Supplementary Information for

A Mediterranean diet does not alter plasma trimethylamine N-oxide concentrations in healthy adults at risk for colon cancer

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SUPPLEMENTAL TABLES

Table S1. Multi-reaction monitoring (MRM) settings for UPLC-MS/MS detection of analytes in plasma

Compound	Retention time (min)	MW (g/mol)	Parent $[M+H]^+$ (m/z)	Daughter (m/z)	Cone voltage (V)	Collision energy (eV)
Carnitine	2.09	161.20	162.26	84.99	84.99	34
Carnitine-d_9	2.08	170.25	171.28	84.99	84.99	34
Betaine	1.25	117.15	118.24	59.42	59	44
γ-Butyrobetaine	0.98	145.20	146.27	87.00	26	16
Betaine-d_9	1.25	126.14	127.30	68.10	68	46
Choline	1.13	103.16	104.20	60.02	60	38
Choline-d_9	1.11	112.16	113.32	69.08	69	40
TMAO	2.01	75.11	76.16	58.91	59	40
TMAO-d_9	1.98	84.12	85.22	68.10	68	40

Table S2. Demographic and dietary characteristics of subjects[a] at baseline classified based on median TMAO plasma concentration[b]

Parameter[c]	Healthy Eating, n=58	Mediterranean Diet, n=58
BMI[d] (kg/m^2)	26.9 (3.6)	27.4 (4.1)
Age (years)	49 (14)	55 (10)
Caucasian	54 (93%)	49 (85%)
Female	41 (71%)	43 (74%)
Current smoker	4 (7%)	9 (16%)
College Graduate	46 (79%)	45 (78%)
Physical Activity, metabolic equivalents/day	20 (15)	19 (15)
Energy intake (kcal/day)	2134 (646)	1954 (446)
Fat (% of energy)	34.3 (5.8)	34.5 (6.5)
Protein (% of energy)	15.8 (2.5)	15.7 (2.8)
Animal Protein (% of energy)	10.1 (2.9)	9.7 (2.8)
Carbohydrate (% of energy)	48.8 (6.5)	49.4 (7.9)
Fruit and vegetables (servings/day)	4.6 (1.9)	4.5 (1.7)
Fiber (g/1000 kcal)	10.7 (3.5)	11.4 (3.7)
Glycemic Index, bread reference	83.7 (5.4)	83.8 (5.9)

[a] Characteristics of 115 subjects at baseline

[b] Data shown are for subjects for whom TMAO plasma concentrations are available at either baseline or post-intervention

[c] Data are given as mean and (SD) or number and percent. None of the differences were statistically significant using either two-sample t-tests for continuous variables or Chi Square tests for categorical variables.

[d] BMI = body mass index

Table S3. Body weight and dietary characteristics of individuals who did or did not decrease in TMAO concentration post-intervention. Data shown is mean and SD except for number of participants assigned to the Mediterranean diet arm.

Parameter	Decreased TMAO (n=41)	Increased TMAO (n=48)	P-value
Percent change TMAO	-35% (26)	77 (77)	**<0.001**
Mediterranean Diet Assignment, n (%)	22 (53%)	24 (50%)	0.746
Percent goals met	87% (22)	82% (20)	0.292
Weight Change (lbs.)	-2.3 (7.8)	-0.34 (4.1)	0.162
Percent weight change post-supplementation versus baseline	-1.18 (4.5)	-0.049 (2.51)	0.155
Change in Animal Protein intake, g/day	-5.5 (21.3)	-1.5 (14.4)	0.297
Change in red meat intake, servings/day	-1.58 (1.80)	-0.98 (1.61)	0.099
Change in fiber intake, g/1000 kcal	5.9 (4.4)	4.4 (4.2)	0.098

Table S4. Baseline dietary intakes in study subjects who had TMAO plasma concentrations above or below the median (2.92 μM) at baseline. Data shown is mean and SD or number and percent.

