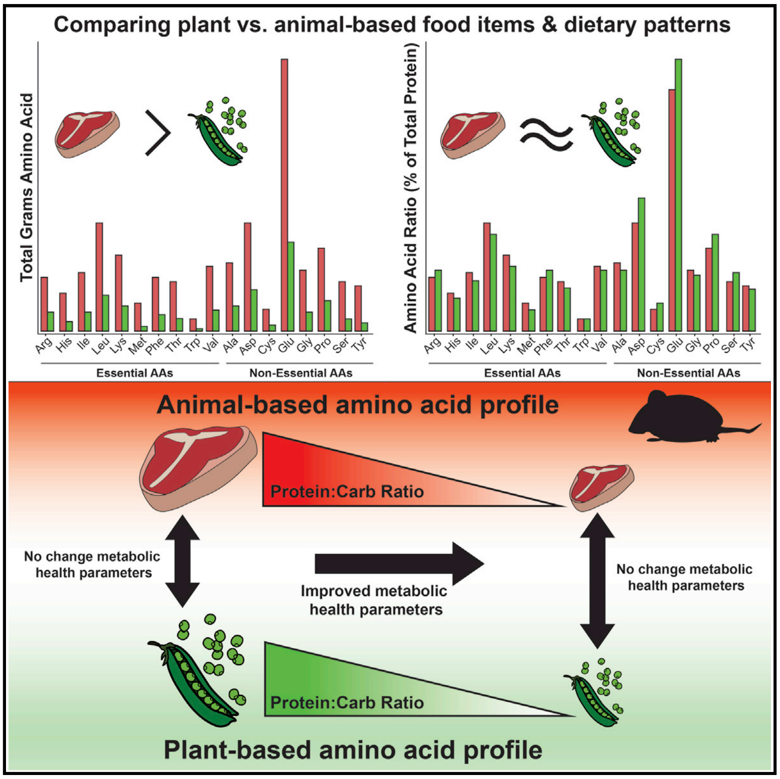
Article

Cell Metabolism

Total protein, not amino acid composition, differs in plant-based versus omnivorous dietary patterns and determines metabolic health effects in mice

Graphical abstract



Highlights

- Plant- versus animal-based food items have different amino acid composition
- Vegans consume less protein but a similar amino acid profile as omnivores
- Total protein, not amino acid composition, drives metabolic health effects in mice

Authors

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In brief

MacArthur et al. show that although vegan diets have lower protein, vegan and omnivorous dietary patterns display similar amino acid intake ratios. They show that total protein, not plant versus animal amino acid composition, drives metabolic health effects in mice, implicating reduced total protein intake in the health benefits of plant-based diets.

MacArthur et al., 2021, Cell Metabolism *33*, 1808–1819
September 7, 2021
https://doi.org/10.1016/j.cmet.2021.06.011

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Total protein, not amino acid composition, differs in plant-based versus omnivorous dietary patterns and determines metabolic health effects in mice

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https://doi.org/10.1016/j.cmet.2021.06.011

SUMMARY

Plant-based dietary patterns are associated with improved cardiometabolic health, but causal dietary components are unclear. Protein has been proposed to play a role, but the importance of protein quantity versus quality remains unknown. We investigated the contributions of total protein amount, amino acid (AA) composition, and plant versus animal source. Analysis of total protein and AA composition of food items and dietary patterns revealed differences between individual food items, but few differences between AA profiles of vegan versus omnivorous dietary patterns. Effects of protein quantity, but not quality, on cardiometabolic health markers were observed in mice using semi-purified diets with crystalline AAs in plant versus animal-based ratios and naturally sourced diets with whole-food ingredients. Our data show relatively little difference in protein quality between plant-based and omnivorous dietary patterns and that reduced total protein intake in plant-based dietary patterns may be a contributor to the benefits of plant-based diets.

INTRODUCTION

Plant-based dietary patterns, including vegetarianism and veganism, are associated with improvements in biomarkers of cardiometabolic health, including body mass index (Bujnowski et al., 2011; Rosell et al., 2006) and insulin sensitivity (Kahleova et al., 2018b), and reduced risk of cardiovascular disease (CVD) (Kelemen et al., 2005), type 2 diabetes mellitus (Patel et al., 2017; Sluijs et al., 2010; van Nielen et al., 2014), and all-cause mortality (Budhathoki et al., 2019; Key et al., 2003; Song et al., 2016).

There are a number of significant differences between vegan or vegetarian and omnivorous dietary patterns that could contribute to the observed benefits of plant-based nutrition (Farmer et al., 2011; Kahleova et al., 2018b). These include but are not limited to differences in energy density, macronutrient content and ratios (protein, carbohydrate, and fat), vitamins (e.g., A, E, C, and folate), micronutrients (e.g., calcium), fiber, and polyphenol abundance. Fiber (Lie et al., 2018) and polyphenols (Scalbert et al., 2005), which are both associated with health benefits, are more abundant in plant-based diets and thus may contribute to benefits of plant-based diets (Kasahara et al., 2018; Roopchand et al., 2015; Zang et al., 2006). Dietary fats including saturated fats and cholesterol, which are lower in plant-based diets, have been studied extensively in association with the detrimental effects of red meat consumption and potential benefits of plant-based diets. However, the specific contribution of fat to health benefits of plant-based diets is difficult to ascertain due to high variability in total fat intake as well as in the specific type of fats consumed with plant-based diets (Schwab et al., 2014).

More recent data from diverse epidemiological and preclinical studies support a role specifically for protein in benefits of plant-based dietary patterns. In epidemiological studies adjusted for other potential confounding dietary and lifestyle factors, plant protein intake is associated with reduced risk of total and CVD mortality (Budhathoki et al., 2019; Song et al., 2016), while total and/or animal protein intake is associated with increased risk of weight gain (Halkjær et al., 2011), diabetes (Levine et al., 2014; Sluijs et al., 2010; van Nielen et al., 2014), and CVD mortality (Song et al., 2016). Importantly, isocaloric substitution of plant protein for animal protein, while controlling for other potentially confounding dietary factors, is associated with reduced CVD (Kelemen et al., 2005) and all-cause mortality (Budhathoki et al., 2019; Song et al., 2016), suggesting an intrinsic difference between animal and plant protein that could contribute to health benefits. Experimentally, isolated plant protein in purified, hydrolysate, or constituent amino acid (AA) form reduces cholesterol and is less atherogenic in rabbits than animal proteins (Huff and Carroll, 1980; Huff et al., 1977; Morita et al., 1997).

An obvious potential difference between plant and animal protein is AA composition, or "protein quality." Plant-based protein sources tend to have lower ratios of essential AAs (EAAs) to non-essential AAs (NEAAs), which have been suggested as mediators of the benefits of plant-based diets. For example, reduced lysine to arginine or methionine to glycine ratios in plant proteins have been associated with reduced glucagon:insulin ratios and cholesterol synthesis (Gorissen et al., 2018; Katan et al., 1982; Sanchez et al., 1988) that could underlie anti-atherogenic effects in preclinical models (Kritchevsky et al., 1982; Morita et al., 1997), although data in humans is equivocal (Vega-López et al., 2010). Dietary restriction of specific AAs that tend to be higher in animal-based food items is also associated with pleiotropic health benefits in rodent models (McCarty, 1999; McCarty et al., 2009). For example, restriction of methionine is associated with health benefits ranging from improved lipid and glucose homeostasis to increased stress resistance and extended longevity in rodent models (Miller et al., 2005; Orentreich et al., 1993). Restriction of branched-chain AAs (BCAAs) is also associated with improved glucose homeostasis and metabolic fitness (Fontana et al., 2016). Thus, differences in AA composition can clearly impact numerous health measures, but whether these differences seen with severely restricted semi-purified diet are causative of benefits in whole-food plant-based dietary patterns remains unclear.

Other studies have not found differences in health outcomes due specifically to protein quality (Lamming et al., 2017; Levine et al., 2014), instead highlighting the non-mutually exclusive role of reduced protein quantity associated with plant-based diets. In rodents, benefits of reduced protein intake without malnutrition include extended longevity (Solon-Biet et al., 2014), reduced tumor growth (Dunaif and Campbell, 1987; Fontana et al., 2013; McCarty, 1999, 2009), and improved markers of metabolic fitness (Laeger et al., 2014; Solon-Biet et al., 2015; Treviño-Villarreal et al., 2018).

