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Nontargeted Metabolite Profiling Discriminates Diet-Specific Biomarkers for Consumption of Whole Grains, Fatty Fish, and Bilberries in a Randomized Controlled Trial[1–3]

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Abstract

Background: Nontargeted metabolite profiling allows for concomitant examination of a wide range of metabolite species, elucidating the metabolic alterations caused by dietary interventions.

Objective: The aim of the current study was to investigate the effects of dietary modifications on the basis of increasing consumption of whole grains, fatty fish, and bilberries on plasma metabolite profiles to identify applicable biomarkers for dietary intake and endogenous metabolism.

Methods: Metabolite profiling analysis was performed on fasting plasma samples collected in a 12-wk parallel-group intervention with 106 participants with features of metabolic syndrome who were randomly assigned to 3 dietary interventions: *1*) whole-grain products, fatty fish, and bilberries [healthy diet (HD)]; *2*) a whole-grain–enriched diet with the same grain products as in the HD intervention but with no change in fish or berry consumption; and *3*) refined-wheat breads and restrictions on fish and berries (control diet). In addition, correlation analyses were conducted with the food intake data to define the food items correlating with the biomarker candidates.

Results: Nontargeted metabolite profiling showed marked differences in fasting plasma after the intervention diets compared with the control diet. In both intervention groups, a significant increase was observed in 2 signals identified as glucuronidated alk(en)-ylresorcinols [corrected *P* value (P_{corr}) < 0.05], which correlated strongly with the intake of whole-grain products ($r = 0.63$, $P < 0.001$). In addition, the HD intervention increased the signals for furan fatty acids [3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF)], hippuric acid, and various lipid species incorporating polyunsaturated fatty acids ($P_{corr} < 0.05$). In particular, plasma CMPF correlated strongly with the intake of fish ($r = 0.47$, $P < 0.001$) but not with intakes of any other foods.

Conclusions: Novel biomarkers of the intake of health-beneficial food items included in the Nordic diet were identified by the metabolite profiling of fasting plasma and confirmed by the correlation analyses with dietary records. The one with the most potential was CMPF, which was shown to be a highly specific biomarker for fatty fish intake. This trial was registered at clinicaltrials.gov as NCT00573781. *J Nutr* 2015;145:7–17.

Keywords: nontargeted metabolite profiling, metabolomics, CMPF, alkylresorcinol, hippuric acid, dietary biomarker

Introduction

The Nordic diet is characterized by many food items with positive nutritional profiles, such as whole-grain products, berries, root vegetables, and fatty fish. Adherence to a healthy Nordic dietary pattern has been associated with lower mortality (1), and controlled interventions using diets modified by single

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[3] Supplemental Tables 1–3 and Supplemental Figures 1–3 are available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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food items or combinations of Nordic foods have shown beneficial cardiovascular health effects (i.e., improved lipid profile and glucose metabolism and anti-inflammatory properties) (2–8).

Targeted analyses on a predetermined set of clinical biomarkers after consumption of specific health-promoting nutrients, foods, or diets can provide important mechanistic information, but they fail to give a broader picture of the altered metabolic status resulting from a variety of interactions between food constituents and endogenous metabolites. Nontargeted metabolite profiling using sensitive and high-throughput analytical technologies offers a wide window to monitor changes in diet-derived and endogenous metabolites, thus aiding the identification of novel biomarkers for both dietary intake and biological effects (9–12).

The Sysdimet-HealthGrain intervention has provided new information of the potential health effects of whole grain, in particular rye, and of a combination of whole grains with bilberries and fatty fish (6, 7). Here we report novel biomarkers for consumption of whole grains, bilberries, and fish by applying an LC-MS–based nontargeted metabolite profiling approach. Unlike the majority of the published nutritional metabolomics studies that focused on urine samples (13), we demonstrate here the impact of dietary intervention on the metabolic profile in fasting plasma. Moreover, the potential biomarkers were linked to specific food groups by correlation analyses with the dietary intake data based on repeated food records.

Methods

Participants, study design, and diets

The study population was from the Sysdimet-HealthGrain intervention, a Finnish randomized controlled dietary intervention (6, 7) aiming to investigate the effects of a diet rich in whole grains, bilberries, and fatty fish on glucose metabolism in high-risk individuals with impaired glucose metabolism and features of the metabolic syndrome. A total of 131 volunteers were recruited for a 12-wk intervention and randomly assigned to 3 parallel interventions: a healthy diet (HD),[9] a whole-grain–enriched diet (WGED), or a control diet (**Figure 1**). The intervention groups were matched for sex and medians of BMI, age, and fasting plasma glucose concentration. A total of 106 participants completed the study. Baseline characteristics of the subjects are shown in **Supplemental Table 1**. The study plan was approved by the Research Ethics Committee, Hospital District of Northern Savo. The intervention was performed in accordance with the Helsinki Declaration.

The intervention diets are described in detail in **Supplemental Figure 1**. In brief, in the HD group, the participants were advised to replace their usual cereal products with whole-grain breads and a bread with low postprandial insulin response and with 3.5 dL (measured uncooked) of whole meal pasta per week. The recommended breads contributed 20–25% of the daily energy intake. In addition, participants were instructed to eat fatty fish (100–150 g fish/meal) 3 times/wk and bilberries (*Vaccinium myrtillus*; frozen, pureed, or dried powder) at 3 portions/d (equivalent to 300 g fresh bilberries/d). In the WGED group, the participants were instructed to consume the same cereal products as in the HD group and not to change their current fish and berry consumption. In the control group, the participants were asked to avoid whole-grain cereals and to consume low-fiber products, intake of bilberries was not allowed, and consumption of fatty fish was restricted to once a week. Other dietary and lifestyle habits were kept unchanged in all groups.

The participants kept a 4-d dietary record (consecutive days including 1 weekend day) during the run-in period (baseline) and at weeks 3, 7, and 11 of the interventions. The dietary data were analyzed by using the MicroNutrica software (Finnish Social Insurance Institute, Turku, Finland) based on the Finnish food composition tables (14).