Baseline Diet	**Above Median, n=57**	**Below Median, n=58**	**P-Value[a]**
Assigned to Mediterranean arm (number, %)	29 (50%)	29 (51%)	0.925
Energy intake (kcal/day)	2134 (641)	1954 (453)	0.086
Fat (% of energy)	34.7 (5.6)	34.1 (6.6)	0.639
Protein (% of energy)	15.8 (2.9)	15.8 (2.7)	0.966
Animal Protein (% of energy)	10.2 (2.8)	9.5 (3.0)	0.203
Carbohydrate (% of energy)	48.1 (5.9)	47.6 (7.6)	0.744
Fruit and vegetables (servings/day)	4.8 (2.0)	4.3 (1.6)	0.126
Fish, Servings/day	0.5 (1.0)	0.5 (0.4)	0.843
Red meat, servings/day	3.0 (1.9)	2.2 (1.8)	0.025
Eggs, servings/day	0.4 (0.5)	0.4 (0.4)	0.814
Dairy, servings/day	3.3 (2.0)	3.1 (1.9)	0.561
Fiber (g/1000 kcal)	10.7 (4.0)	11.5 (3.2)	0.259
Glycemic Load, bread reference	203 (67)	180 (53)	0.042

[a]P-values are from independent samples t-tests (two-sided), from Chi square tests for categorical variables, or from Fisher's Exact test for current smoking. None are significant after corrections for multiple comparisons.

Table S5. Cytokine concentrations in 116 subjects with TMAO concentrations above or below the median at baseline. The median TMAO concentration was 2.92 μmol/L. Data shown is mean and SD.

Parameter	**Above Median*, n=57** (pg/mL)	**Below Median*, n=58** (pg/mL)	**P-Value**
IL-1β	436 (1528)	499 (1650)	0.830
IL-6	536 (1671)	557 (1514)	0.942
IL-8	670 (3540)	568 (3393)	0.875
TNFα	884 (2714)	717 (1863)	0.700
IL-4	415 (1112)	370 (1027)	0.821
IL-10	1718 (6428)	1642(5361)	0.945
IL-13	12973 (57741)	11460 (45854)	0.876
INFɣ	616 (2640)	643 (3586)	0.964
CRP	2.31 (3.08)	2.60 (3.24)	0.631

*Mean TMAO was 5.8 μmol/L (SD 5.6) for subjects above the median and 2.0 μmol/L (SD 0.6) for subjects below the median.

Table S6. Spearman correlations (rho) of serum measures with bacterial diversity measures at baseline, major phyla and ratios in colon biopsy tissues (n=86) at baseline. Starred coefficients had p<0.05 without adjustment for multiple comparisons.

Bacterial populations in colonic biopsies	**TMAO µM**	**Betaine µM**	**Choline µM**	**Carnitine µM**	**γ-Butyro-betaine µM**	**TMAO: Betaine**	**TMAO: Choline**	**TMAO: Carnitine**	**TMAO: γ-Butyro-betaine**
Shannon Diversity Index	-0.088	0.022	**-.286***	-0.136	0.042	-0.087	0.066	0.086	-0.083
Inverse Simpson Index	-0.045	0.014	-0.203	-0.078	0.049	-0.041	0.069	0.053	-0.069
θ_{YC} Distances	-0.121	-0.031	-0.171	-0.159	-0.087	-0.136	-0.036	0.025	-0.05
Actinobacteria	-0.047	0.052	-0.083	-0.078	-0.029	-0.094	-0.015	0.027	-0.06
Bacteroidetes	0.118	-0.051	-0.043	0.013	-0.078	0.211	0.19	0.068	0.202
Firmicutes	-0.009	0.043	-0.058	-0.009	0.105	-0.05	0.012	0.024	-0.054
Proteobacteria	0.004	0.041	0.085	0.056	0.044	-0.044	-0.075	-0.097	-0.036
Verrucomicrobia	**-.230***	**-.249***	**-.263***	-0.183	-0.159	-0.084	-0.109	-0.058	-0.105
Firmicutes:Bacteroidetes	-0.093	0.015	-0.089	-0.034	0.103	-0.16	-0.087	-0.028	-0.175
Prevotella:Bacteroides	0.023	0.119	-0.102	-0.139	-0.1	0.048	0.053	0.119	0.124
Prevotella	0.016	0.14	-0.082	-0.082	-0.121	0.034	0.04	0.077	0.14
ClostridiumXIVa	-0.193	-0.194	-0.115	-0.054	-0.158	0.003	-0.08	-0.131	-0.003
Eubacterium	-0.132	0.097	-0.109	-0.072	-0.04	-0.163	-0.085	0.001	-0.024
Desulfovibrio	-0.032	0.071	-0.015	0.107	-0.089	-0.06	-0.032	-0.132	0.019
Akkermansia mucinophilia	**-.231***	**-.248***	**-.262***	-0.182	-0.158	-0.085	-0.11	-0.059	-0.106