Epidemiological studies are also consistent with benefits of low-protein intake on all-cause mortality (Levine et al., 2014). Individuals consuming plant-based diets consume less protein when adjusted for both calorie intake and body weight; however, the relative contribution of protein quantity versus quality remains unclear, as correction for plant versus animal source attenuates health differences in some studies (Budhathoki et al., 2019; Levine et al., 2014) but not others (Song et al., 2016) and most experimental studies on reduced protein intake use only a single source of protein.

Here, we sought to critically evaluate the potential of both protein quantity and quality to contribute to health benefits of plant-based dietary patterns. To this end, we investigated whether individuals adhering to plant-based dietary patterns consume a different AA profile versus those who do not. We also directly assessed effects on health markers in controlled rodent studies using isocaloric diets in which we modulated both total protein and AA composition using either crystalline AA or naturally sourced protein ingredients. We found surprisingly few differences between AA profiles of vegans and vegetarians versus omnivores, but large effects of total protein amount independent of source in controlled rodent studies, suggesting a potential effect of total protein rather than AA composition in health benefits of plant-based diets.

RESULTS

To quantify differences between plant- and animal-based AA profiles on the level of individual food ingredients, we compiled data from the USDA National Nutrient Database (NDB) for standard reference (release SR28). Of 4,676 food items with available protein (by weight and % energy) and AA content, 2,328 were animal based (sub-categories including dairy and egg, poultry, beef, pork, finfish and shellfish, and lamb) and 1,162 were plant based (sub-categories including fruits, vegetables, nuts and seeds, legumes, and grains). In the database there is a high number of redundant food items with nearly identical AA profiles (e.g., yogurt, Greek, nonfat, vanilla versus yogurt, Greek, nonfat, strawberry). To reduce these redundancies, we performed hierarchical clustering on AA profiles within food item sub-categories. This resulted in 37 animal and 38 plant-based food item clusters that represent the diversity of amino acid profiles across food item subgroups. These clusters were then used as the units of analysis for further comparisons.

Animal-based food items tended to have more total protein by weight (g protein/100-g food item; Figure 1A) and as a percent of energy (Figure S1A), and significantly more of all AAs by weight (g AA/100-g food item; Figures 1B and S1B). Following normalization of each AA to total protein (ratio of g AA/g total AA) (Figure 1C), seven AAs had a significantly higher proportional abundance in animal-based food items (Ile, Leu, Lys, Met, Ala, Gly, and Tyr) and two in plant-based food items (Asp and Glu) (Figure 1D).

When AAs were grouped as essential versus non-essential, plant-based food items had significantly less of both groups on a weight basis, but on a ratio basis, plant-based food items had proportionally less EAAs and more NEAAs; however neither was significantly different between groups (Figure S1C).

When food items were broken out into their subgroups, greater variability was evident in the AA profiles of plant-based food groups compared with animal-based groups. In principal component plots, much greater spread in plant-based food groups/items is evident across the first principal component, which captures approximately 91% of the variance in the data (Figure 1E).

Between subgroups, animal-based food groups tended to have higher content of each AA on a weight basis compared with plant groups, with plant-based groups showing greater variability (Figure S1D). When expressed as ratios relative to total protein, the plant-based groups also showed greater variability with all animal-based groups showing similar AA composition except dairy and eggs (Figure S1E). Strong positive and negative correlations between individual AA ratios were evident across all food items (Figure 1F), with the first clustering split discriminating AAs tending to be proportionally higher in animal-based items (Leu, Ile, Met, and Lys) versus plant (Asp and Glu).

The *in silico* analysis above is inherently limited by the potential for bias within the database due to a lack of weighting; all food item clusters receive equal weight even though actual human intakes likely strongly skew toward specific food items/food

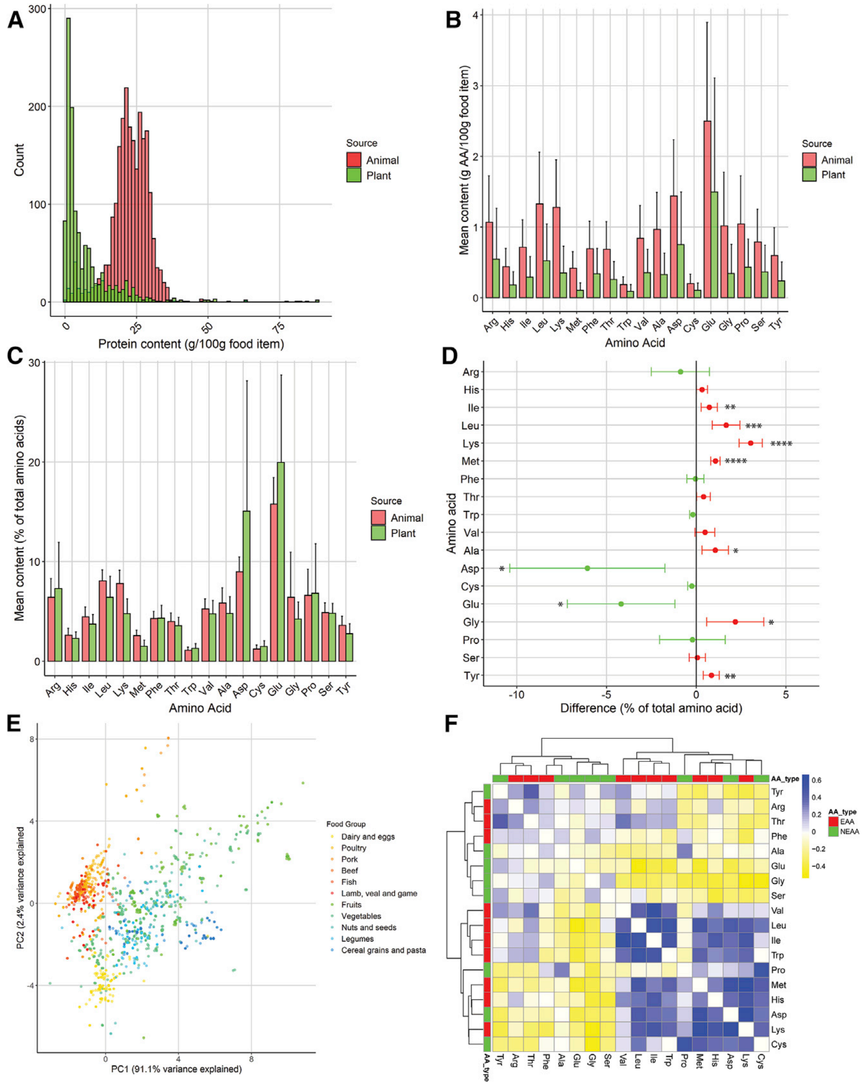


Figure 1. Amino acid profiles of plant- and animal-based food items

(A) Histogram of average protein content (g protein/100 g food item) of plant- and animal-based food items.

(B and C) Amino acid (AA) content of plant- versus animal-based food items presented (B) as g AA/100 g food item and (C) as percentage of total AAs.

(D) Difference in percent of total AA (±95% confidence interval) for animal versus plant items. Positive difference (red) indicates higher relative amount in animal food items; negative difference (green) indicates higher in plant food items.

(E) Principal component plot of AA ratios across multiple plant- and animal-based food item subgroups.

(F) Correlation of individual AA proportions across all food items in the database.

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Benjamini-Hochberg adjusted for multiple comparisons. See also Figure S1.

groups versus others. To account for this potential bias, we next analyzed total protein and AA ratios of typical dietary patterns utilizing data from the National Health and Nutrition Examination Survey (NHANES) from 2005 to 2012 including two 24-h dietary recalls per person on a representative sample of the United States population every 2 years (four distinct cycles of data collection). We first stratified participants (n = 23,245) into three groups based on their dietary intakes over two 24-h recalls: individuals consuming only plant-based food (vegans; n = 67; 0.3%), individuals consuming only dairy or egg animal-based foods (vegetarians; n = 1,996; 8.6%), and individuals consuming animal-based foods including meat (omnivores; n = 21,182; 91.1%). These proportions of American participants self-reporting a vegan or lacto-ovo vegetarian dietary pattern are similar to those reported independently in a Gallup poll (Reinhart, 2018).