Nontargeted LC-MS metabolite profiling analysis

Fasting EDTA plasma samples were collected for the LC-MS metabolite profiling analysis (106 samples at baseline and 106 samples at the end of the study). An aliquot of the sample, 100 μL, was mixed with 400 μL of acetonitrile (VWR International), mixed on a vortex at maximum speed 15 s, incubated on an ice bath for 15 min to precipitate the proteins, and centrifuged at 16,000 × *g* for 10 min to collect the supernatant. The supernatant was filtered through 0.2-μm polytetrafluoroethylene filters in a 96-well plate format. Aliquots of 2 μL were taken from at least half of the plasma samples, mixed together in 1 tube, and used as the quality control (QC) sample in the analysis; a solvent blank was prepared in the same manner.

The samples were analyzed by the liquid chromatography quadrupole time-of-flight mass spectrometry (LC-qTOF-MS) UHPLC-qTOF-MS system (Agilent Technologies), which consisted of a 1290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6540 UHD accurate-mass qTOF spectrometer. The samples were analyzed by using 2 different chromatographic techniques, i.e., reversed phase (RP) and hydrophilic interaction (HILIC) chromatography. The sample tray was kept at 4°C during the analysis. The data acquisition software was the MassHunter Acquisition B.04.00 (Agilent Technologies). The QC and the blank samples were injected after every 12 samples and at the beginning of the analysis. The order of the analysis of the samples was randomized.

In the RP technique, 4 μL of the sample solution was injected onto the column (Zorbax Eclipse XDB-C18, 2.1 × 100 mm, 1.8 μm; Agilent Technologies) and maintained at 50°C. The mobile phases, delivered at 0.4 mL/min, consisted of water (eluent A, Milli-Q purified; Millipore) and methanol (eluent B; Sigma-Aldrich), both containing 0.1% (vol:vol) of formic acid (Sigma-Aldrich). The following gradient profile was used: 2%→100% B (0–10 min), 100% B (10–15 min), 100%→2% B (15–15.1 min), 2% B (15.1–18 min).

In the HILIC technique, 3 μL of the sample solution was injected onto the column (Acquity UPLC BEH Amide column, 2.1 × 100 mm, 1.7 μm; Waters Corporation) and maintained at 45°C. The mobile phases, delivered at 0.6 mL/min, consisted of 50% acetonitrile (vol:vol; eluent A) and 90% acetonitrile (vol:vol; eluent B), respectively, both containing 20 mmol/L ammonium formate, pH 3 (Sigma-Aldrich). The following gradient profile was used: 0–2.5 min, 100% B; 2.5–10 min, 100% B→0% B; 10–10.1 min, 0% B→100% B; 10.1–14 min, 100% B.

The MS conditions after both chromatographic analyses were as follows: Jetstream ESI source, operated in positive and negative ionization mode, conditions were a drying gas temperature of 325°C and flow of 10 L/min, a sheath gas temperature of 350°C and flow of 11 L/min, a nebulizer pressure of 45 pounds per square inch, capillary voltage of 3500 V, nozzle voltage of 1000 V, fragmentor voltage of 100 V, and a skimmer of 45 V. For data acquisition, a 2-GHz extended dynamic range mode was used, and the instrument was set to acquire over the *m/z* 50–1600. Data were collected in the centroid mode at the acquisition rate of 2.5 spectra/s (i.e., 400 ms/spectrum) with an abundance threshold of 150. For the automatic data-dependent MS/MS analyses performed on the QC samples, the 4 most abundant ions were selected for fragmentation from every precursor scan cycle. These ions were excluded after 2 product ion spectra and released again for fragmentation after a 0.25-min hold. The precursor scan time was based on ion intensity, ending at 20,000 counts or after 300 ms. The product ion scan time was 300 ms. The collision energies were 10, 20, and 40 V in subsequent assays. The continuous mass axis calibration was performed by monitoring 2 reference ions from an infusion solution throughout the assays. The reference ions were *m/z* 121.050873 and *m/z* 922.009798 in positive mode and *m/z* 112.985587 and *m/z* 966.000725 in negative mode.

Data analysis

Collection and statistical analysis of the LC-MS data. The data were collected by using the vendor's software (MassHunter Qualitative Analysis B.05.00; Agilent Technologies), and the output was transferred in compound exchange format (cef.files) into the Mass Profiler

[9] Abbreviations used: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; ESI, electrospray ionization; FDR, false discovery rate; HD, healthy diet; HILIC, hydrophilic interaction; HA, hippuric acid; LC-qTOF-MS, liquid chromatography quadrupole time-of-flight mass spectrometry; P_{corr}, corrected P value; PLS-DA, partial least squares discriminant analysis; QC, quality control; RP, reversed phase; rt, retention time; VIP, variable influence on projection; WGED, whole-grain–enriched diet.

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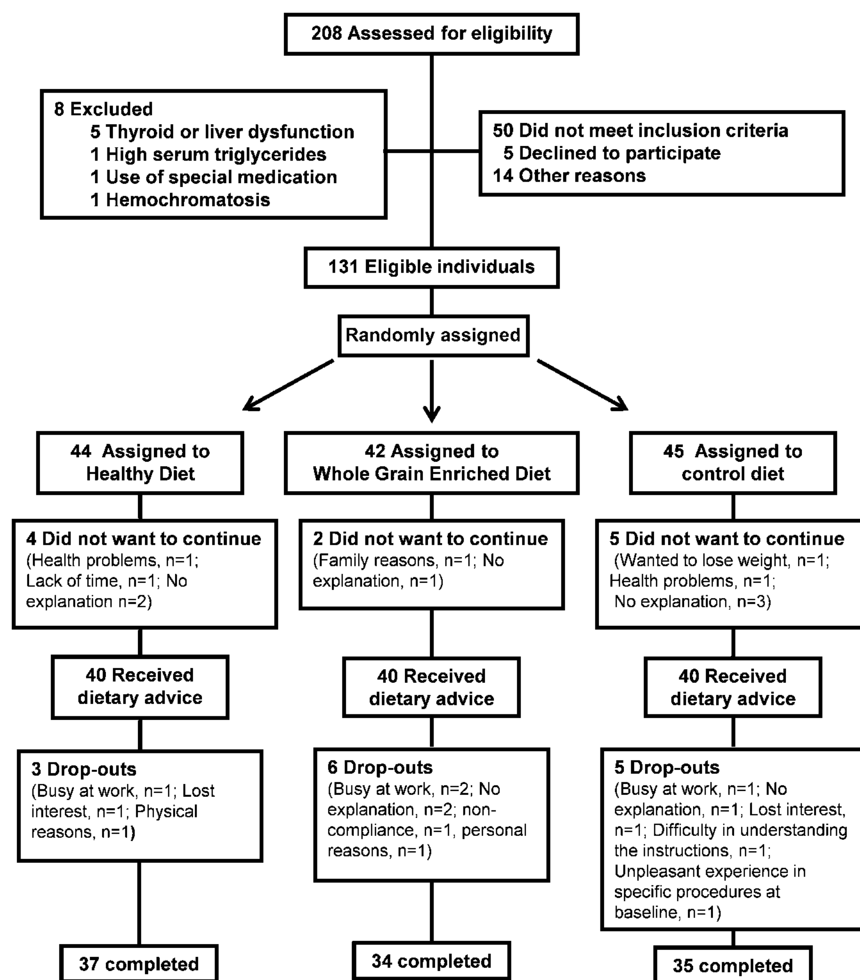

