

Supplemental Information

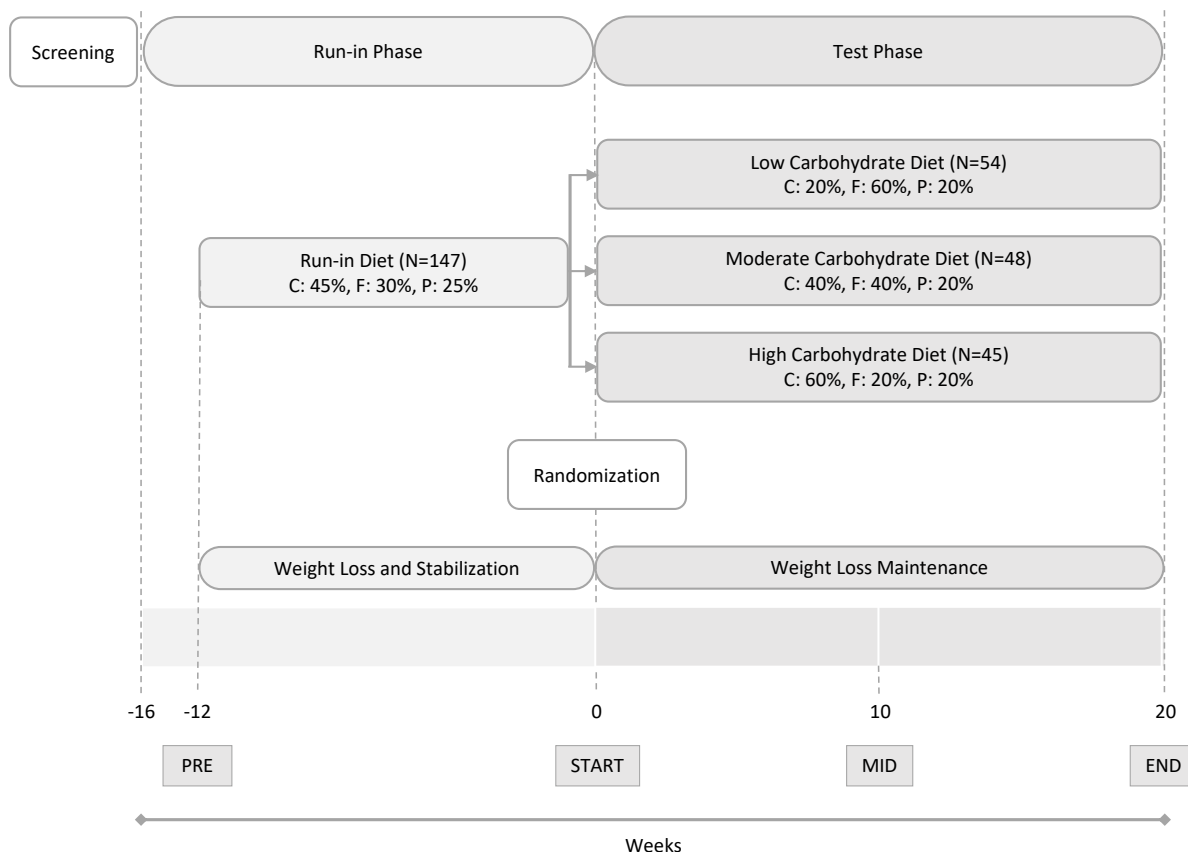
Weight-independent effects of dietary carbohydrate-to-fat ratio on metabolomic profiles: secondary outcomes of a 5-month randomized controlled feeding trial

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Supplementary Fig. 1. Study design.