SUPPLEMENTAL FIGURES

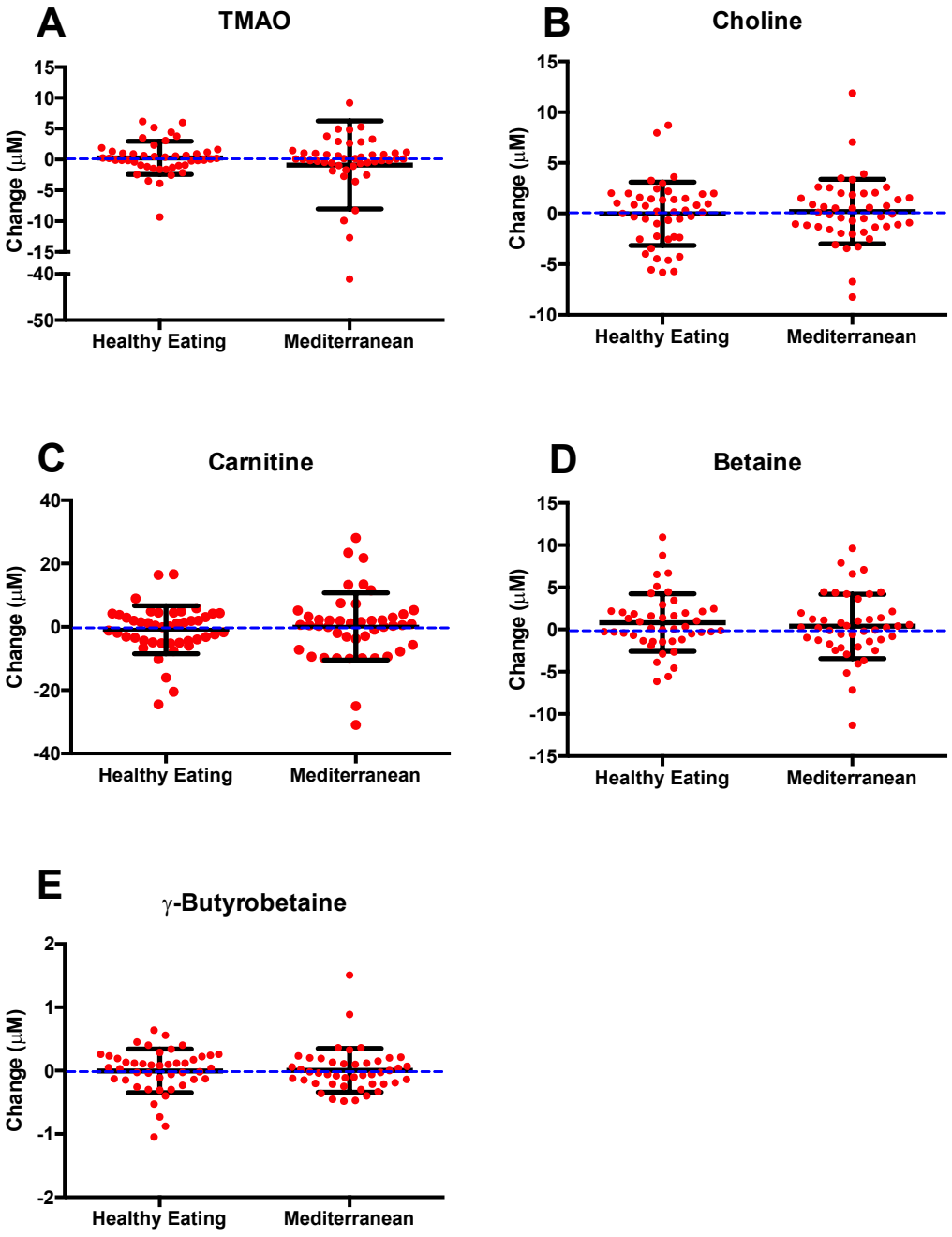

Figure S1. Absolute changes in fasting serum levels of TMAO (**A**), choline (**B**), carnitine (**C**), betaine (**D**) and γ-butyrobetaine (**E**) over 6 months of each respective intervention [changes were calculated as (concentration at 6 months – concentration at baseline)]. Lines represent mean ± SD. Note: subjects who did not complete the intervention were excluded from this calculation.