FIGURE 1 Sysdimet-HealthGrain intervention study design. The intervention groups were matched for sex and medians of BMI, age, and fasting plasma glucose concentration. A total of 106 participants with features of metabolic syndrome completed the study.

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Professional software (MPP 2.2; Agilent Technologies) for compound alignment and data preprocessing. Further data processing and selection for the most discriminating compounds between diets were performed in Excel (Microsoft 2007). To remove insignificant features, only the features found in at least 80% of the samples at either or both the beginning and/or the end of the HD or WGED interventions were included in the analysis. This resulted in a data set comprising a total of 3130 features (1415 in RP ESI−, 362 in RP ESI+, 632 in HILIC ESI+, and 721 in HILIC ESI−).

The difference in the peak area value between the beginning and end of the intervention for each participant was determined for each feature in all the 3 diet groups. Student's *t* test was used to compare the HD and WGED groups with the control group to find the discriminative features affected by either or both of the test diets when compared with the control. The Benjamini-Hochberg false discovery rate (FDR) was used to adjust the results for multiple comparisons (15) in each of the 4 analytical approaches separately, taking into account all of the features included in each of the 4 preprocessed data sets, resulting in a total of 90 features with a corrected P value (P_{corr}) < 0.05 (the list of the differential features is shown in **Supplemental Table 2**).

In addition, the preprocessed data sets from each of the 4 analytical approaches were subjected to supervised clustering algorithm partial least-squares discriminant analysis (PLS-DA; Simca-13; Umetrics) including only the samples collected at the end of the interventions. The data were $\log_{10}$-transformed, pareto-scaled, and the model was validated by the Simca-13 internal cross-validation. Further visualization of the data were performed on the RP ESI(−) data matrix containing the features exhibiting P_{corr} < 0.05 (74 metabolic features) by the K-means cluster analysis with the Pearson correlation as the distance metric, followed by hierarchical clustering within each of the K-means clusters (16).

Identification of the differential features in the LC-MS data. Identification of the metabolites was based on the MS/MS spectral comparison of the pure standard compounds and on the search of the candidate compounds in the published databases, including the Human Metabolome database, Metlin, ChemSpider, and SciFinder, and the results were verified with the MS/MS spectral features included in the databases or reported in earlier publications. Lipids were identified on the basis of the MS/MS fragmentation when the metabolite was intensive enough to be detected in the automatic data-dependent MS/MS analysis. The fragmentation pattern for the phosphocholines and phosphoetanolamines followed what has been published previously (17–19). The key elements for identification were the protonated head group (*m/z* 184.07 for phosphocholines and *m/z* 196.03 for phosphoetanolamines) as well as the deprotonated PUFA fragments visible in the negative ionization mode (the MS/MS fragmentation data for all of the identified metabolites are shown in **Supplemental Table 3**). The plasmalogen P-16:0/20:4 was identified based on the head group indicating phosphocholines as well as the *m/z* 303 corresponding to arachidonic acid (20:4), with the links in Metlin solely for plasmalogens for the mass 765.579. Glucuronidated alk(en)ylresorcinols were identified based on the MS/MS fragmentation and compared with the published data as described in the **Supplemental Figure 2**.

Clinical characteristics and dietary intake. Linear mixed-effects models were used to analyze the group differences in the clinical characteristics and dietary intake at baseline and during the intervention as reported earlier (6). The analyses were performed by using the R Project for Statistical Computing version 2.7.2 and nlme R-package version 3.1-96 (20). The interaction term between the group and intervention time point (before or after the intervention) was used to examine the group-related changes during the intervention. The control group was used as a reference group when comparing the group differences. The Benjamini-Hochberg FDR was used to adjust the results for multiple comparisons (15).

TABLE 1 Metabolites putatively identified in LC–MS analysis including compounds with significant fold changes ($P < 0.05$) in the healthy diet or the whole-grain–enriched diet groups compared with the control group of participants with features of metabolic syndrome[1]