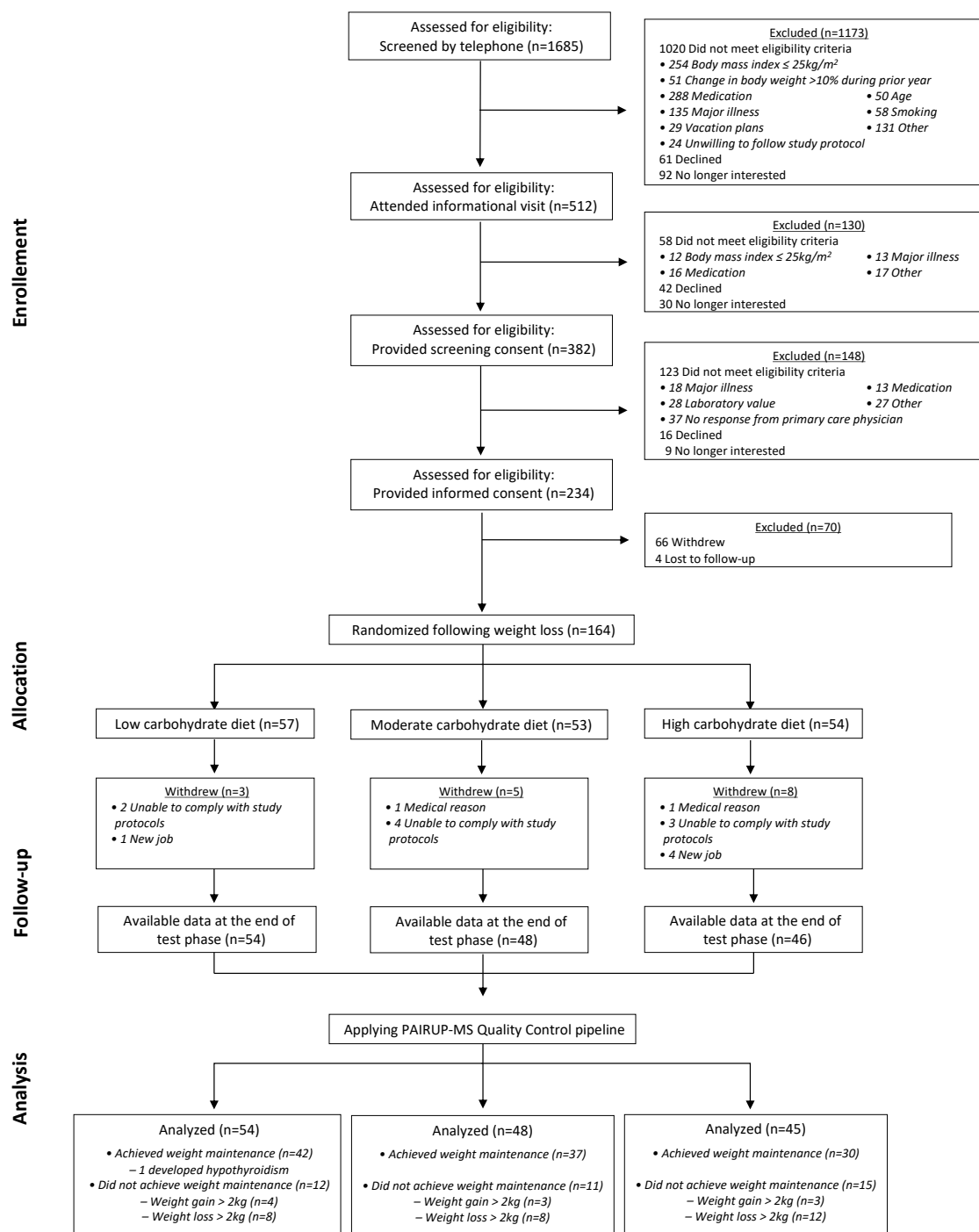


Study design overview: This randomized controlled trial comprised run-in and test phases. During both phases, feeding protocols were implemented to promote compliance with specified dietary targets for carbohydrate, fat, and protein.

The run-in phase involved obtaining PRE-weight-loss measurements, reducing energy intake to achieve weight loss of $12 \pm 2\%$ relative to baseline body weight, stabilizing body weight after weight loss, and then obtaining measurements prior to random assignment (START of trial). During the test phase, focus shifted to comparing effects of low-, moderate-, and high- carbohydrate diets during weight-loss maintenance. Energy intake was adjusted to maintain body weight within ± 2 kg of that achieved after weight loss. Measurements were repeated at 10 (MID) and 20 (END) weeks of the test phase. Metabolomics profiles were generated at PRE, START, MID, and END.

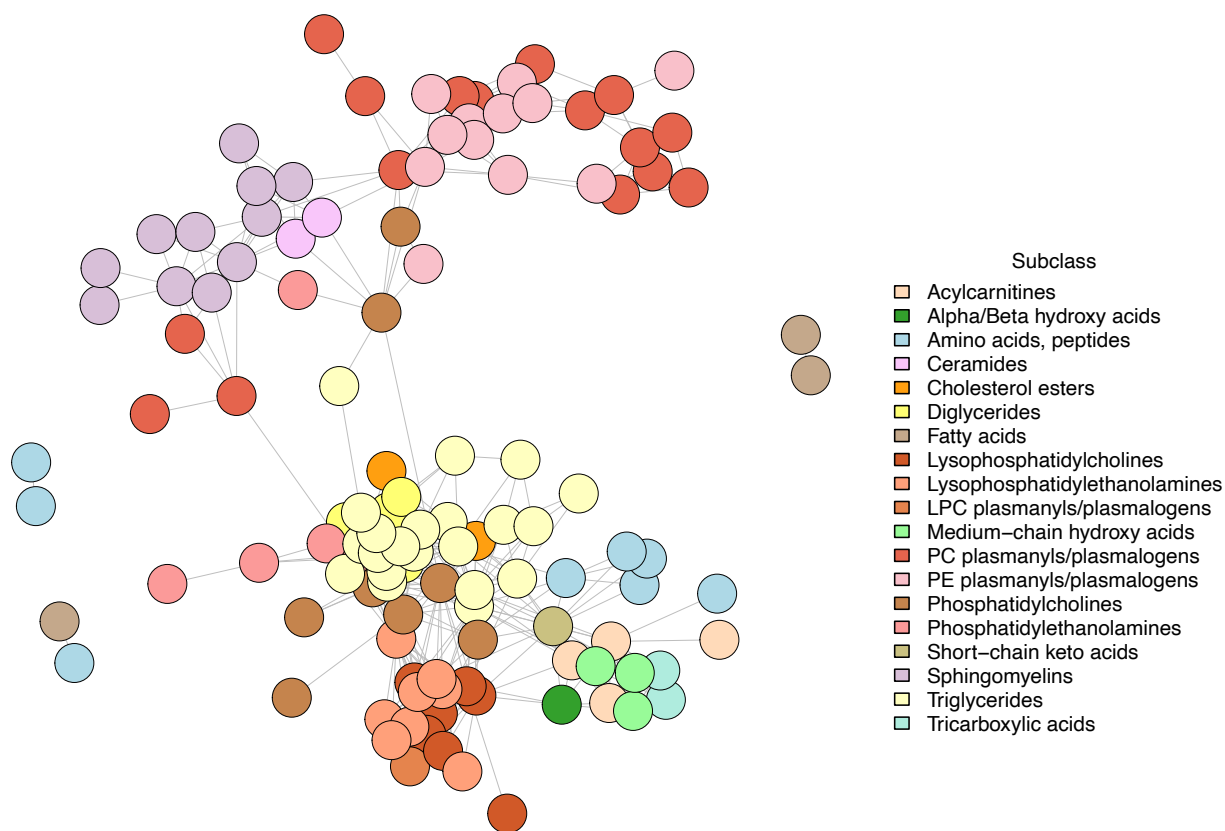
C, carbohydrate; END, end of test phase; F, fat; MID, middle of test phase; P, protein; PRE, start of run-in phase; START, start of test phase.