Individuals adhering to a vegan dietary pattern consumed less protein both as a percentage of energy and as grams normalized to body weight than either vegetarians or omnivores (% energy, 11.4%, 16.5%, and 16.2%; g/kg, 0.64, 0.95, and 1.04 g/kg, respectively; Figures 2A and 2B). Individuals consuming a vegan diet replaced calories from protein entirely with calories from carbohydrates and also tended to consume fewer calories from fat than vegetarians or omnivores (carbohydrate, 62.3%, 49.6%, and 49.9%; fat, 26.1%, 31.9%, and 33%, respectively; Figures S2A–S2C). Individuals consuming a vegan diet consumed significantly less of all AAs than omnivores on a weight basis (Figures 2C and S2D).

When AAs were normalized to total AA intake, AA intake ratios between all three diet groups showed a similar general pattern (Figure 2D). Relative intake of nine AAs was significantly higher in omnivores (His, Ile, Leu, Lys, Met, Thr, Val, Ala, and Tyr), and two were higher in vegans (Phe and Glu) (Figure 2E). Despite these statistically significant differences, it is notable that the differences in relative intake were less than 0.5% for all but two AAs (Lys and Glu). This suggests that, although the differences are consistent enough to detect statistical significance, the magnitude of the differences are small. This observation was confirmed by a lack of segregation by group in principal component analysis (Figure 2F).

Because the magnitude of variation in AA composition between these distinct dietary patterns was so small, we next stratified participants by total protein intake to see if larger differences in AA profiles could be identified in high- versus low-protein consumers. When participants were stratified by decile of % energy intake from protein, distinct trends emerged but with the same broad patterns intact (Figure 3A). Upon principal component analysis of AA ratios, the first and tenth deciles of percent energy intake from protein showed moderate segregation along PC1 (Figure 3B). Interestingly, individuals consuming low protein tended to replace protein exclusively with carbohydrate, similar to the trend seen with vegans (Figures 3C and 3D). The trends in differences in proportional AA intake were very consistent and all AAs showed

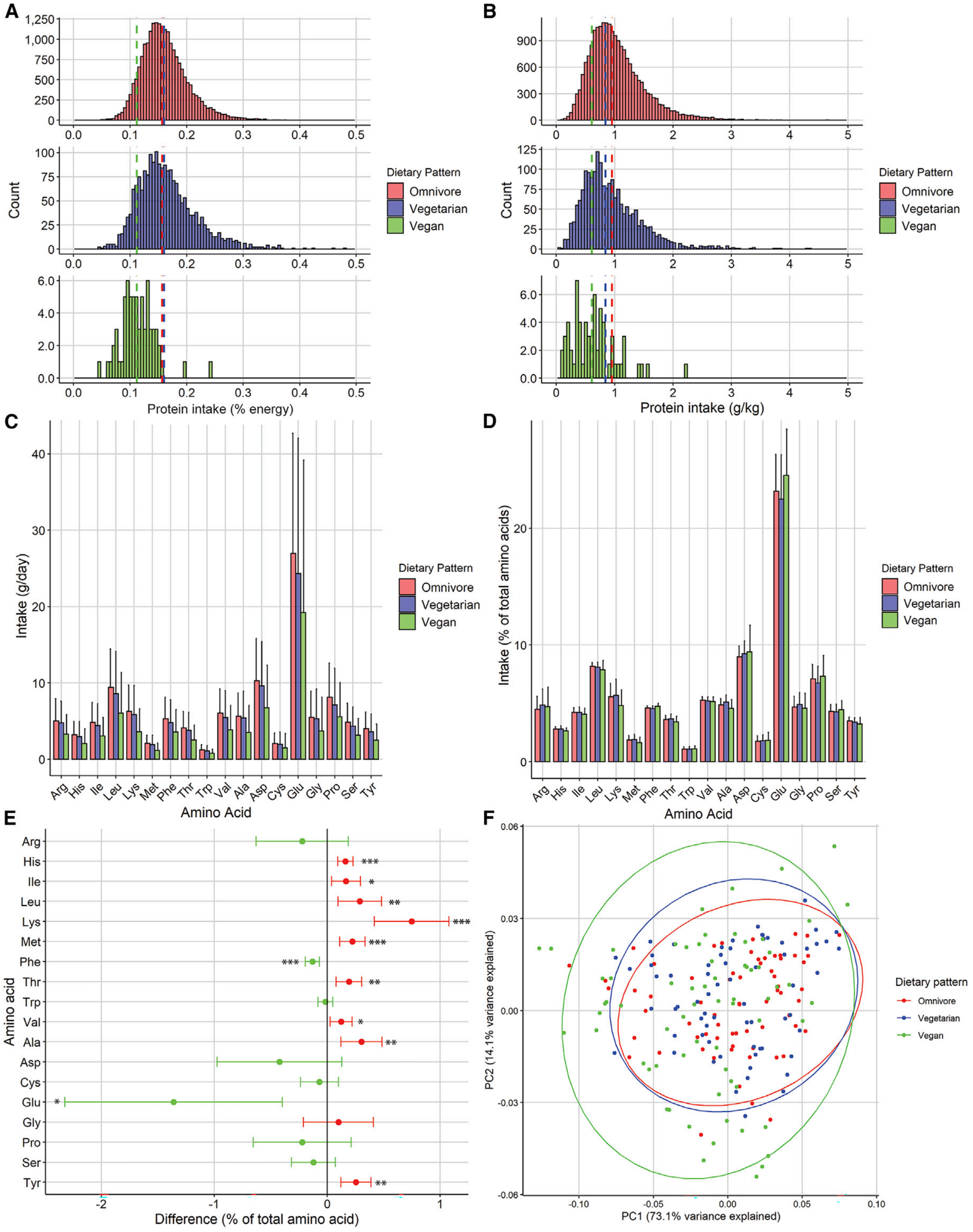


Figure 2. Amino acid profiles of dietary patterns

(A and B) Protein intake for vegans (green), lacto-ovo vegetarians (blue), and meat-consuming omnivores (red) presented as (A) g/kg body weight and (B) % of energy intake.

(C and D) Amino acid (AA) profiles of the three dietary patterns presented as (C) g/day intake and (D) % of total AA intake.

(E) Difference in percent of total AA (±95% confidence interval) for omnivore versus vegan dietary pattern. Positive difference (red) indicates higher relative amount in omnivore; negative difference (green) indicates higher vegan.

(F) Principal component plot of AA ratios with observations colored by dietary pattern.

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Benjamini-Hochberg adjusted for multiple comparisons. See also Figure S2.