						Healthy diet		Whole-grain–enriched diet	
Column	Mode	MW	*m/z*	rt, min	Putative annotation	P^2	Fold change[3]	P^2	Fold change[3]
HILIC	ESI−	246.019	245.011	0.46	Sulfated phenol derivative	0.015	1.36	0.119	1.24
HILIC	ESI−	220.004	218.996	0.47	Sulfated phenol derivative	0.003	1.80	0.079	1.38
HILIC	ESI−	104.047	103.039	1.04	Hydroxyisobutyric acid	0.009	1.12	0.43	2.16
RPLC	ESI+	181.074	182.082	1.11	L-Tyrosine	0.032	1.20	0.009	1.20
HILIC	ESI+	113.059	114.067	1.25	Creatinine	0.59	0.94	0.007	1.23
HILIC	ESI+	136.038	137.046	1.41	Hypoxanthine	0.002	1.16	0.011	1.28
HILIC	ESI+	312.147	313.155	1.76	Phenylalanine-phenylalanine	0.79	1.63	0.044	1.71
RPLC[4]	ESI−	189.993	188.985	1.91	Pyrocatechol sulfate	5.6×10^{-9}*	1.96	0.13	1.12
HILIC	ESI+	217.131	218.139	2.05	Propionylcarnitine	0.63	0.95	0.043	1.21
RPLC[4]	ESI−	213.009	212.002	2.39	Indoxylsulfuric acid	0.044	1.01	0.29	1.13
HILIC	ESI+	157.110	158.118	2.95	Pipecolic acid betaine	0.035	1.32	0.008	1.90
HILIC	ESI+	145.110	146.117	3.10	γ-Butyrobetaine	0.43	0.98	0.012	1.12
RPLC[4]	ESI+	179.058	180.067	3.14	Hippuric acid	9.89×10^{-6}*	3.31	0.64	1.19
HILIC	ESI+	129.079	130.087	3.66	Pipecolic acid	0.038	2.08	0.07	2.09
HILIC	ESI+	143.094	144.102	3.95	Unknown betaine (proline betaine analogue)	0.45	0.99	0.012	1.32
HILIC	ESI−	216.040	215.032	4.94	Hexose (chloride adduct)	0.86	1.11	0.022	1.27
HILIC	ESI+	117.054	118.061	5.53	Unknown betaine (glycine-betaine analogue)	0.09	1.04	0.012	1.08
HILIC	ESI+	187.168	188.176	6.03	N-acetyl spermidine	0.14	0.93	0.020	1.20
HILIC	ESI+	169.085	170.093	6.18	3-Methyl-L-histidine	0.43	0.98	0.040	1.13
RPLC	ESI+	362.210	363.217	6.61	Cortisol	0.36	1.04	0.030	1.14
HILIC	ESI+	174.112	175.118	6.79	L-Arginine	0.45	0.97	0.010	1.19
HILIC	ESI+	146.105	147.112	6.94	L-lysine	0.24	0.97	0.010	1.63
HILIC	ESI+	132.089	133.097	6.99	L-Ornithine	0.13	0.96	0.004	1.24
RPLC[4]	ESI−	240.100	239.092	7.45	CMPF	5.4×10^{-9}*	2.56	0.002	1.13
RPLC[4]	ESI−	268.131	267.123	8.52	3-Carboxy-4-methyl-5-pentyl-2-furanpropionic acid	0.00157*	1.22	0.12	1.33
RPLC	ESI+	541.317	542.325	9.57	LPC (20:5) minor isomer	1.52×10^{-6}*	1.73	0.23	1.13
RPLC	ESI+	541.317	542.325	9.69	LPC (20:5)	1.42×10^{-6}*	1.74	0.21	1.12
RPLC	ESI−	499.270	498.260	9.69	LPE (20:5)	0.00003*	1.57	0.29	1.20
RPLC	ESI−	525.285	524.208	9.84	LPE (22:6)	0.001*	1.13	0.43	1.03
RPLC	ESI+	567.333	568.341	9.84	LPC (22:6)	0.023	2.10	0.36	1.44
RPLC	ESI−	302.224	301.217	10.30	EPA	5.34×10^{-5}*	1.46	0.38	1.08
RPLC	ESI−	328.240	327.240	10.48	DHA	0.0001*	1.30	0.47	1.05
RPLC	ESI−	552.366	551.358	10.84	Nonadecyl-benzenediol glucuronide (AR 19:0-Gln)	4.61×10^{-6}*	1.20	8.6×10^{-7}*	1.46
RPLC	ESI−	578.382	577.373	10.89	Heneicosenyl-benzenediol-glucuronide (AenR 21:1-Gln)	3.68×10^{-9}*	1.18	4.19×10^{-10}*	1.38
RPLC	ESI+	779.560	780.568	11.84	PC (16:0/20:5)	0.0009	1.45	0.23	1.11
RPLC	ESI+	805.576	806.584	12.03	PC (16:0/22:6)	0.024	2.35	0.71	0.94
RPLC	ESI+	731.558	732.566	12.04	PC (16:0/16:1)	0.46	2.04	0.024	1.43
RPLC	ESI+	765.579	766.587	12.14	PC (P-16:0/20:4)	0.052	1.60	0.11	1.34
RPLC	ESI+	807.592	808.600	12.34	PC (20:5/18:0)	0.002	1.41	0.08	1.05

[1] Parameters for the LC-MS analysis include chromatography (Column), ionization mode in MS (Mode), MW, identified ion (*m/z*), and rt. n = 106 study participants with features of metabolic syndrome. *False discovery rate-corrected $P < 0.05$. AenR, alkenylresorcinol; AR, alkylresorcinol; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; ESI, electrospray ionization; Gln, glucuronide; HILIC, hydrophilic interaction; LPC, lysophosphatidylcholine; LPE, lysophosphatidyl-ethanolamine; MW, molecular weight; PC, phosphatidylcholine; RPLC, reversed-phase liquid chromatography; rt, retention time.

[2] Student's t test comparing the fold change during intervention with the control group.

[3] Average intraindividual fold change during the intervention.

[4] Metabolite was detected in both chromatographic analyses with $P < 0.05$.

Correlations between biomarker candidates and recorded dietary intake. Spearman rank correlation coefficients were calculated for the relations between the change in the daily intake of whole-grain bread, berries, and fish and the change in the peak abundance of the selected biomarker candidates resulting from the statistical analysis. All subjects were included in the correlation analyses (n = 106). Furthermore, ANCOVA was used to study associations between the selected biomarkers and food intake with the use of the intervention group as a covariate. The stepwise linear regression model was applied to test the individual and combined associations of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid (CMPF), EPA, and DHA (independent variables) with the consumption of fish (dependent variable). The level of statistical significance was set at $P < 0.05$. The correlations were analyzed by using the Statistical Package for the Social Sciences (SPSS) for Windows (19.0 version; IBM).

Results

Clinical characteristics and dietary intake. Clinical characteristics and dietary intakes were reported in detail earlier (6, 7). There were no statistically significant changes in the measured characteristics during the study (Supplemental Table 1). During the intervention, the HD and WGED groups consumed 7.7 portions (182 g) and 7.9 portions (187 g)/d of the whole-grain

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breads and low-insulin-response rye breads, respectively; the control group consumed 6.8 portions (197 g)/d of white-wheat breads, resulting in significantly higher fiber intake in the HD and WGED groups compared with the control group (Supplemental Table 1). In the HD group, mean fish consumption was 3.3 fish meals (100–150 g fish/meal)/wk, resulting in increased intake of EPA and DHA when compared with the control group (Supplemental Table 1). The intake of bilberries in the HD group was 3.2 portions/d (Supplemental Figure 1).