Supplementary Fig. 2. CONSORT flow diagram.



The CONSORT flow diagram outlines the progress through the phases of enrolment, intervention allocation, follow-up, and data analysis in the Framingham State Food Study, (FS)2, parallel randomized trial, involving the three dietary groups: low-, moderate-, and high-carbohydrate.

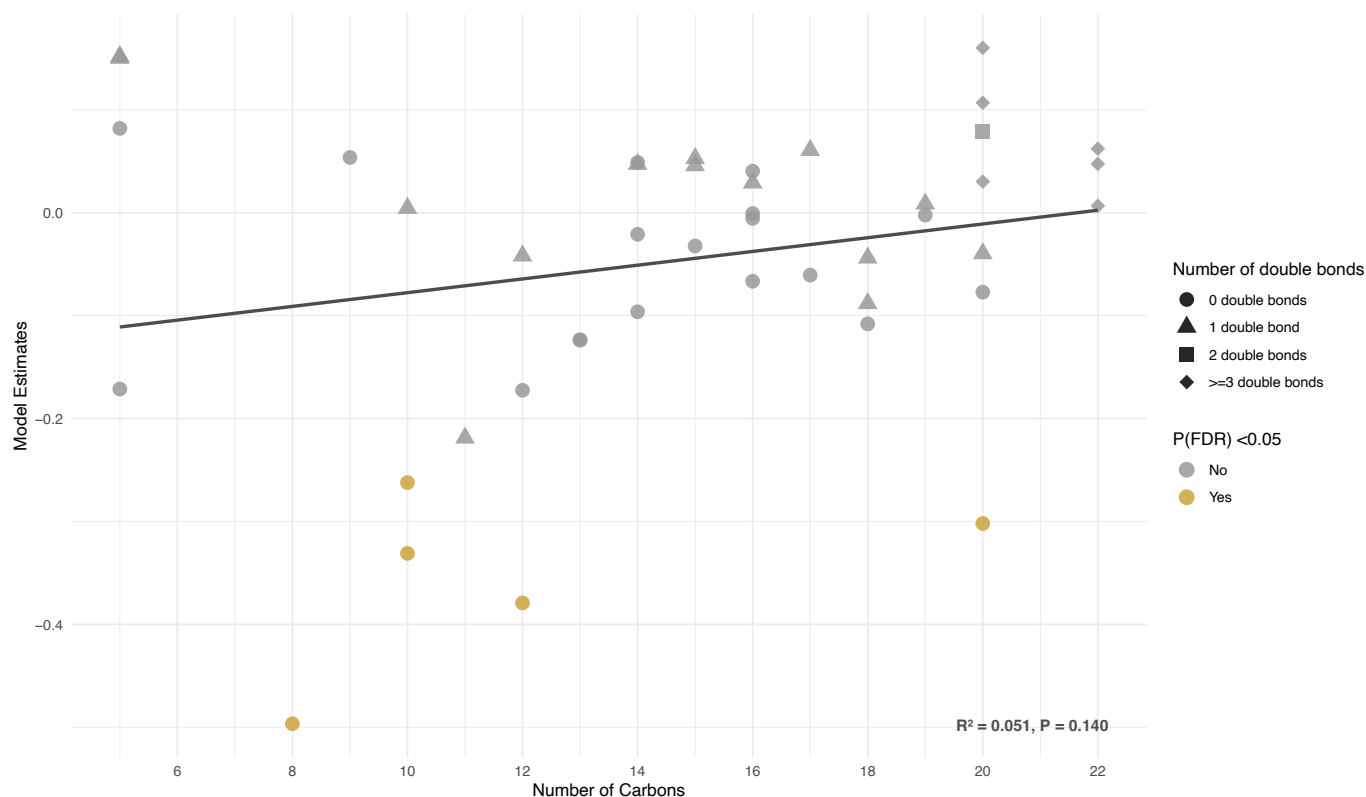
Supplementary Fig. 3. Network analysis of metabolites with FDR significant trends across low-, moderate-, and high-carbohydrate diets.



The network plot's output utilizes the Fruchterman-Reingold force-directed layout algorithm for network visualization. The metabolites that exhibited FDR significant level changes with higher CFR were included in the analysis. Each circle node in the plot corresponds to a specific metabolite. The edges denote significant correlations ($r > 0.6$) between the delta level changes (END minus START) of two metabolites. The distance between nodes reflects the strength of their relationships. Nodes with stronger relationships (e.g., higher correlation values) are drawn closer together, whereas nodes with weaker relationships are positioned farther apart. The color of each node indicates its subclass group based on HMDB categorization. Metabolites from the same group share the same color. The labels on the nodes indicate the metabolite names. Source data are provided as a Source Data file.