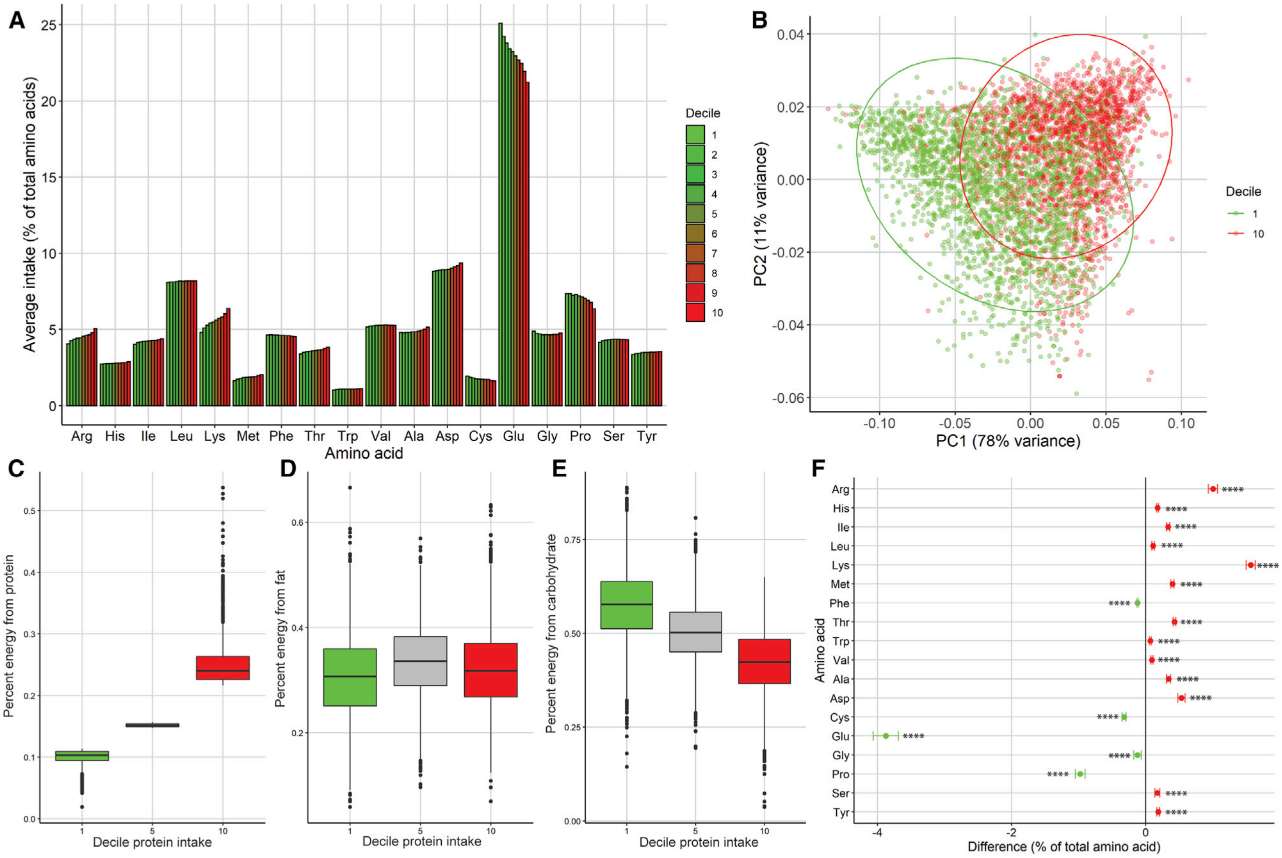


Figure 3. Amino acid ratios in high- versus low-protein consumers

(A) Amino acid ratios in individuals across deciles of protein intake as a percent of total energy intake with low-consuming individuals in decile 1 and high consumers in decile 10.

(B) Principal component plot of amino acid ratios in individuals in the highest (red) and lowest (green) deciles of protein intake.

(C–E) Average percent energy intake from protein (C), fat (D), and carbohydrate (E) for the 1st, 5th, and 10th deciles of protein intake.

(F) Difference in percent of total AA (±95% confidence interval) for 1st versus 10th decile of protein intake. Positive difference (red) indicates higher relative amount in 10th decile; negative difference (green) indicates higher in 1st decile.

****$p < 0.0001$. Benjamini-Hochberg adjusted for multiple comparisons.

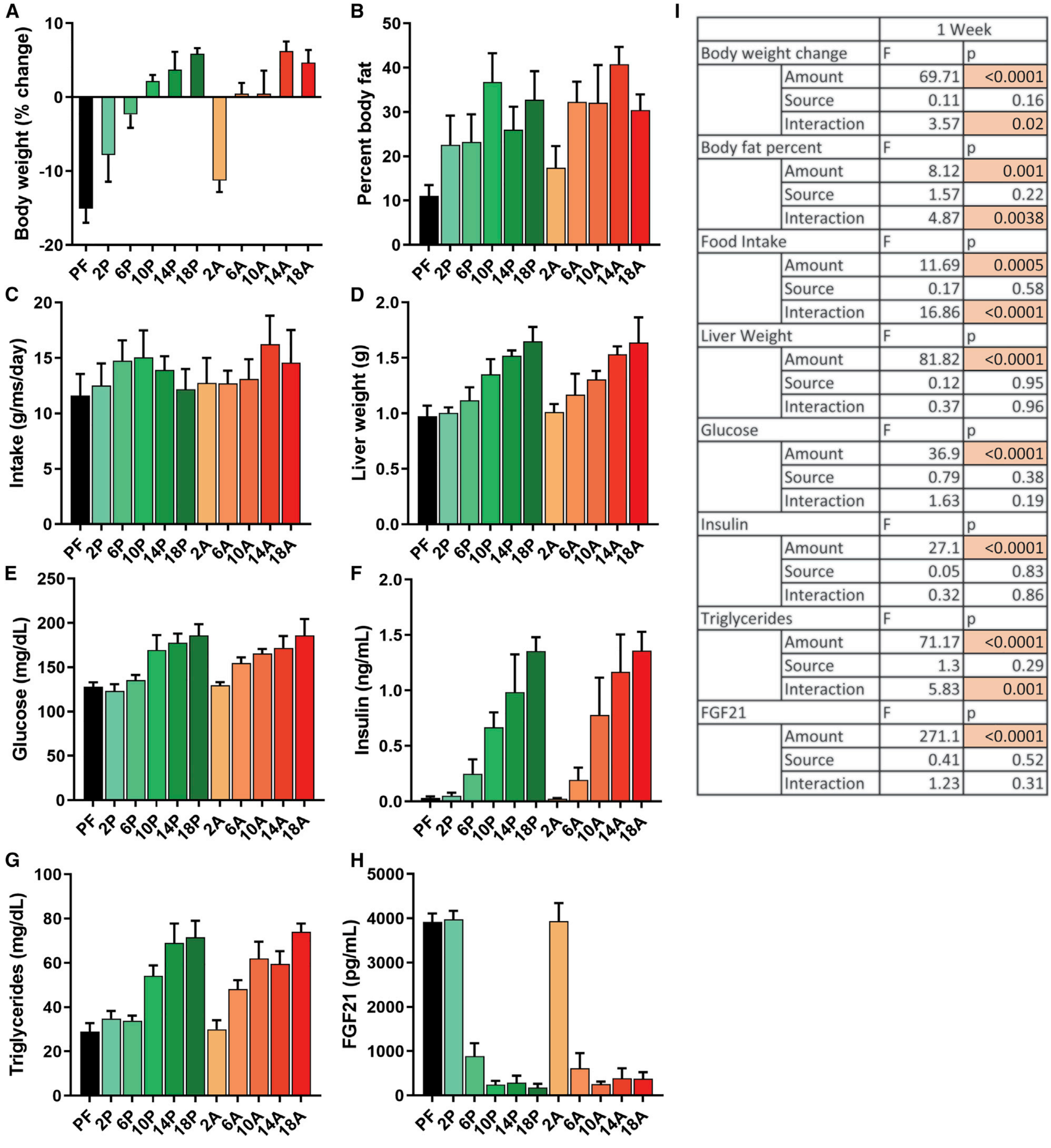
highly statistically significant proportional differences between the first and tenth decile groups with Arg, Hist, Ile, Leu, Lys, Met, Thr, Typ, Val, Ala, Asp, Ser, and Tyr higher in high-protein consumers and Phe, Cys, Glu, Gly, and Pro higher in low-protein consumers (Figure 3F). Despite these highly statistically significant differences, there were again only small differences in magnitude of differences, with only three AAs having >1% difference (Lys, Glu, and Pro).

Our combined *in silico* analyses of food items and dietary patterns revealed differences in total protein amount in plant- versus animal-based food items and dietary patterns. It also revealed differences in the AA profiles of plant- versus animal-based food items and dietary patterns and high- versus low-protein dietary patterns. However, only two AAs had >0.5% difference in relative abundance in vegan versus omnivorous. Thus, we identified AA profiles corresponding to plant- versus animal-based dietary patterns; however, we sought to understand whether such small relative differences could explain health differences. To experimentally test the potential contribution of these differences in AA composition relative to differences in total protein intake to the health benefits of plant-based diets, we turned to controlled feeding studies in rodents.

We designed AA profiles corresponding to plant versus animal-based dietary patterns using results from the analysis of the USDA food item database. Diets were made with total AA content between 0% and 18% of energy and AA ratios corresponding to either a plant- or animal-based profile by adding crystalline AAs of the desired content and ratio to a semi-purified, protein-free base mixture (Table S1). As dietary protein content was reduced, sucrose was added to maintain isocaloric diets and to reflect the observed tendency of vegans to replace calories from protein with carbohydrate. All diets were fed *ad libitum*.