Identification of the biomarker candidates changed significantly during the intervention(s) when compared with the control. Nontargeted metabolite profiling showed metabolic changes in the fasting plasma of the participants in the HD and the WGED groups when compared with the control group. Of the 3130 molecular features collected in the 4 different LC-MS modes, a total of 400 were found to have significant changes after the intervention with either one or both of the intervention diets when compared with the fold-change values obtained from the control group (Student's *t* test, $P < 0.05$). After correcting for the multiple measurements within each of the 4 analytical approaches, the number of metabolic features with a significant change during the intervention when compared with the control group was 90 ($P_{corr} < 0.05$; Supplemental Table 2). The metabolites amenable for identification based on the automatic data-dependent MS/MS analysis are listed in **Table 1**, and the MS/MS spectral fragmentation data can be found in Supplemental Table 3. The metabolites with $P_{corr} < 0.05$, herein discussed as statistically significant features, changed significantly during the interventions when compared with the control, whereas those having $P < 0.05$ but that were not statistically significant after the FDR correction are termed as metabolites having a trend.

A particularly clear change in the fasting plasma lipid profile after the HD intervention was the accumulation of CMPF (2.56-fold; $P_{corr} = 1.98 \times 10^{-6}$). It was identified based on fragmentation match in Metlin and verified with the pure standard compound (**Figure 2**). Interestingly, in the near vicinity of the CMPF metabolite, there was another compound with *m/z* 267.1234 exhibiting the very same fragmentation pattern and also regarded as a significant marker in the RP ESI(−) analysis (1.22-fold; $P_{corr} = 0.03$). The fragmentation and the elemental composition suggested a compound with an additional C_2H_4 in the molecular structure, which would refer to a two-carbon units longer side chain in the position 1 in the furan backbone of the metabolite. SciFinder browsing suggested such a compound, namely 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid, reported earlier in uremic serum (21) as well in plant material (22).

Another prominent change in the fasting plasma in the HD group was an increase in hippuric acid (HA) (3.3-fold; $P_{corr} = 0.0006$) (23). Other phenolic metabolites, such as pyrocatechol sulfate, were also increased in the HD group (2.0-fold; $P_{corr} = 1.98 \times 10^{-6}$). The other metabolites affected by the HD intervention were phosphatidylcholines, lysophosphatidylcholines, and lysophosphatidyl-ethanolamines with long-chain PUFAs. The HD intervention clearly increased, e.g., lysophosphatidylcholine (20:5) with 2 isomers (both 1.7-fold; $P_{corr} = 1.00 \times 10^{-4}$), lysophosphatidyl-ethanolamine (20:5; 1.6-fold; $P_{corr} = 1.60 \times 10^{-3}$) and lysophosphatidyl- ethanolamine (20:6; 1.1-fold; $P_{corr} = 0.024$). As expected, EPA (20:5) and DHA (22:6) FAs also increased in the HD group [1.5-fold ($P_{corr} = 2.50 \times 10^{-3}$) and 1.3-fold ($P_{corr} = 3.90 \times 10^{-3}$), respectively].

Whereas multiple metabolic markers were found in the HD group, in the WGED group there were only 2 metabolites with significant *P* values after FDR correction. These also significantly changed during the HD intervention when compared with

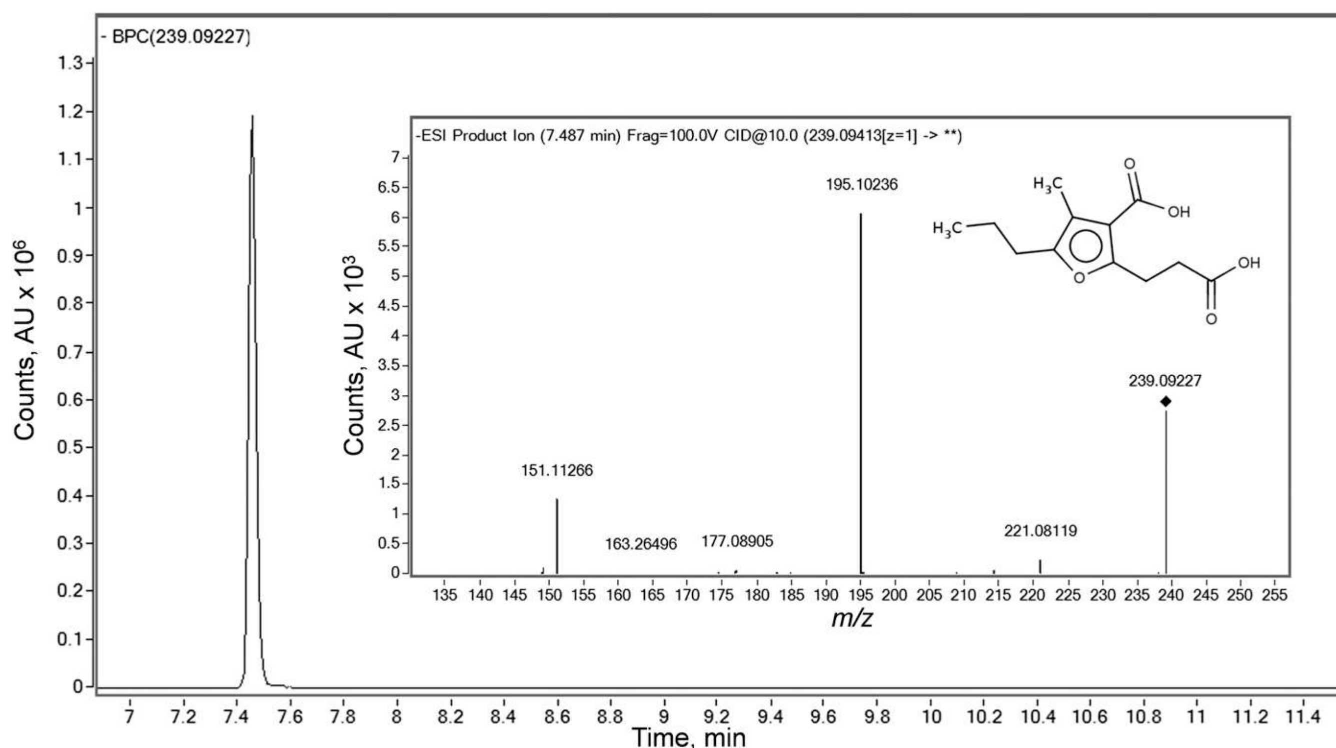

FIGURE 2 Base peak chromatogram for the ion *m/z* 239.092 identified as CMPF with fragmentation spectra obtained in tandem MS with negative ESI. AU, arbitrary units; BPC, base peak chromatogram; CID, collision-induced dissociation; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; ESI, electrospray ionization; Frag, fragmentor voltage.