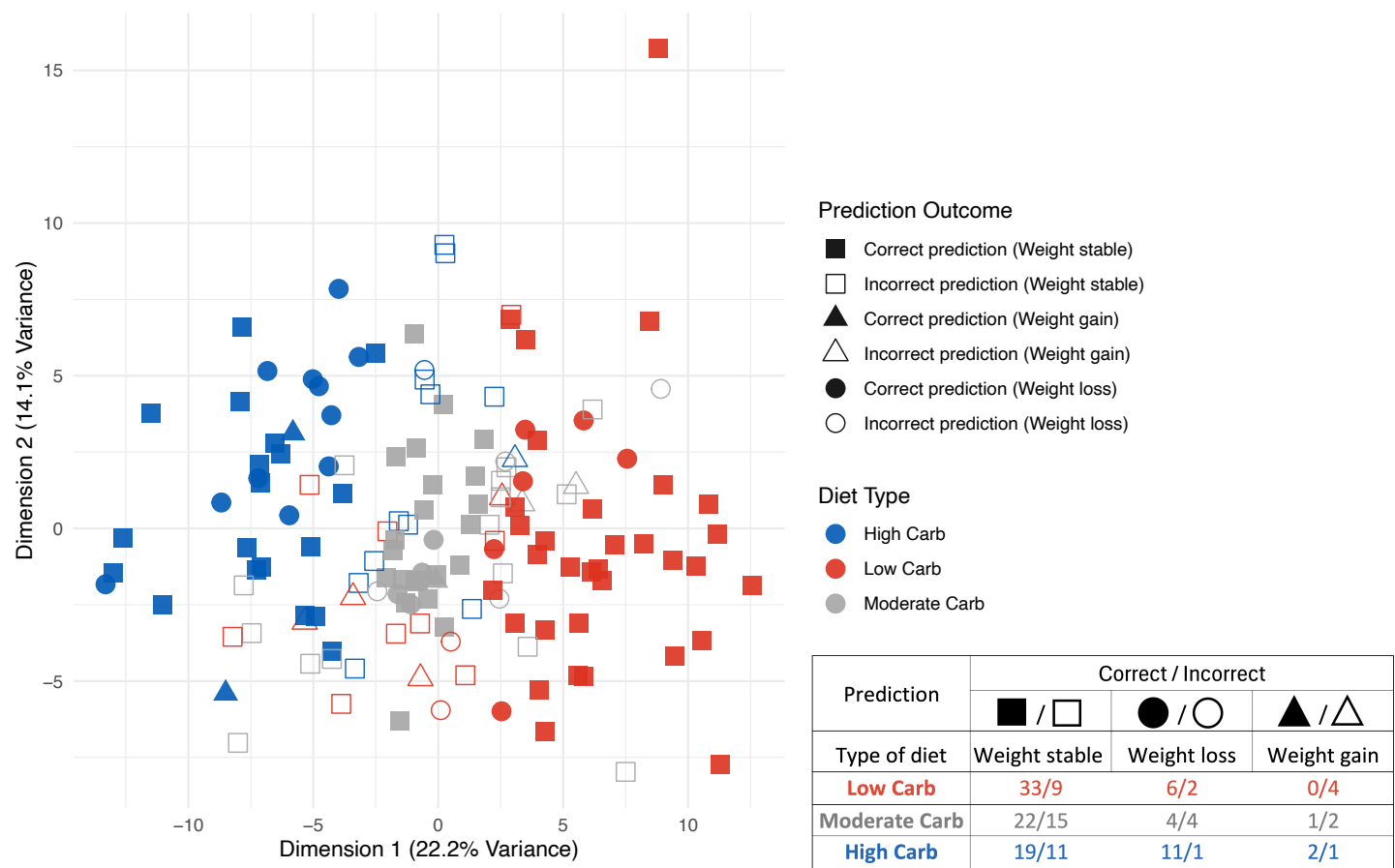
END, end of test phase; FDR, False Discovery Rate; HMDB, Human Metabolome Database identification number; START, start of test phase.

Supplementary Fig. 4. Associations between carbon chain length in fatty acids with metabolite trends from low- to moderate- to high-carbohydrate diet.



The scatterplot illustrates the association between carbon chain length in fatty acids and dietary patterns, as assessed by the carb-to-fat ratio increase (i.e., from low- to moderate- to high-carbohydrate diets). The symbol shapes reflect the number of double bonds in the molecules: circles for saturated, triangles for one double bond, squares for two double bonds, and diamonds for three or more double bonds. Yellow symbols indicate metabolites that changed significantly with increasing carb-to-fat ratio diets, as determined by False Discovery Rate (FDR) adjustment, while grey symbols indicate non-significant changes. The horizontal axis represents the number of carbon atoms in the fatty acids, and the vertical axis displays the beta coefficients, which are the model's estimates for each metabolite. R^2 value and corresponding two-sided P values were obtained from a linear regression model. Source data are provided as a Source Data file. FDR, False Discovery Rate.

Supplementary Fig. 5. PLS-DA score plot for diet-related metabolites: predictive outcomes and weight-loss maintenance annotations for low, moderate, and high carbohydrate diets.



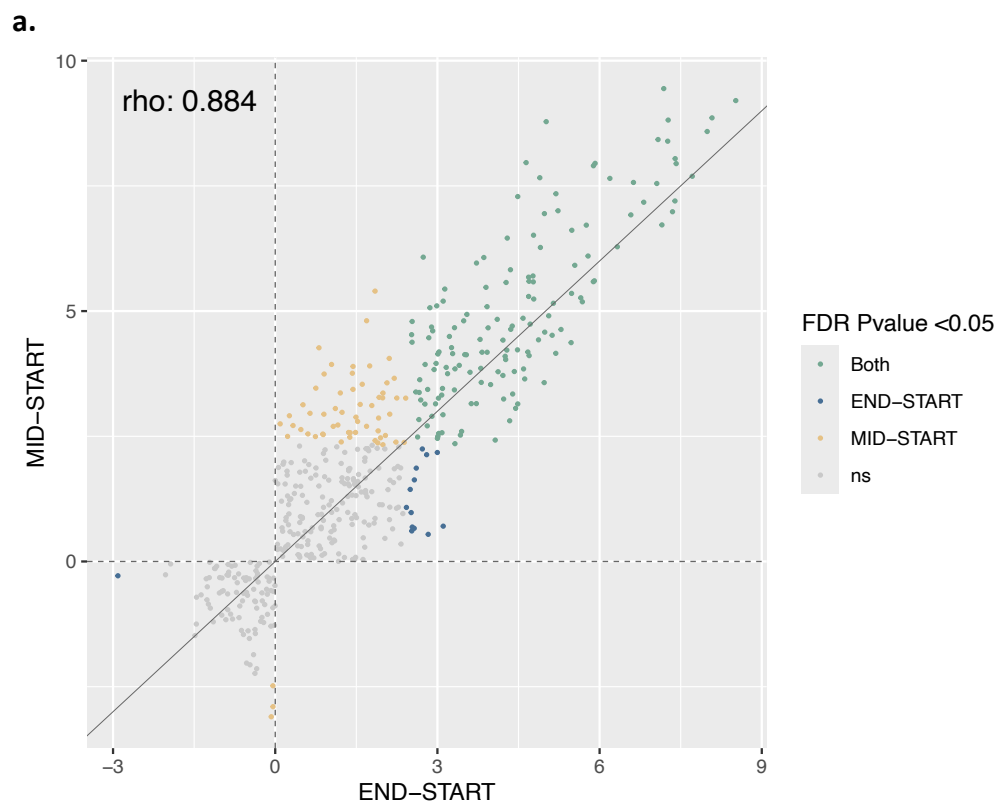
The PLS-DA score plot demonstrates the separation of participants by diet, based on metabolites exhibiting FDR-significant changes between the diets. Scores are derived from the data of the diet groups. Predictive outcomes result from a 5-fold cross-validation process, utilizing the Mahalanobis distance to measure model efficacy. Further participant annotations are based on weight-loss maintenance during the test phase of the study, which was used as a surrogate marker of adherence. "Weight stable" refers to participants who maintained their weight loss, while the "weight loss" and "weight gain" categories identify individuals who experienced a change of more than 2 kg. The accompanying table provides a summary of the correct and incorrect diet predictions for participants in each class.