We fed these diets to male B6D2/F1 mice for either 1 week or 6 weeks and measured multiple biomarkers of cardiometabolic health. Data were then analyzed for main effects of total AA amount, AA composition (plant versus animal), and AA amount by composition interaction by two-way ANOVA.

After 1 week of feeding, body weight, body fat percent, fasting blood glucose, insulin, and triglycerides were all significantly decreased as a function of reduced dietary AA intake (Figures



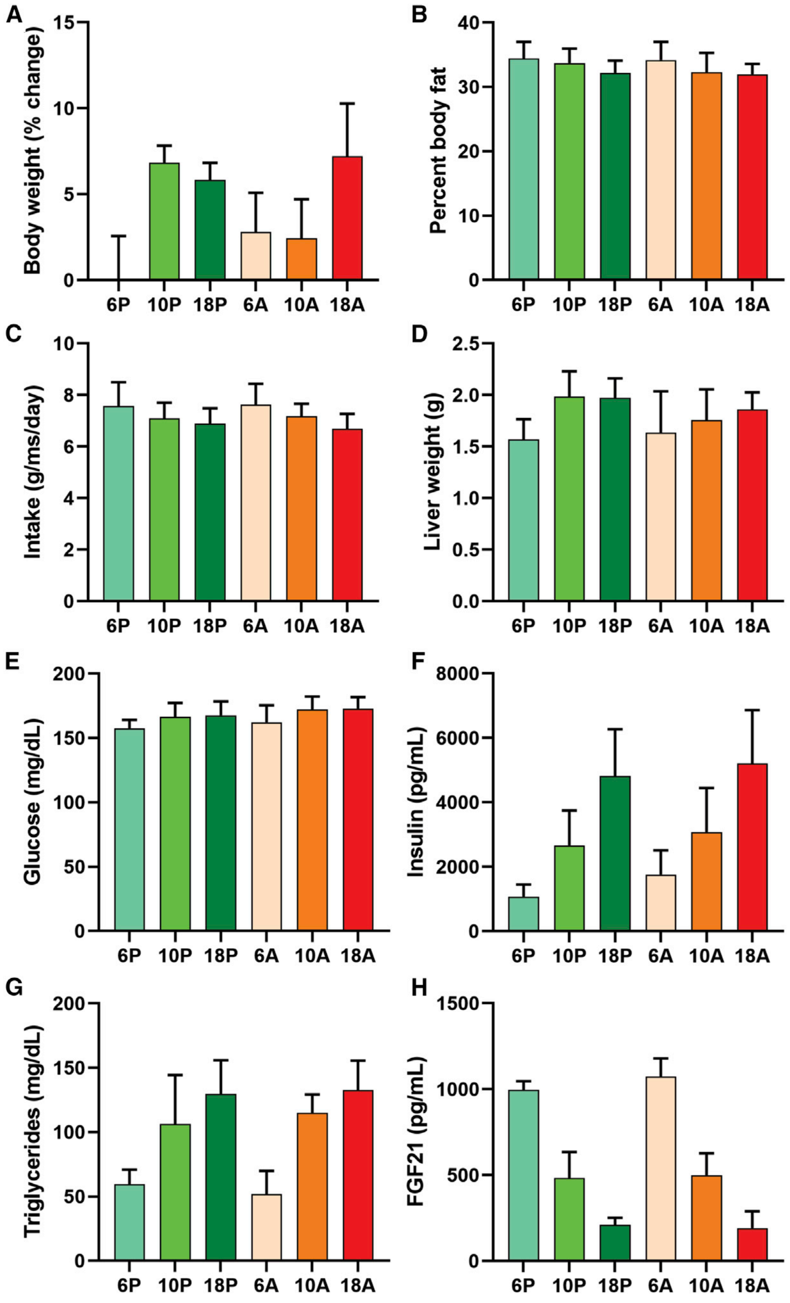
		1 Week	
Body weight change		F	p
	Amount	69.71	<0.0001
	Source	0.11	0.16
	Interaction	3.57	0.02
Body fat percent		F	p
	Amount	8.12	0.001
	Source	1.57	0.22
	Interaction	4.87	0.0038
Food Intake		F	p
	Amount	11.69	0.0005
	Source	0.17	0.58
	Interaction	16.86	<0.0001
Liver Weight		F	p
	Amount	81.82	<0.0001
	Source	0.12	0.95
	Interaction	0.37	0.96
Glucose		F	p
	Amount	36.9	<0.0001
	Source	0.79	0.38
	Interaction	1.63	0.19
Insulin		F	p
	Amount	27.1	<0.0001
	Source	0.05	0.83
	Interaction	0.32	0.86
Triglycerides		F	p
	Amount	71.17	<0.0001
	Source	1.3	0.29
	Interaction	5.83	0.001
FGF21		F	p
	Amount	271.1	<0.0001
	Source	0.41	0.52
	Interaction	1.23	0.31

Figure 4. Titration of protein in plant- and animal-based ratios using semi-purified diets for 1 week

(A–H) Percent change in body weights (A), body fat percentage (B), food intake (C), liver weight (D), blood glucose (E), serum insulin (F), serum triglycerides (G), and serum FGF21 (H) in male B6D2F1 mice fed for 7 days with semi-purified diets containing varying total AA content.

(I) Two-way ANOVA statistics for main effects of protein amount and protein source (plant versus animal composition), and for protein amount by source interaction. p, p value; F, F statistic; P, plant; A, animal-based amino acid profiles from crystalline amino acids. PF = 0% protein. 2–18 indicate percent of energy from protein. n = 4 mice per group. Error bars indicate SD. See also Table S1.

4A–4E) with significant main effects of AA amount, but not composition (Figure 4I). Interestingly, there was a significant interaction effect ($p < 0.05$) between AA content and composition for body weight, body fat, and triglycerides, although in each case AA amount accounted for the majority of variation (Figure 4I). Serum FGF-21 was significantly increased as a function of reduced AA content (Figure 4F), but without significant effects of AA composition or interaction (Figure 4H).



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		6 Weeks	
Body weight change		F	p
	Amount	2.84	0.07
	Source	0.001	0.96
	Interaction	1.28	0.28
Body fat percent		F	p
	Amount	3.43	0.04
	Source	0.75	0.39
	Interaction	0.28	0.75
Food Intake		F	p
	Amount	13.95	<0.001
	Source	0.01	0.95
	Interaction	0.59	0.56
Liver Weight		F	p
	Amount	6.51	0.003
	Source	1.33	0.25
	Interaction	1.14	0.32
Glucose		F	p
	Amount	4.74	0.01
	Source	2.76	0.1
	Interaction	0.009	0.99
Insulin		F	p
	Amount	18.5	<0.001
	Source	0.98	0.33
	Interaction	0.04	0.96
Triglycerides		F	p
	Amount	67.9	<0.001
	Source	0.33	0.86
	Interaction	0.78	0.69
FGF21		F	p
	Amount	93.27	<0.001
	Source	0.13	0.56
	Interaction	0.32	0.64

Figure 5. Titration of protein in plant- and animal-based ratios using semi-purified diets for 6 weeks

(A–H) Percent change in body weights (A), body fat percentage (B), food intake (C), liver weight (D), blood glucose (E), serum insulin (F), serum triglycerides (G), and serum FGF21 (H) in male B6D2F1 mice fed for 6 weeks with semi-purified diets containing varying total AA content.

(I) Two-way ANOVA statistics for main effects of protein amount and protein source (plant versus animal composition), and for protein amount by source interaction. p, p value; F, F statistic; P, plant; A, animal-based amino acid profiles from crystalline amino acids. 6–18 indicate percent of energy from protein. n = 8 mice per group. Error bars indicate SD. See also Table S1.