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the fold change after the control diet. The signals were 2 closely eluting peaks with *m/z* values of 551.358 (1.2-fold; $P_{corr} = 4.00 \times 10^{-4}$) and 577.373 (1.2-fold; $P_{corr} = 1.98 \times 10^{-6}$) (Table 1). Under MS/MS fragmentation, both metabolites showed a neutral loss of glucuronide, and the aglycone masses matched with alkylresorcinol 5-nonadecyl-1,3-benzenediol for the compound with *m/z* 551.358 and alkenylresorcinol 5-(16-heneicosenyl)-1,3-benzenediol for the aglycone of the compound with *m/z* 577.373. The MS/MS spectra of these candidate metabolites showed identical fragmentation with the published spectra (24, 25), and although the exact position of the double bond in the molecule was not determined, the compounds were identified as nonadecyl-benzenediol glucuronide (alkylresorcinol 19:0-Gln) and heneicosenyl-benzenediol-glucuronide (alkenylresorcinol 21:1-Gln) (Table 1, Supplemental Figure 2).

In addition to the glucuronidated alk(en)ylresorcinols, there was a trend toward altered metabolism of various betaine compounds in the WGED group (Table 1). The retention between 2 and 3 min in the HILIC ESI(+) had numerous signals exhibiting betaine-like fragments in the MS/MS analysis, out of which only a few could be identified based on known occurrence of betaines in human biofluids (23, 26, 27). Among the identified betaine metabolites was pipecolic acid betaine, which showed an increased trend in both the HD and WGED groups [1.3-fold ($P = 0.035$) and 1.9-fold ($P = 0.008$), respectively]. The peaks of 2 main betaine compounds, glycine-betaine and proline-betaine, had a very large interindividual variation and did not differ between the control and HD or WGED groups. Interestingly, 2 metabolites eluting shortly after both glycine betaine and proline betaine peaks with exactly the same mass and similar fragmentation pattern, namely *m/z* 144.103, retention time (rt) 3.95, and *m/z* 118.062, rt 5.53 (Table 1), showed a differential trend in the WGED group when compared with the control. Among the betaine metabolite family, propionylcarnitine and γ-butyrobetaine were also increased in the WGED group when compared with the control group [1.2-fold ($P = 0.043$) and 1.1-fold ($P = 0.012$), respectively]. Furthermore, there were also increases in certain amino acids such as L-lysine, ornithine, and L-arginine in the WGED group when compared with the control group [1.6-fold ($P = 9.40 \times 10^{-3}$) 1.2-fold ($P = 4.30 \times 10^{-3}$), and 1.2-fold ($P = 9.70 \times 10^{-3}$), respectively].

Classification of the LC-MS data by chemometric analyses. The endpoint samples from all of the intervention participants were subjected to the chemometric algorithm PLS-DA, as exemplified with the data from the RP ESI(−) mode (**Figure 3**). The clustering of the samples in the PLS-DA analysis was in line with the statistical analyses, because the samples collected at the end of the HD intervention differed from the other diets (Figure 3A). Furthermore, as visualized by the loadings plot, the most

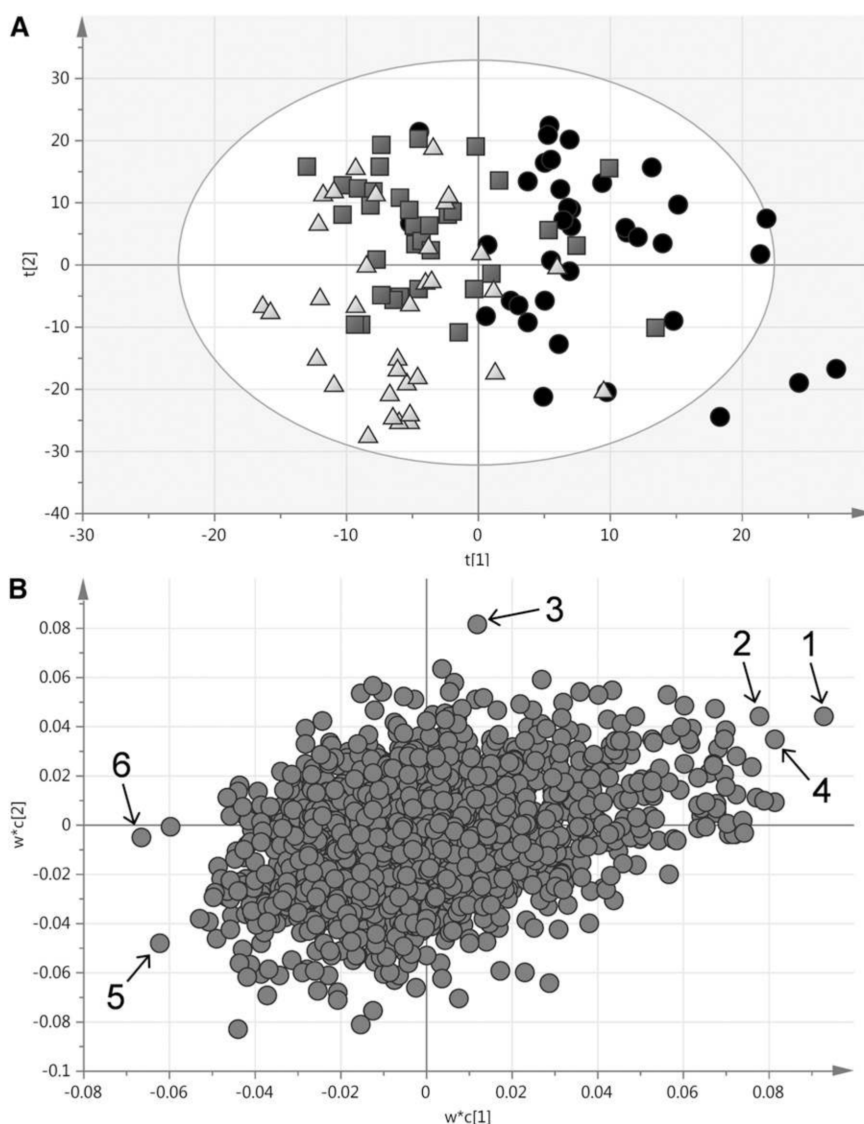

FIGURE 3 Partial least squares discriminant analysis of the reverse phased negative mode data. (A) Score plot showing the individual endpoint samples. Healthy diet (black circles). (B) Whole-grain–enriched diet (gray squares). (C) control diet (light gray triangles). (B) Loading scatterplot showing the individual compounds. The depicted metabolites are as follows: pyrocatechol sulfate, VIP 2.56 (1); CMPF, VIP 1.93 (2); AenR C21:1-Gln, VIP 1.79 (3); hippuric acid, VIP 2.46 (4); unknown, MW 188.094, rt 3.3, VIP 1.81 (5); unknown lipid, MW 869.539, rt 11.8, VIP 1.76 (6). AenR, alkenylresorcinol; CMPF, 3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid; MW, molecular weight; rt, retention time; VIP, variable influence on projection.