The orientation of components 1 and 2 has been inverted for visual consistency by multiplying the respective component scores by -1. This adjustment, made solely for illustration purposes, does

not affect the relative positioning of subjects within the plot. Source data are provided as a Source Data file.

FDR, False Discovery Rate; PLS-DA, Partial Least Squares Discriminant Analysis.

Supplementary Fig. 6. Metabolite trends from low- to moderate- to high-carbohydrate diet: comparative results between early vs. entire test phase among participants excluding those who gained >2 kg weight during the test phase.

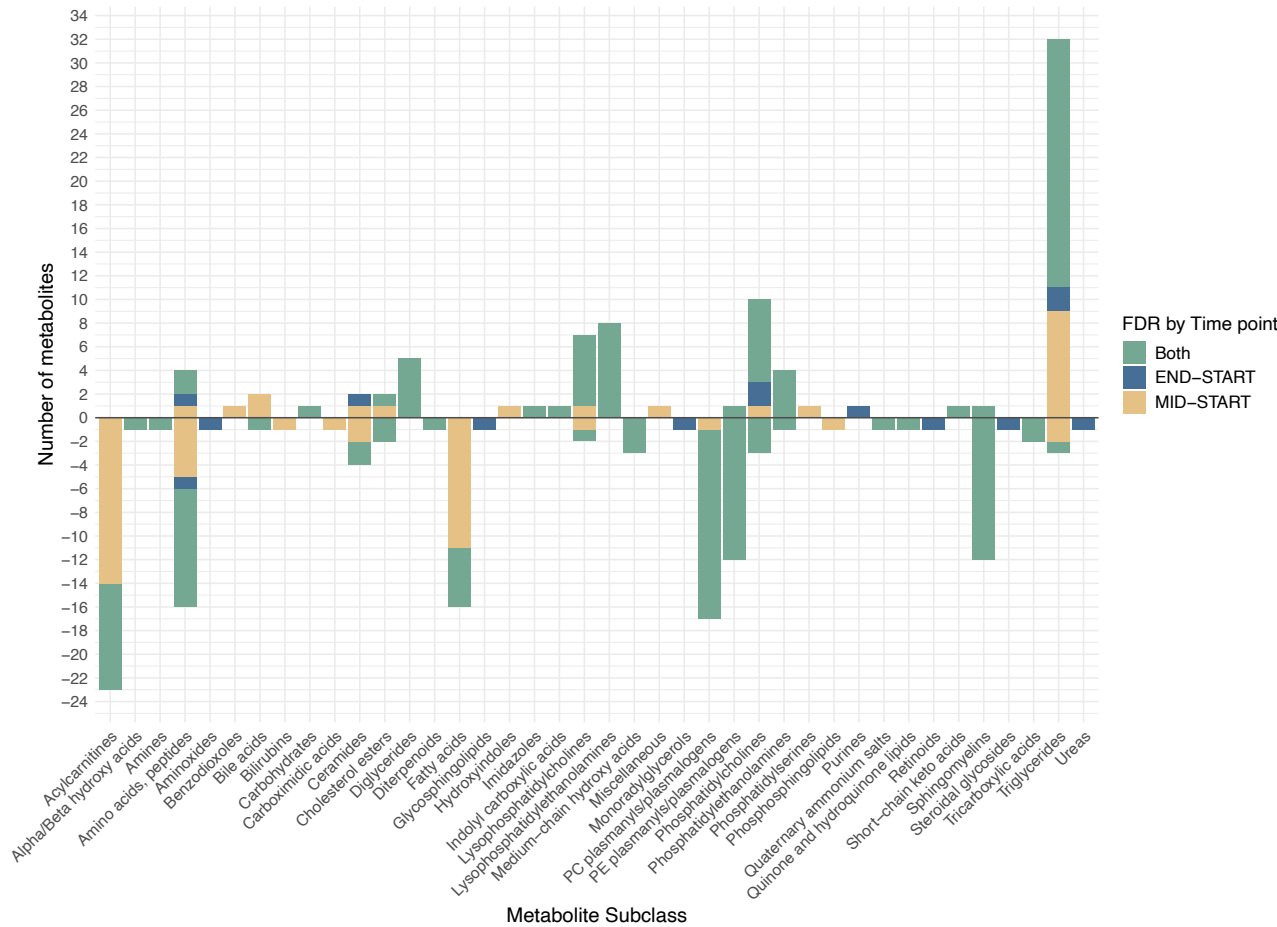


a. Metabolite changes: entire vs. early test phase

This “fish” plot (see Fig. 3 for explanation), illustrates the metabolite changes, in participants excluding those who gained >2 kg weight during the test phase ($n = 137$), over two distinct timeframes. The first analysis was conducted for the entire test phase (END-START), and the second analysis focused on metabolite changes during the early test phase (MID-START). The x- and y-axes represent values derived from t-statistics of the corresponding linear regression analyses. The R coefficient indicates Spearman’s correlation ($\rho = 0.884$; $P < 2.2e-16$). Each point corresponds to a specific metabolite. Metabolites on the plot are color-coded based on their FDR P -value significance. Blue (“END-START”) indicates FDR-significant metabolite changes only during the entire test phase, while yellow (“MID-START”) represents FDR-significant metabolite changes