Over the course of a longer 6-week treatment period, we only used levels of total AA that were sustainable (6%, 10%, or 18% of energy), as the very low levels (0% and 2%) result in non-sustainable reductions in lean mass. At these sustainable levels, body weight tended to decrease, while food intake and adiposity tended to increase as a function of decreasing dietary protein content (Figures 5A–5C). Liver weight, fasting blood glucose, insulin, and triglycerides also all tended to decrease as a function of decreasing protein (Figures 5D–5G). FGF21 tended to increase as a function of decreasing protein (Figure 5H). There were no significant main effects of source or interaction effects for any of these variables (Figure 5I).

Having observed that reduced AA content was the significant driver of cardiometabolic benefits, with only very minor effects of plant versus animal AA composition in the context of highly controlled semi-purified diet experiments, we next tested whether these two major findings apply to protein derived from naturally sourced ingredients with their numerous potential confounding factors (e.g., different lipid species, differing polyphenol content, etc.).

To this end, we made diets using up to 16 whole-food items varying in total protein amount and source (Figures S3A–S3C). This number of food items was chosen as it approximately represents the number of unique food items consumed by an individual per day in the NHANES database. Animal or plant-based protein mixtures were added to a low-protein base consisting of ~2.5% of energy from plant-based protein sources to achieve diets with 6%, 10%, or 18% of energy from protein primarily from plant or animal sources (Figure S3D). Food items were chosen which could be found in normal human intake but also had to be selected to control for extreme differences in energy density which are often present between plant- and animal-based food items. Calculated diet compositions were relatively well matched for macronutrient composition, energy density, and fiber content (Figure S3A) and with AA ratios emulating previously observed differences in plant- versus animal-based dietary patterns particularly with regard to the observed Glu versus Lys trade-off (Figure S3C). Distinct visual appearances were clear that could not be controlled for (Figure S3B). Chemical analysis of the highest protein plant and animal diets confirmed the accuracy of our calculations in terms of macronutrient breakdown, energy density (Figure S3E), and AA ratios (Figure S3F). All diets were fed *ad libitum*.

Male B6D2/F1 mice were fed one of the six naturally sourced diets varying in total protein content and source for 6 weeks and cardiometabolic markers were analyzed as before. Mice on lower protein diets tended to have similar body weights but higher adiposity (Figures 6A and 6B), with a significant main effect of protein amount on adiposity (Table S1). There was not an observable significant effect of protein amount on food intake, but there was a significant effect of source and a borderline significant ($p = 0.05$) source by amount interaction effect, with the high-protein animal group tending to eat more (Figure 6C). There were significant main effects of protein amount and source on liver weight with higher protein increasing liver weight and plant source tending to be higher than animal (Figure 6D). There were significant main effects of protein amount for glucose, insulin, and triglycerides with higher protein tending to increase all three (Figures 6E–6G). There was also a significant interaction effect for insulin and trend to significant interaction effect for glucose and triglycerides ($p < 0.1$), with higher animal protein tending to increase values more than higher plant protein (Figures 6E–6G). FGF21 showed a significant effect of protein amount with no effects of source or interaction (Figure 6H). Glucose tolerance measured by oral glucose tolerance test showed a significant effect of protein amount and trend to significant effect of source ($p = 0.06$) and amount by source interaction ($p = 0.1$) with higher animal protein groups having lower glucose tolerance (Figure 6I).

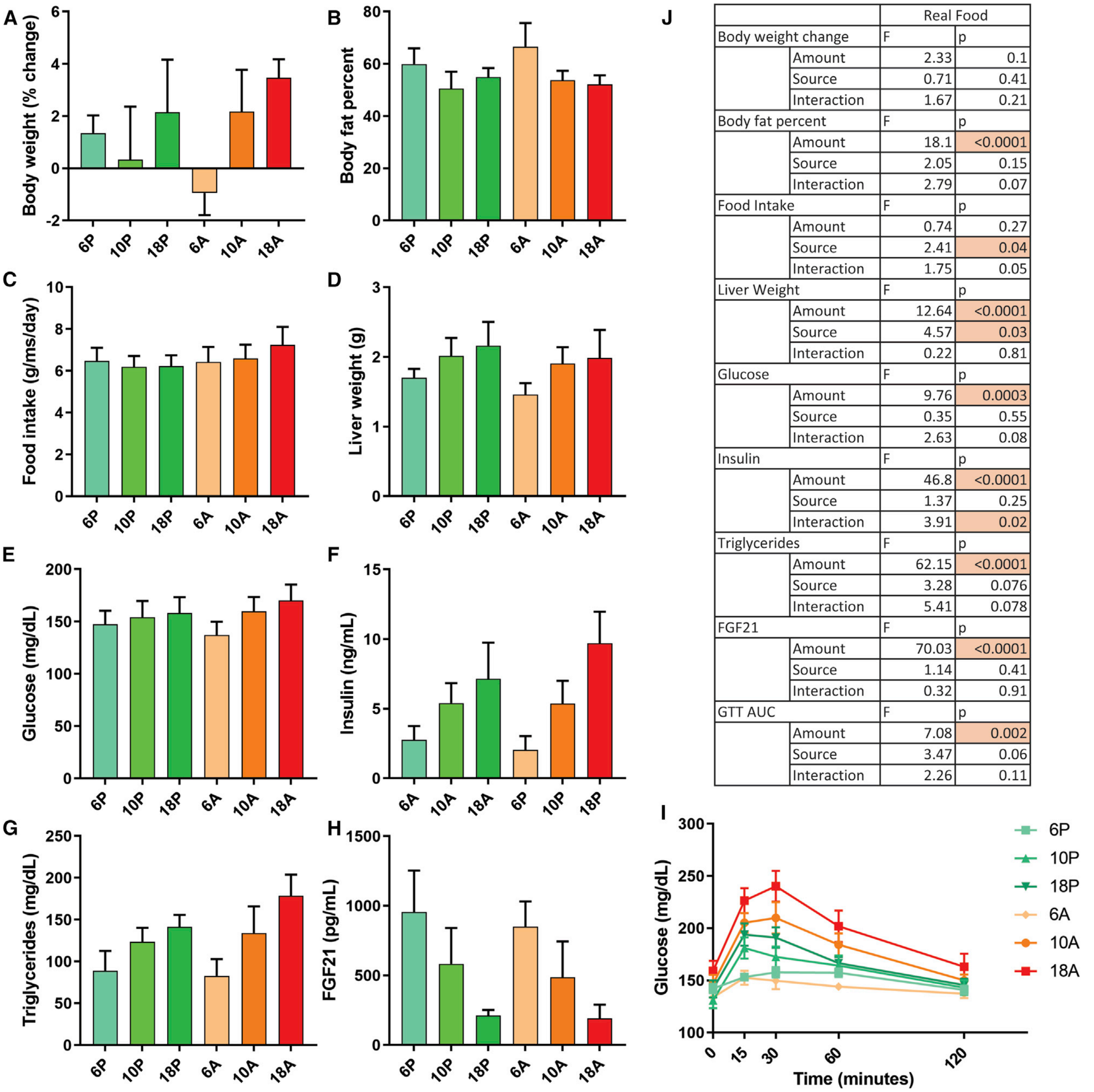
DISCUSSION

Benefits of plant-based diets on cardiometabolic health are widely acknowledged, but the underlying mechanisms, including the potential role of protein quantity and/or quality, remain unclear. Here, we used *in silico* and experimental approaches to interrogate the specific contributions and potential interactions between total protein content and AA composition of plant- versus animal-based dietary patterns. *In silico* analyses demonstrated that while consumers of plant-based dietary pattern consume less protein and more carbohydrate, there are only small differences in AA composition between plant- and animal-based dietary patterns. Animal studies demonstrated the outsized role of protein:carbohydrate ratio (protein amount) versus protein source or AA composition on cardiometabolic markers. These main effects of protein amount were confirmed under highly controlled experimental conditions in mice using semi-purified diets with crystalline AAs in plant- versus animal-based ratios as the sole protein source. For some parameters, a significant interaction effect between AA profile and AA amount was observed, but the variance explained by these interactions was consistently much lower than that explained by total AA amount, and these significant interactions were only apparent at 1 week and tended go away after 6 weeks on diet.