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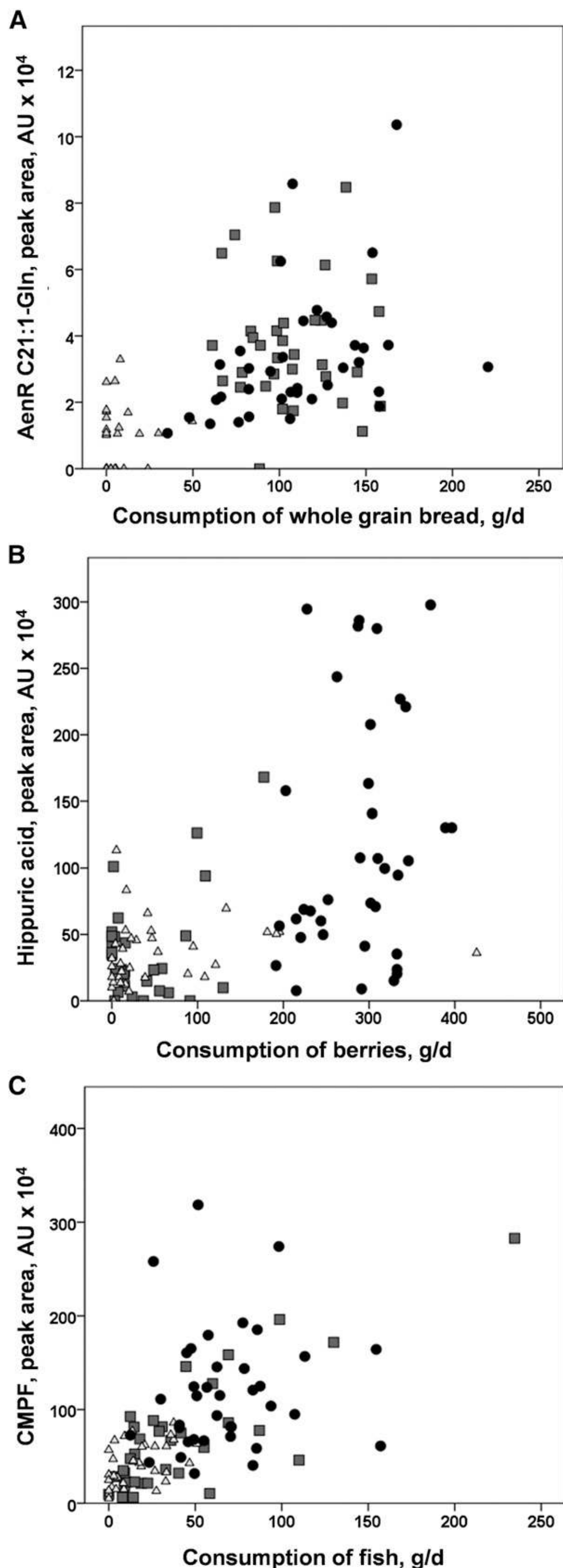

FIGURE 4 Plot charts showing the peak areas of the putative biomarkers as a function of reported consumption of selected foods in the healthy diet (black circles), the whole-grain–enriched diet (dark gray squares), and the control diet (light gray triangles) groups. (A) AenR C21:1-Gln and the consumption of whole-grain bread (containing at least 50% whole grain); (B) hippuric acid and the consumption of berries (includes whole berries, berry purées, and berry juices). Three outliers in the whole-grain–enriched diet group were excluded because their berry consumpion included mainly juices with low polyphenol content and was >2 SDs from the mean consumption of berries. (C) CMPF and the consumption of fish. AenR, aleknylresorcinol; AU, arbitrary units; CMPF, 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid; Gln, glucuronide.

discriminating metabolites for the study groups in the multivariate PLS-DA model were the same compounds that were also identified as putative biomarkers by the univariate statistical analysis (Figure 3B). The variable influence on projection (VIP) values for, e.g., HA, pyrocatechol sulfate, and CMPF were 2.46, 2.56, and 1.94, respectively, indicating that these compounds are among the most powerful discriminators between the HD group and the 2 other groups. In addition, from the loadings plot, several unknown lipid metabolites were spotted based on the mass and rt, e.g., molecular weight 869.539, rt 11.8, VIP 1.76, which contributed to the clustering of the control group, as well as a smaller metabolite of unknown identity with molecular weight 188.094, rt 3.3, VIP 1.81. These metabolites are most likely endogenous compounds with higher concentrations in the control group than in the test groups.

Seventy-four features in the RP ESI(−) had P_{corr} < 0.05 and were subjected to the K-means cluster algorithm followed by the hierarchical cluster analysis with the heat map output (**Supplemental Figure 3**). When the fold-change values for the significant features were included in the analysis, 7 clusters were formed on the basis of the similar response profile throughout the study participants. Cluster 1 contained 2 metabolites, HA and pyrocatechol sulfate, and cluster 2 held the 2 alk(en)ylresorcinol glucuronides. In cluster 3, a group of unknown lipids with a retention time >12 min were increased in the HD group but also tended to be increased in the WGED group. The fourth group clustered mainly the EPA and DHA FAs with several fragment ions, as well as the CMPF metabolite, which also was represented by several fragment ions. Cluster 5 contained various lipid metabolites that were increased in particular after the HD intervention, including the identified lipids lysophosphatidyl-ethanolamine (20:5), lysophosphatidyl-ethanolamine (22:6), and 2 isomers of lysophosphatidylcholine (20:5). Cluster 6 had unknown lipid metabolites that were increased in the HD group and were completely undetected in the majority of the participants in the WGED and control groups. Cluster 7 was formed from unknown metabolites, mostly lipids, that seemingly decreased after the HD intervention.