solely during the early test phase. Green ("Both") represents FDR-significant metabolite changes in both periods (early and entire test phases), and grey ("ns") indicates absence of FDR-significant metabolite changes in either period. Source data are provided as a Source Data file.

b.



b. FDR significant metabolite changes: entire vs. early test phase

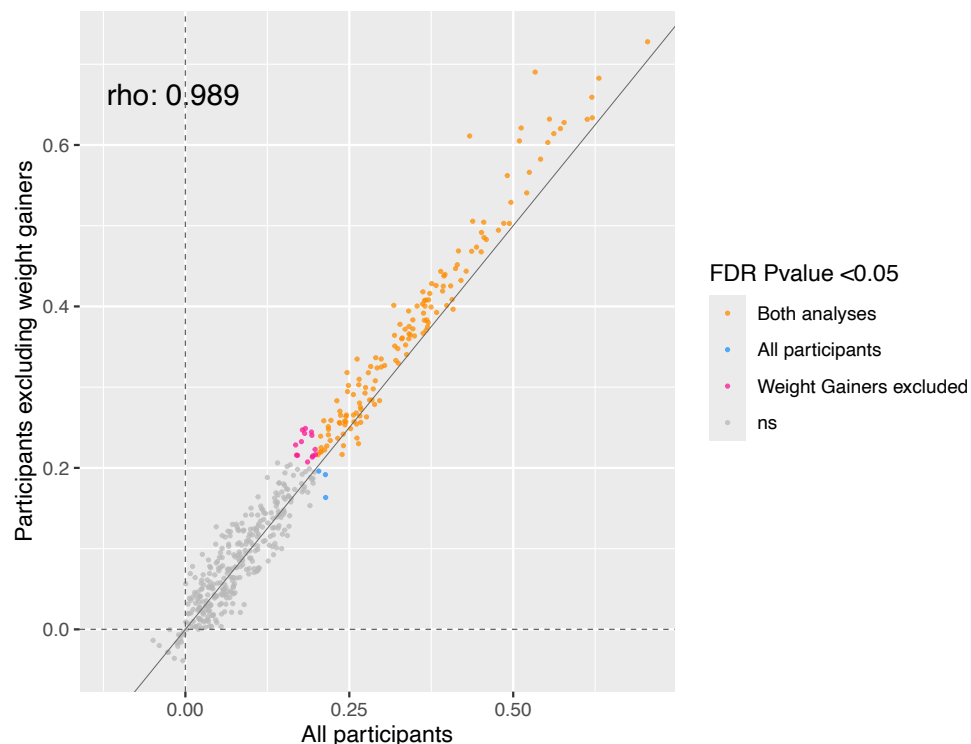
This bar plot illustrates the number of metabolites that exhibited FDR-significant changes from low- to moderate- and high-carbohydrate diets (increasing Carbohydrate to Fat ratio) across different intervals of the test phase. Each bar represents a group of metabolites belonging to a specific subclass. The bars indicate the number of metabolites showing statistically significant changes, corrected for False Discovery Rate (FDR) using a cutoff of < 0.05 . The y-axis of the plot represents the Carbohydrate to Fat ratio Trend, where negative values indicate a decreasing trend

as the Carbohydrate to Fat ratio increases, and positive values indicate an increasing trend as the Carbohydrate to Fat ratio increases. The absolute values on the y-axis represent the count of metabolites exhibiting these trends.

"END-START" represents FDR-significant metabolite changes only throughout the entire test phase. "MID-START" represents FDR-significant metabolite changes only during the early test phase. "Both" signifies FDR-significant metabolite changes in both periods (early and entire test phases). Source data are provided as a Source Data file.

END, end of test phase; FDR, False Discovery Rate; (FS)², Framingham State Food Study; MID, middle of test phase; ns, not significant; START, start of test phase.