The main effects of protein quantity were also robustly observed using naturally sourced diets composed of at least 15 different whole-food ingredients. This result was surprising given how many uncontrolled variables were introduced in this approach, including in non-caloric components such as phytonutrients as well as different lipid profiles. While these results are not mutually exclusive with roles of non-proteinaceous components in benefits of plant-based diets, they do support the notion of protein intake as a major factor in cardiometabolic benefits especially at low levels of total protein intake.

Our data are consistent with and expand upon findings regarding the larger effect sizes of protein quantity over quality specifically at low levels of total protein intake (Brandhorst and Longo, 2019). Levine et al. found that protein amount is more important than single plant (soy) versus single animal (casein) source in promoting tumor growth in mice (Levine et al., 2014), while Fontana et al. found evidence of a threshold between 10% and 20% protein below which single plant versus single animal source is less important than the protein content in a mouse model of human xenograft prostate tumor growth (Fontana et al., 2013). One of the few prospective clinical trials comparing vegan with control diets with a specific focus on the protein component found benefits on body composition and insulin sensitivity in a 16-week intervention (Kahleova et al., 2018a, 2018b). The authors attributed these benefits to reduced animal protein intake and increased plant protein intake, and in particular a proportional reduction in leucine (8.0% versus 7.7% protein energy in control versus vegan diets, respectively) and histidine (2.7% versus 2.6% protein energy in control versus vegan diets, respectively). However, while these associations were corrected for BMI and energy intake, they were not corrected for total protein intake, which was also significantly reduced (17% versus 12% total daily energy), and thus more likely to explain changes in fat mass and insulin resistance than the small changes in AA composition (Kahleova et al., 2018a).

It is important to note that while our model places reduced protein quantity over altered quality (plant versus animal protein source or AA composition) in relative importance when comparing health effects of plant-based versus omnivorous dietary patterns, the presumed mechanism is still one of restriction of one or more EAAs below some threshold, rather than total AA



		Real Food	
Body weight change		F	p
	Amount	2.33	0.1
	Source	0.71	0.41
	Interaction	1.67	0.21
Body fat percent		F	p
	Amount	18.1	<0.0001
	Source	2.05	0.15
	Interaction	2.79	0.07
Food Intake		F	p
	Amount	0.74	0.27
	Source	2.41	0.04
	Interaction	1.75	0.05
Liver Weight		F	p
	Amount	12.64	<0.0001
	Source	4.57	0.03
	Interaction	0.22	0.81
Glucose		F	p
	Amount	9.76	0.0003
	Source	0.35	0.55
	Interaction	2.63	0.08
Insulin		F	p
	Amount	46.8	<0.0001
	Source	1.37	0.25
	Interaction	3.91	0.02
Triglycerides		F	p
	Amount	62.15	<0.0001
	Source	3.28	0.076
	Interaction	5.41	0.078
FGF21		F	p
	Amount	70.03	<0.0001
	Source	1.14	0.41
	Interaction	0.32	0.91
GTT AUC		F	p
	Amount	7.08	0.002
	Source	3.47	0.06
	Interaction	2.26	0.11

Figure 6. Titration of protein in plant- and animal-based ratios using naturally sourced diets for 6 weeks

(A–I) Percent change in body weights (A), body fat percentage (B), food intake (C), liver weight (D), blood glucose (E), serum insulin (F), serum triglycerides (G), serum FGF21 (H), and oral glucose tolerance test curves (I) in male B6D2F1 mice fed for 6 weeks with naturally sourced diets containing varying total protein content from plant or animal natural sources.

(J) Two-way ANOVA statistics for main effects of protein amount and protein source (plant versus animal products), and for protein amount by source interaction. p, p value; F, F statistic; P, plant; A, animal food sources. 6–18 indicate percent of energy from protein. n = 6–8 mice per group. Error bars indicate SD.

See also Figure S3 and Table S2.

or nitrogen restriction (Yap et al., 2020). Clearly changes in AA composition, which invariably result in alteration of AA ratios, can affect health outcomes even at relatively high total protein intake. For example, restriction of sulfur AA at 14% total protein, or restriction of BCAA at 21% total protein (Fontana et al., 2016), both improve cardiometabolic endpoints in mice, similar to total protein restriction. Although future work is required to identify which specific AAs are implicated in the case of low-protein diets, the observation here suggests that AA composition does not vary widely enough between dietary patterns enriched in plant versus animal protein sources to cause such a restriction threshold to be met.

An unexpected finding from our *in silico* analyses was the lower than expected variation in aggregate AA profiles of different food items and intake patterns. Despite observing the expected increase in the NEAA:EAA ratio in plant-based protein (Figure S1C), and some variability between AA ratios in plant-based food groups (Figures 1E and S1E), the overall pattern of AA ratios between plant- and animal-based food items on the whole was striking. More importantly, a similar phenomenon was observed in the context of dietary patterns, where we found strikingly little variation in the AA profiles of different dietary patterns from participants across multiple cycles of NHANES. Whether by comparing vegans and omnivores who had substantial differences in the macronutrient profile of their diets, or by stratifying individuals by their percent energy intake from protein, we observed that most AAs only varied by less than 0.5% in terms of their relative abundance despite the considerable variation in total protein intake. We also note that comparing the extremes of dietary protein intake (top and bottom 10% of protein energy intake), neither of which is typical for the general US population, probably serves to overestimate the true differences in AA profiles between dietary patterns but still only showed differences of less than 1% in terms of relative abundance of most AAs.

Limitations of study

We note the following limitations of our study that impact our interpretation that the low-protein content of plant-based diets is a major driver of health benefits. First, our experimental data in mouse models show that the most drastic changes in biomarkers of cardiometabolic health occur at protein levels below 10% of energy. In human cohorts, protein intake is largely fixed between 12% and 18% with little deviation outside this range. While there are some exceptions, including Okinawan populations that eat less than 10% protein calories in a largely plant-based diet (Le Couteur et al., 2016), limited sample sizes available in population studies make it difficult to assess if humans react similarly to mice when exposed to low-protein intake. Interestingly, some short-term prospective clinical interventions with protein content around 10% are consistent with improved metabolic markers (Fontana et al., 2016). Although it has been hypothesized that increased calorie consumption with low-protein diets due to protein leverage contributes to obesity and metabolic disease, it is not clear whether this model holds true in humans consuming meals with less than 10% of energy from protein (Gosby et al., 2011, 2016).

Second, as with any epidemiologic study, the association of a single macronutrient with health parameters is tenuous due to extreme confounding by variable lifestyle and other dietary factors including which macronutrient is substituted when protein is reduced. In the NHANES data, vegans tended to substitute protein for carbohydrate, which informed our mouse models. However, the effect of protein for fat substitution is less well studied. Furthermore, although protein energy intake can be treated as a single variable, it is in reality a combination of AA ratios that cannot be fixed, even experimentally, and thus represent a complex multidimensional landscape which complicates drawing conclusions about the effects of dietary protein in free-living humans.

Third, because the maximum amount of dietary protein intake studied here in mice was 18%, which is in the standard range for commercial, naturally sourced chow for rodents and close the median intake for humans according to the NHANES database, our data say nothing about the potential of plant versus animal protein to affect health outcomes at higher levels of protein intake. Very high levels of protein intake in plant-based dietary patterns have previously been unusual. However, the recent rise in popularity of high-protein plant-based meat alternative makes this a newly relevant question.

Our mouse data using naturally sourced diets do suggest that some interactions between protein source and amount may exist at the higher end of the protein:carbohydrate ratio. A number of studies support the notion of health benefits at high levels of protein intake, in part due to suppression of overall energy intake (Berryman et al., 2016; Eisenstein et al., 2002), although other studies point out that benefits on cardiometabolic markers such as insulin sensitivity do not match those expected from the resultant weight loss (Berryman et al., 2016; Smith et al., 2016) and that high-protein diets may not have robust health effects in individuals with metabolic disease (Raben et al., 2021). Furthermore, experimental studies using high-protein diets (25% energy) in rabbits provide clear evidence of differential atherogenic and cholesterolemic effects of single protein sources depending on plant versus animal origin (Huff and Carroll, 1980; Huff et al., 1977), which could be in part attributed to differences in NEAA/EAA ratios or more specifically to differences in arginine and lysine levels in casein versus soy protein, respectively (Vahouny et al., 1985). What molecular mechanisms underlie these anti-atherogenic effects and whether they require high plant protein intake both remain unclear, but with important implications given the recent increase in popularity of plant-based meat substitutes engineered to resemble the taste, texture, and macronutrient profile of meat. Whether these high-energy-density, high-protein options will increase total protein intake and whether this will be associated with the benefits of plant-based dietary patterns, or loss of benefits due to increased protein intake as predicted by our data here, remains to be seen.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2021.06.011.

