Methods

Study samples and metabolite profiling

Data were acquired using a system comprised of a Shimadzu Nexera X2 U-HPLC (Shimadzu Corp.) coupled to a Q Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Fisher Scientific). Metabolite extracts were prepared from plasma samples (10 µL) via protein precipitation with the addition of 9 volumes of 74.9:24.9:0.2 (volume:volume:volume) of acetonitrile:methanol: formic acid–containing stable isotope-labeled internal standards [valine-d8 (Sigma-Aldrich) and phenylalanine-d8 (Cambridge Isotope Laboratories)]. The samples were centrifuged (10 min; 9000 x g; 4°C), and the supernatant fluid was injected directly onto a 150 x 2-mm, 3-µm Atlantis HILIC column (Waters). The column was eluted isocratically at a flow rate of 250 µL/min with 5% mobile phase A (10 mmol ammonium formate/L and 0.1% formic acid in water) for 0.5 min followed by a linear gradient to 40% mobile phase B (acetonitrile with 0.1% formic acid) over 10 min. MS analyses were carried out using electrospray ionization in the positive-ion and full-scan spectra were acquired over 70-800 m/z at 70,000 resolution and 3-Hz data-acquisition rate. Other MS settings were as follows: sheath gas, 40; sweep gas, 2; spray voltage, 3.5 kV; capillary temperature, 350°C; S-lens, 40; heater temperature, 300°C; microscans, 1; automatic gain control target, 1E6; and maximum ion time, 250 ms. Metabolite identities were confirmed using authentic reference standards. To enable assessment of data quality and to facilitate data standardization across the analytical queue and sample batches, pairs of pooled plasma reference samples were analysed at intervals of 20 study samples. One sample from each pair of pooled references served as a passive QC sample to evaluate the analytical reproducibility for measurement of each metabolite while the other pooled sample was used to standardize data using a "nearest neighbour" approach. Standardized values were calculated using the ratio of the

value in each sample over the nearest pooled plasma reference multiplied by the median value measured across the pooled references. After standardization, the coefficients of variation measured among 92 pooled QC samples were 2.8% for valine, 1.8% for leucine, 1.7% for isoleucine, 2.32% for phenylalanine and 2.26% for tyrosine. Raw data were processed using TraceFinder 3.1 (Thermo Fisher Scientific) and Progenesis QI (Nonlinear Dynamics).

The data were acquired using high resolution-accurate mass profiling and metabolites were identified on the basis of accurate mass, retention time, and product ion spectra matching to authentic reference standards, and therefore meet the "Level 1 – identified metabolites" guideline of the Metabolomics Standards Initiative (MSI). The LC method achieves separation of metabolite peaks over approximately 13 minutes (the total method time is 32 minutes including wash and equilibration time) and this relatively "long" method was developed toward optimal separation and to mitigate the types of ionization suppression and interferences as found in both short LC and flow injection methods. Leucine and isoleucine are nearly baseline-separated in this method.

Results

ESM Table 1. Changes in individual amino acids and scores after 1 year of intervention adjusted for changes in HOMA-IR or insulin

	Control group	MedDiet+EVOO	MedDiet+nuts
Multivariable* model additionally adjusted for 1-year change of HOMA-IR	**1-year mean change in SD units (95% CI)**	**1-year mean change in SD units (95% CI)**	**1-year mean change in SD units (95% CI)**
Leucine	0 (ref)	-0.21 (-0.38 to -0.05)	-0.06 (-0.22 to 0.10)
Isoleucine	0 (ref)	-0.47 (-0.54 to -0.40)	-0.10 (-0.26 to 0.06)
Valine	0 (ref)	-0.17 (-0.33 to -0.10)	-0.11 (-0.27 to 0.04)
Phenylalanine	0 (ref)	-0.06 (-0.23 to 0.11)	0.06 (-0.11 to 0.22)
Tyrosine	0 (ref)	0.00 (-0.17 to 0.18)	-0.01 (-0.18 to 0.17)
BCAA score	0 (ref)	-0.21 (-0.37 to -0.05)	-0.09 (-0.25 to 0.07)
AA score	0 (ref)	-0.06 (-0.23 to 0.11)	0.04 (-013 to 0.21)
Multivariable* model adjusted additionally for 1-year change of insulin			
Leucine	0 (ref)	-0.26 (-0.45 to -0.08)	-0.13 (0.32 to 0.05)
Isoleucine	0 (ref)	-0.29 (-0.47 to -0.10)	-0.16 (-0.35 to 0.02)
Valine	0 (ref)	-0.19 (-0.38 to -0.01)	-0.17 (-0.36 to 0.01)
Phenylalanine	0 (ref)	-0.08 (-0.27 to 0.11)	0.05 (-0.14 to 0.23)
Tyrosine	0 (ref)	0.00 (-0.18 to 0.19)	-0.04 (-0.22 to 0.14)
BCAA score	0 (ref)	-0.23 (-0.40 to -0.07)	-0.12 (-0.28 to 0.04)
AA score	0 (ref)	-0.08 (-0.25 to 0.010)	0.02 (-0.15 to 0.19)

Abbreviations: AA, Aromatic Amino acids; BCAA, branched-chain amino acids; CI, confidence interval; SD, Standard Deviation

* Adjusted for age (years), sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidaemia, hypertension, baseline fasting glucose and baseline metabolite levels (or baseline score).