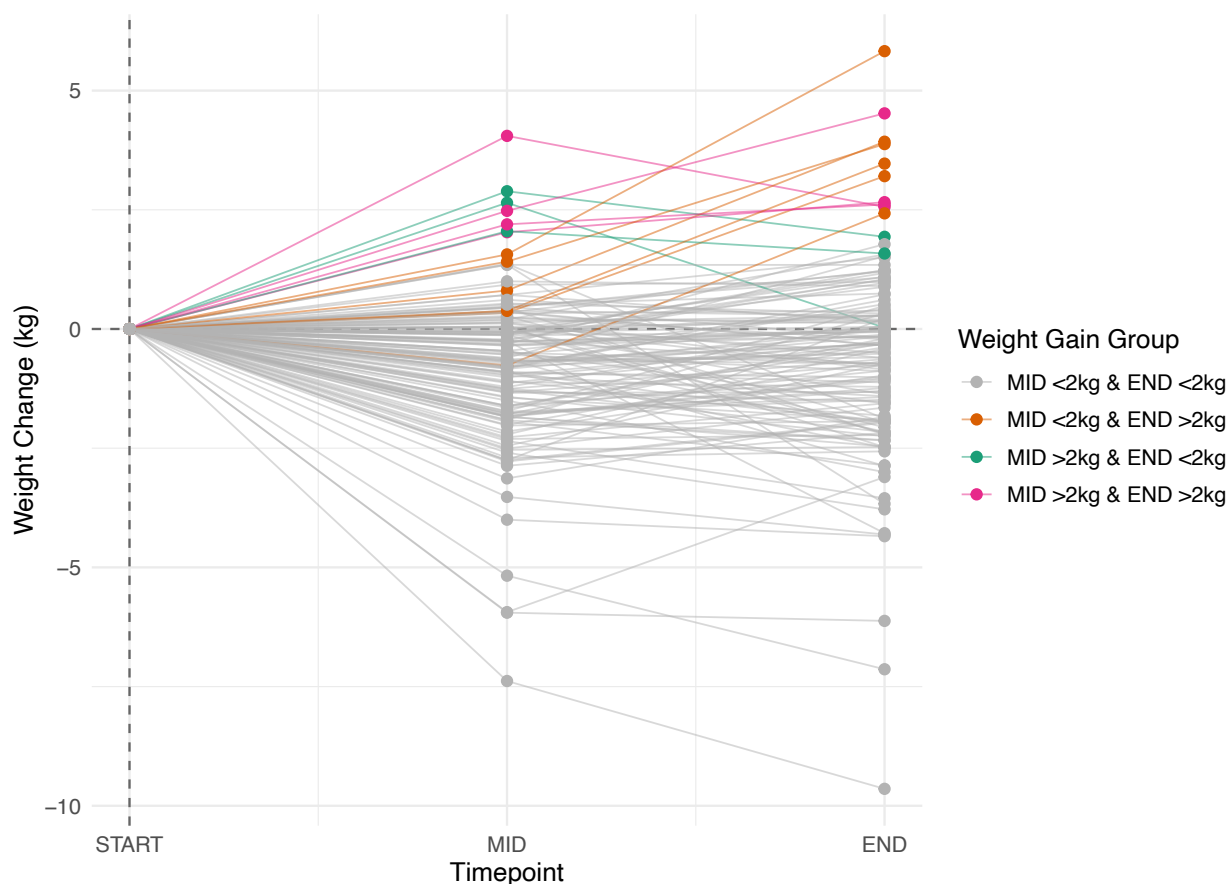
Supplementary Fig. 7. Metabolite level changes from low- to moderate- to high-carbohydrate diet: Comparative results between primary analysis and sensitivity analysis excluding participants who gained >2 kg weight during the test phase.



This “fish” plot (see Fig. 3 for explanation), illustrates the metabolite changes between the primary analysis (including all participants, $n = 147$) and the sensitivity analysis (excluding those who gained weight >2 kg during the test phase, $n = 137$). The x- and y-axes represent values derived from the regression t-statistics for each metabolite, adjusted by dividing by the square root of the number of participants in the respective analysis. The R coefficient indicates Spearman’s correlation ($\rho = 0.989$; $P < 2.2e-16$). Each point corresponds to a specific metabolite. Metabolite changes are color-coded based on their FDR significance. Yellow (“Both analyses”) signifies metabolites identified as FDR-significant in both analyses. Blue (“All participants”) represents FDR-significant metabolites identified solely in the primary analysis. Pink (“Weight Gainers excluded”) represents FDR-significant metabolites identified only in the sensitivity analysis and grey (“ns”) refers to metabolites that were not significant in either analysis. Source data are provided as a Source Data file.

FDR, False Discovery Rate; ns, not significant.

Supplementary Fig. 8. Individual trajectories of weight change during the test phase.



The plot illustrates weight changes for all individuals ($n = 147$) across three time points: START (start of the test phase), MID (middle of the test phase), and END (end of the test phase). The vertical axis represents weight change in kilograms, and the horizontal axis indicates study time points. Each line represents an individual trajectory. Colors denote weight change categories: grey (MID-PWL < 2 kg and END-PWL < 2 kg), orange (MID-PWL < 2 kg and END-PWL > 2 kg), green (MID-PWL > 2 kg and END-PWL < 2 kg), and pink (MID-PWL > 2 kg and END-PWL > 2 kg). Source data are provided as a Source Data file.

END, end of test phase; kg, kilograms; MID, middle of test phase; START, start of test phase.

Supplementary Table 1. Metabolite level changes in relation to higher Carbohydrate-to-Fat ratio: assessing consistency across (FS)2 and Popular Diets Study.

Metabolite changes in (FS)2 vs PDS	Concordant change in metabolite levels			Discordant change in metabolite levels			Total n of significant metabolites	% of metabolites with concordant change in metabolite levels
	(FS)2>0 & PDS>0	(FS)2<0 & PDS<0	Sum	(FS)2<0 & PDS>0	(FS)2>0 & PDS<0	Sum		
Significant in both	20	17	37	1	0	1	38	97.4%
Significant only in (FS)2	4	5	9	1	6	7	16	56.3%

The table summarizes whether metabolites common to both (FS)2 and the Popular Diets Study (external validation crossover trial) exhibit consistent changes in metabolite levels (either increasing or decreasing) when assessed from low- to moderate- to high-carbohydrate diets. Only the common metabolites that were found to be statistically significant (FDR) in the (FS)2 are included.

(FS)2, Framingham State Food Study; FDR, False Discovery Rate; n, number; PDS, Popular Diets Study.

Supplementary Table 2. Performance metrics comparison of PLS-DA models for 2 extreme diets and 3 diet types analyses.