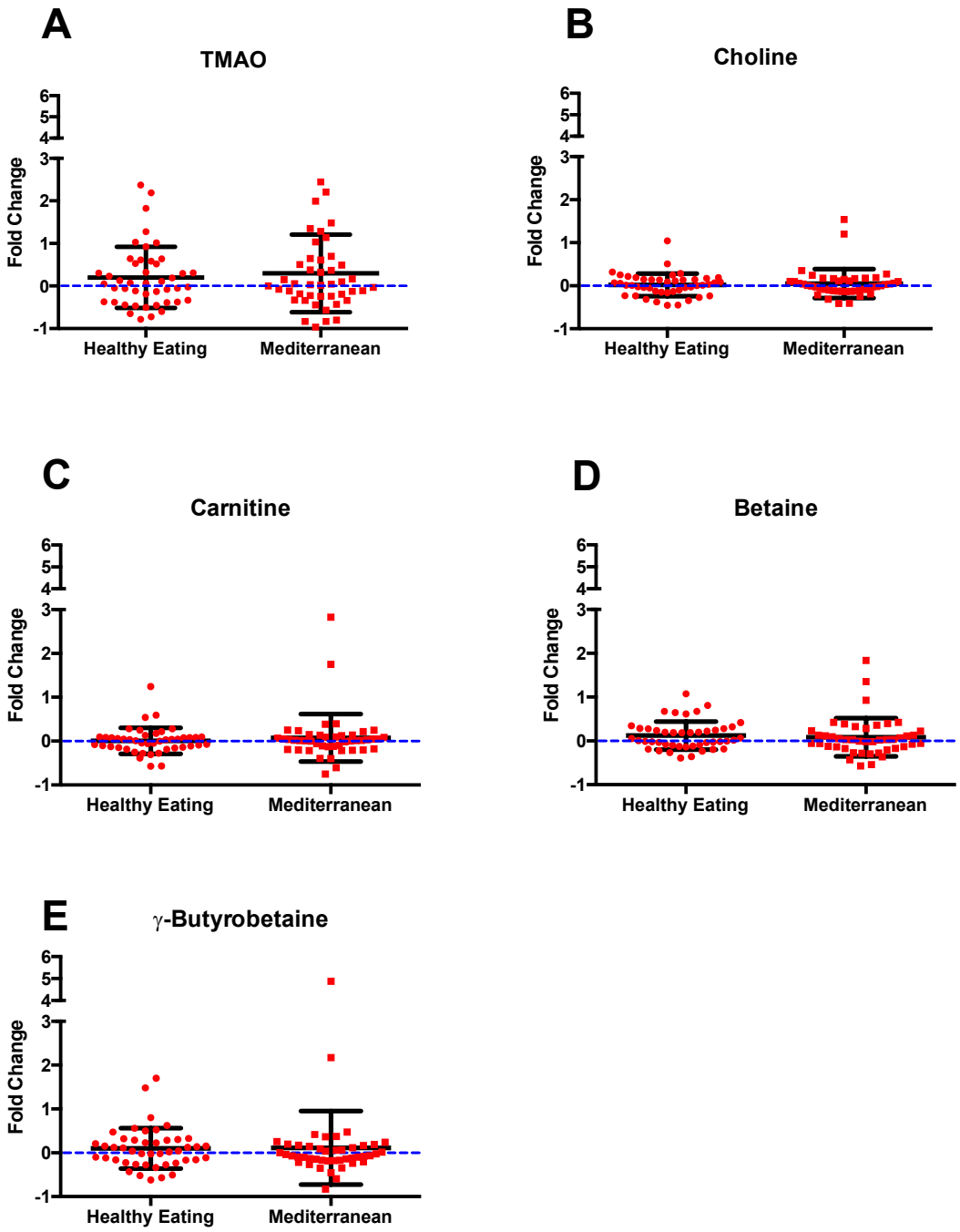

Figure S2. Fold changes in fasting serum levels of TMAO (**A**), choline (**B**), carnitine (**C**), betaine (**D**) and γ-butyrobetaine (**E**) over 6 months of each respective intervention [fold changes were calculated as (concentration at 6 months) / concentration at baseline]. Lines represent mean ± SD. Subjects who did not complete the intervention are excluded from this calculation. Note: scales are the same for all compounds in order to facilitate comparisons.