Multiple imputation methods were used to account for missing values of HOMA-IR and insulin.

ESM Table 2. Associations of changes in amino acid levels after 1 year with the risk of incident type 2 diabetes. The PREDIMED trial, 2003-2010.

	HR (95%CI)		
	No change*	**Decrease***	**Increase***
Leucine	1 (ref.)	1.42 (0.84 - 2.38)	1.16 (0.70 - 1.94)
Isoleucine	1 (ref.)	1.20 (0.70 - 2.04)	1.88 (1.20 - 2.96)
Valine	1 (ref.)	1.06 (0.62 - 1.80)	0.97 (0.59 - 1.58)
Phenylalanine	1 (ref.)	0.55 (0.33 - 0.93)	0.87 (0.53 - 1.41)
Tyrosine	1 (ref.)	0.74 (0.44 - 1.23)	0.93 (0.54 - 1.59)
BCAA score	1 (ref)	0.87 (0.49 - 1.53)	2.01 (1.27 - 3.18)
AA score	1 (ref)	0.68 (0.42 - 1.09)	1.16 (0.70 - 1.94)

* No change includes changes less than 1 SD, decrease are changes lower than 1 SD and increase are changes higher than 1 SD

† Including 158 type 2 diabetes cases and 505 non-cases in the overall subcohort (36 overlapping cases).

‡ An inverse normal transformation was applied to raw values. Model adjusted for metabolite (or score) level at baseline, age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidaemia, hypertension, baseline fasting glucose (mean + quadratic term of centred mean) and stratified by recruitment centre.

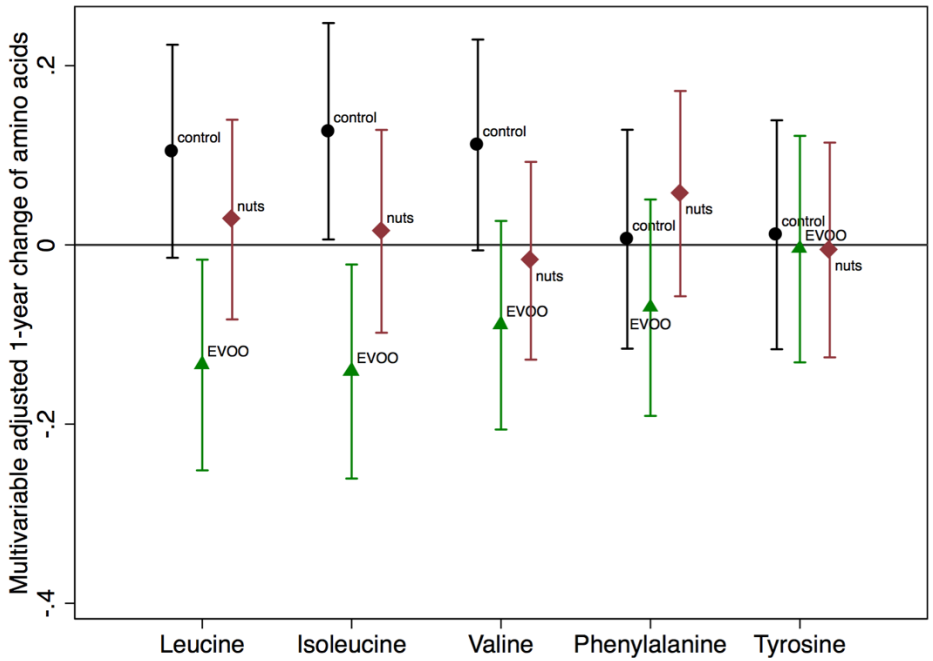
ESM Table 3. Change of HOMA-IR Index (95% confidence intervals) after 1 year by Quartiles of Baseline Plasma Branched-Chain and Aromatic Amino Acids in the PREDIMED trial, 2003–2010.

	1 year change of HOMA-IR	
	Mean (95% CI)[a]	***P* value**
Baseline BCAA score		
Q1	0.12 (-0.34 to 0.59)	0.596
Q2	0.14 (-0.26 to 0.55)	0.484
Q3	0.46 (0.07 to 0.85)	0.022
Q4	0.77 (0.35 to 1.19)	<0.001
Baseline AA score		
Q1	0.16 (-0.26 to 0.57)	0.459
Q2	0.38 (-0.01 to 0.77)	0.055
Q3	0.47 (0.04 to 0.90)	0.031
Q4	0.55 (0.14 to 0.96)	0.009

Abbreviations: AA, Aromatic Amino acids; BCAA, branched-chain amino acids; CI, confidence interval;

[a] Adjusted for age (years), sex (male, female), intervention group (MedDiet+EVOO, MedDiet+nuts), body mass index (kg/m^2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidaemia, hypertension and baseline fasting glucose

ESM Figure 1. Changes in amino acids after 1 year of intervention, by intervention group



Changes are adjusted for age (years), sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidaemia, hypertension, baseline fasting glucose and baseline metabolite levels.