Including the total population

Analyses:	2 extreme diets	3 diet types		
Positive Class:	High Carb	Low Carb	Moderate Carb	High Carb
Sensitivity	0.867	0.722	0.563	0.711
Specificity	0.833	0.828	0.788	0.882
Positive Predictive Value	0.813	0.709	0.563	0.727
Negative Predictive Value	0.882	0.837	0.788	0.874
Balanced Accuracy	0.850	0.775	0.675	0.797

Excluding the participants who gain weight >2kg during the test phase from the testing dataset

Analyses:	2 extreme diets	3 diet types		
Positive Class:	High Carb	Low Carb	Moderate Carb	High Carb
Sensitivity	0.881	0.740	0.600	0.714
Specificity	0.880	0.851	0.772	0.905
Positive Predictive Value	0.861	0.740	0.563	0.769
Negative Predictive Value	0.898	0.851	0.798	0.878
Balanced Accuracy	0.881	0.795	0.686	0.810

The table presents performance metrics of Partial Least Squares Discriminant Analysis (PLS-DA) models performed by including the 2 extreme diets (Low Carbohydrate and High Carbohydrate) and all 3 diet types (Low Carbohydrate, Moderate Carbohydrate, High Carbohydrate). PLS-DA, Partial Least Squares Discriminant Analysis.

Supplementary Table 3. Descriptive and clinical characteristics at randomization of participants who did or did not achieve weight-loss maintenance during the test phase.

Characteristics	Weight-loss maintenance	Non weight-loss maintenance	P-value
N	109	38	
Age (years)	39.84 (14.05)	35.13 (15.28)	0.052
Female, Yes (%)	76 (69.72)	26 (68.42)	1
<i>Race*:</i>			
White (%)	83 (76.15)	32 (84.21)	0.168
Black (%)	11 (10.09)	5 (13.16)	
Asian (%)	4 (3.67)	1 (2.63)	
Other/Not Sure (%) **	11 (10.09)	0 (0.00)	
Hispanic ethnicity, Yes (%)	17 (15.60)	3 (7.89)	0.359
BMI (kg/m ²)	28.45 (3.83)	29.79 (5.34)	0.318
<i>Body composition:</i>			
Lean body mass (% of total mass)§	60.26 (6.78)	60.04 (5.21)	0.782
Body fat mass (% of total mass)	36.77 (7.08)	37.06 (5.45)	0.816
Weight loss (% of pre-weight loss)	-10.45 (1.52)	-10.53 (1.77)	0.810
delta Weight change during test phase (kg)	-0.25 (0.99)	-1.53 (3.44)	1.44E-05

Data presented as mean (Standard Deviation) for continuous variables and n (%) as categorical variables. Weight-loss maintenance: participants whose weight changed by more than two kilograms (either increased or decreased by >2 kg) from their weight at the START of the test phase (study randomization). Comparisons between groups were conducted using ANOVA or Kruskal-Wallis tests, depending on the distribution of the continuous variables. For categorical variables, the Chi-squared test with Yates' continuity correction was used, or Fisher's Exact Test when expected cell counts were less than 5. All statistical tests were two-sided.

* Race was determined by self-report using predefined categories that were standard when the trial was implemented: White, Black/African American, Asian, American Indian/Alaska Native, Native Hawaiian/Other Pacific Islander, Other, and Not Sure.

** Among these 11 participants, 10 reported “Other/Not Sure” for race (and also reported Hispanic ethnicity), and one specified multiple races (White, Black/African American, American Indian/Alaska Native)

§ Lean body mass does not include bone mineral content.

HC, High Carbohydrate group; LC, Low Carbohydrate group; MC, Moderate Carbohydrate group; N, number; START, start of test phase.

Supplementary Table 4. Concordant and discordant changes in metabolite levels (across diets with higher Carbohydrate-to-Fat ratio) and T2D risk.

Metabolite	SubClass
Concordant increase in metabolite levels (with higher Carbohydrate-to-Fat ratio) and T2D risk	
DG 32:0	Diglycerides
DG 32:1	Diglycerides
DG 34:1	Diglycerides
DG 34:2	Diglycerides
DG 38:5	Diglycerides
PC 38:3	Phosphatidylcholines
PE 34:1	Phosphatidylethanolamines
TG 48:0	Triglycerides
TG 48:1	Triglycerides
TG 48:2	Triglycerides
TG 50:0	Triglycerides
TG 50:1	Triglycerides
TG 50:2	Triglycerides
TG 50:3	Triglycerides
TG 50:5	Triglycerides
TG 50:6	Triglycerides
TG 51:1	Triglycerides
TG 51:2	Triglycerides
TG 52:1	Triglycerides
TG 52:3	Triglycerides
Concordant decrease in metabolite levels (with higher Carbohydrate-to-Fat ratio) and T2D risk	
LPC 18:2	Lysophosphatidylcholines
Discordant increase in metabolite levels (with higher Carbohydrate-to-Fat ratio) and T2D risk	
Indole-3-propionate	Indolyl carboxylic acids and derivatives
LPC 18:3	Lysophosphatidylcholines
Discordant decrease in metabolite levels (with higher Carbohydrate-to-Fat ratio) and T2D risk	
CAR 4:0;OH	Acylcarnitines
Cer 18:1;O2/24:0	Ceramides
Alpha-hydroxybutyrate/Beta-hydroxybutyrate	Alpha/Beta hydroxy acids

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51 The table summarizes both concordant and discordant changes in metabolite levels observed in
52 the FS(2) study (when assessed from low- to moderate- to high-carbohydrate diet) and their
53 association with T2D risk as indicated in the meta-analysis.

54 Only metabolites demonstrating FDR significance in both studies were included.

55 (FS)2, Framingham State Food Study; FDR, False Discovery Rate; T2D, Type 2 Diabetes.

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