ACKNOWLEDGMENTS

This work was supported in part by the NIH/NIDDK/NIA (5R01DK090629-08 to J.R.M. and 1F31AG064863-01 to M.R.M.).

AUTHOR CONTRIBUTIONS

M.R.M., J.R.M., C.K.O., and S.J.M. conceived of and designed the experiments. M.R.M., S.J.M., J.S.R., J.H.T.-V., P.K., J.J., and K.T. performed animal experiments. M.R.M. and J.G. performed computational analyses. M.R.M. and J.R.M. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: December 10, 2020
Revised: April 14, 2021
Accepted: June 16, 2021
Published: July 15, 2021

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Ultra-sensitive mouse insulin ELISA kit	Crystal Chem	Cat# 90080
Serum triglyceride colorimetric assay kit	Millipore Sigma	Cat# TRO100-1KT
Mouse FGF21 ELISA kit	R&D Systems	Cat# MF2100
Experimental models: Organisms/strains		
Mouse strain: B6D2F1/J	The Jackson Laboratory	Cat# JAX:100006
Software and algorithms		
GraphPad Prism v. 9.1	GraphPad	N/A
R v4.0.3	N/A	N/A
Other		
Mouse diets (see Tables S1 and S2)	This paper	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Michael R. MacArthur (mmacarthur@ethz.ch)

Materials availability

This study did not generate new unique reagents.

Data and code availability

Code relating to database analysis will be made available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

For all experiments 16-week-old male B6D2/F1 hybrids were purchased from Jackson Labs (strain no. 100006). Mice were acclimated in the facility for at least one week prior to experiments and allowed ad libitum access to food (Purina 5053 chow) and water during acclimation. Mice were maintained at 22°C with 12 hr light-dark cycles and 30-50% relative humidity. All experiments were approved by the respective IACUC. Mouse numbers were n=4 per group for the 1 week semi-purified titration experiment and n=6-8 per group for the 6 week semi-purified titration and naturally sourced titration experiments. Mice were observed daily to ensure good health status. Specific pathogen checks were performed quarterly in the facility.

Diets

For semi-purified titration experiments, isocaloric diets were made with crystalline AA added to generate either a ‘plant’ or ‘animal’ based profile which was determined by taking the average AA ratios of plant vs animal food items from the USDA NDB. For titration studies, AAs were isocalorically replaced with sucrose. The AAs/sucrose were added to Research Diets D12450BSpx base in final 1% agar to form a homogenous solid diet. The exact compositions of semi-purified diets are described in Table S1.

For naturally sourced titration experiments, whole-food items listed in Figure S3D were mixed to achieve the macronutrient profiles listed in Figure S3A. The items were blended in water/agar to achieve a final 0.25% agar concentration to form a homogenous solid diet. The exact compositions of naturally sourced diets are described in Table S2. Calculated dietary composition including AA profiles, macronutrient composition and energy density were confirmed by HPLC and bomb calorimetry measurements at N.P. Analytical Laboratories.

All diets in all studies were fed *ad libitum*. Food intake was measured daily at approximately ZT22. Mice were fasted for four hours prior to sacrifice and tissue collection which took place at approximately ZT20.

METHOD DETAILS

Body composition

Body mass was determined by daily measurement at approximately ZT22. Lean and fat mass were measured in awake mice using an EchoMRI analyzer system.

Metabolic health markers

Glucose was measured using a Clarity BG1000 handheld glucometer. Insulin was measured using by ELISA following manufacturer protocol (Crystal Chem #90080). FGF21 was measured by ELISA following manufacturer protocol (R&D Systems #MF2100). Serum triglycerides were measured using a colorimetric kit according to manufacturer protocol (MilliporeSigma TRO100-1KT).

Glucose tolerance test (GTT)

Following a 16 hr fast, 1.5 g/kg of D-glucose solution (30%, dissolved in sterile water, Sigma Aldrich, St Louis MO) was administered by oral gavage and blood glucose measured from a small tail nick prior to (time 0) and at 15, 30, 60 and 120 minutes post-injection using a handheld glucometer (Clarity BG1000, VWR, Radnor PA).

QUANTIFICATION AND STATISTICAL ANALYSIS

Food item and database analyses

AA profiles of individual food items were analyzed using the USDA Food and Nutrient Database SR-28 (NDB). Food items with the food groups codes 100 (dairy and eggs), 500 (poultry), 1000 (pork), 1300 (beef), 1500 (finfish and shellfish) and 1700 (lamb, veal and game) were considered "animal" food items. Food items with the food group codes 900 (fruits), 1100 (vegetables), 1200 (nuts and seeds), 1600 (legumes) and 2000 (cereal grains and pasta) were considered "plant" food items.

AAs in the USDA NDB were extracted in three groups for measurement. The three groups were tryptophan, sulfur-containing AAs and all other AAs. Tryptophan was measured by alkaline hydrolysis followed by HPLC. Sulfur-containing AAs were measured by performic oxidation followed by HPLC. All other AAs were measured by acid hydrolysis followed by HPLC. Values in the database are represented as grams of AA per 100g of food item. Full details on measurement methods and calculation are available in the USDA NDB SR28 documentation.

Food item intake analyses

The National Health and Nutrition Examination Survey is a large observational survey program which releases data from a nationally representative sample of Americans every 2 years. These data include dietary, anthropometric and clinical measures. Dietary data include two 24-hour dietary recalls conducted by trained dietary interviewers. To analyze AA composition of dietary records from the NHANES, AA data from SR28 were linked to NHANES Food and Nutrition Database for Dietary Studies (FNDDS) food items using SR link codes. Individual records were obtained from survey rounds covering from 2005 to 2012, comprising four rounds of data release. Individuals who had two complete 24-hour dietary recalls, were not pregnant and were over the age of 18 were included, leaving at total of 23,245 participants. For all nutrient intake variables including AAs and total protein, the mean value of the two days of dietary records was used. Vegans were categorized as individuals who reported eating no animal products on either of the 24-hour recalls. Vegetarians were categorized as individuals who reported consuming eggs or dairy products, but no meat, fish or poultry on 24-hour recalls. A full description of survey protocols and all data are available at the US Centers for Disease Control (CDC) NHANES website.

Statistical methods

Database analyses were performed in R (4.0.3). USDA NDB SR28 ASCII data files were downloaded from the USDA website and imported in R. NHANES XPT data files were downloaded from the CDC NHANES website. Data were imported into R using the sasxport.get function from the Hmisc package. For NHANES AA analysis, nutrient variables from each individual's two 24 hour recalls were averaged. For food item analysis, food items within each food subgroup (beef, dairy and eggs, finfish and shellfish, lamb, pork, poultry, fruit, grains, legumes, nuts and seeds, vegetables) were clustered based on their relative AA profile in order to account for high levels of redundancy in the database. Optimal cluster numbers were determined using the NbClust package. 37 clusters were identified from animal-based food item groups and 38 were identified from plant-based food item groups. All clustering was performed using a Euclidean distance matrix and unweighted pair group method with arithmetic mean agglomeration method. These clusters were then used as the unit of analysis for further comparisons between plant and animal-based food items. For both USDA NDB and NHANES analyses, significance was assessed by performing Welsh's t-test between animal/plant food items or omnivore/vegan dietary patterns. Tests were adjusted for multiple comparisons using the Benjamini-Hochberg correction. Principal component analyses were performed using the prcomp function.

For mouse studies, data were analyzed using Graphpad Prism (8.4.3). All experiments were analyzed with two way ANOVA and main effects of total protein amount and protein source/AA profile as well and protein content by protein source/profile interaction were presented. Statistical parameters are listed in the figure legends. Two way ANOVA tests met assumptions for homogeneity of variance and normality using the Levene and Shapiro-Wilk tests respectively.