Association between the changes in plasma biomarkers and consumption of the selected food items. After identifying the strongest putative biomarker metabolites for the intervention groups, we wanted to determine which foods specifically were associated with increases in these metabolites by conducting correlation analyses with the food intake data collected during the interventions. CMPF was among the most clearly changed metabolites in the HD intervention, and we found very strong positive associations between plasma CMPF and recorded fish intake (**Table 2**). The association was stronger with the total intake of fish than with the intake of fatty fish only. However, there was no significant association between plasma CMPF and the intake of lean fish, possibly because the consumption of lean fish was limited due to the design of the original study.

Cross-sectionally, plasma CMPF was also associated with the intake of berries at baseline (r = 0.29, P = 0.003) and at the end of the intervention (r = 0.36, P = 0.001) (**Table 3**). However, there was no association between the changes in the intake of berries and the changes in plasma CMPF when adjusted for the

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TABLE 2 Associations between plasma CMPF peak areas detected in LC–MS analysis and food intake calculated based on 4-d food records of participants with features of metabolic syndrome (n = 104–105)[1]

	CMPF (peak area)					
	Baseline[2]		End[3]		Change[3]	
Dietary intake, g/d	r	P	r	P	r	P
All fish	0.25	0.009	0.47	6×10^{-7}	0.34	4×10^{-5}
Fatty fish	0.23	0.19	0.45	2×10^{-5}	0.28	0.001
Lean fish[4]	0.10	0.32	0.16	0.07	0.11	0.12
Whole-grain products	0.15	0.12	0.10	0.51	−0.20	0.047
Other grain products	−0.06	0.54	−0.18	0.20	0.17	0.09
Vegetables and root vegetables	0.14	0.17	0.07	0.44	−0.04	0.60
Fruit[5]	0.06	0.55	0.08	0.35	−0.21	0.02
Berries[5]	0.29	0.003	0.36	0.001	0.06	0.51
Milk products	−0.02	0.84	−0.08	0.36	0.08	0.34
Red meat	−0.05	0.63	0.09	0.31	−0.11	0.20
Poultry	0.10	0.31	−0.12	0.16	0.04	0.47
Potatoes	−0.05	0.60	−0.08	0.35	0.02	0.85
Nuts	−0.07	0.48	−0.06	0.45	0.06	0.46
Eggs	−0.04	0.70	−0.01	0.99	0.05	0.58
Vegetable oils and spreads	0.02	0.85	−0.06	0.52	−0.01	0.93
Dairy fat–based spread	−0.06	0.57	−0.06	0.52	0.06	0.49

[1] CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.
[2] Spearman rank correlation.
[3] ANCOVA, intervention group included into the model.
[4] Consumption was low.
[5] Includes whole fruit/berries, purée, and juice.

effect of intervention group (Table 2). Plasma CMPF was not positively associated with the intakes of grain products, fruit, vegetables, milk products, meat, potatoes, nuts, eggs, or oils and spreads (Table 2). The intakes of berries and fish correlated especially at the end of the interventions (r = 0.52, P = 8.2 × 10^{-9} vs. baseline r = 0.10, P = 0.328), because the participants in the HD group were advised to increase their intakes of both fish and berries during the intervention. To account for this, the effect of the intervention group was included into the model when analyzing associations between dietary intake and plasma CMPF cross-sectionally at the end of the study, and when comparing the respective changes during the intervention period. Furthermore, we applied a linear regression model in which both intakes of berries and fish were included. In this analysis, changes in fish intake had an independent effect on changes in plasma CMPF when the intake of berries was included in the model (r = 0.47, P = 7 × 10^{-8}), but berries also seemed to have an independent effect on plasma CMPF (r = 0.29, P = 0.001). However, in this model, the effect of fish intake on plasma CMPF concentration was 5-fold that of the effect of berries.

We also tested the associations of plasma CMPF with the calculated dietary intakes of EPA and DHA and with plasma EPA and DHA peak areas, the established biomarkers of fish intake. We found a strong positive correlation between the plasma CMPF and EPA and DHA at baseline, at the end of the study, and between the respective changes during the interventions (Table 3). Using the stepwise linear regression model, we observed that plasma CMPF is an even stronger independent predictor of fish consumption than plasma EPA (for CMPF: r = 0.40, P = 7 × 10^{-5}; for EPA: r = 0.21, P = 0.031). DHA did not predict fish consumption significantly. The adjusted R^2 for the model that included only plasma CMPF was 0.26 and for the model including both CMPF and EPA was 0.29, indicating that EPA improves the prediction of fish consumption by only 3%.

Associations between the consumption of specific food items were also evaluated for several other biomarker candidates, including HA and alk(en)ylresorcinol glucuronides (**Table 4**). The associations between the respective biomarkers at the end of the intervention period are illustrated by plotting the peak area as a function of the reported consumption of the specific food (**Figure 4**). A significant correlation was observed between the change in the peak abundance of plasma HA and the change in the daily intake of bilberries but not with the change in the intake of whole grains (Table 4). The alk(en)ylresorcinol glucuronides, which increased both in the HD and WGED groups, were strongly positively associated with the intake of whole-grain bread, and also with the intake of berries (Table 4). The correlation with berry consumption was, however, a consequence of the concurrent consumption of whole-grain bread and berries in the HD group, because after inclusion of the intervention group in the model, there was no longer an association between alk(en)ylresorcinol glucuronides and the intake of berries (r = 0.07, P = 0.510). In addition, the changes in betaines correlated positively with the changes in the intake of whole-grain bread (Table 4). Of all of the betaines, pipecolic acid betaine had the strongest correlation with the intake of whole-grain bread (r = 0.34, P = 4 × 10^{-4}).

TABLE 3 Associations between plasma CMPF peak areas detected in LC–MS analysis and markers of fish intake of participants with features of metabolic syndrome (n = 104–106)[1]

	CMPF					
	Baseline[2]		End[3]		Change[3]	
	r	P	r	P	r	P
Calculated dietary EPA intake,[4] mg/d	0.27	0.005	0.42	3×10^{-5}	0.19	0.06
Calculated dietary DHA intake,[4] mg/d	0.25	0.009	0.39	1×10^{-4}	0.31	2×10^{-4}
Plasma EPA[5]	0.68	6×10^{-16}	0.38	1×10^{-5}	0.33	1×10^{-4}
Plasma DHA[5]	0.67	8×10^{-15}	0.36	2×10^{-5}	0.23	0.006

[1] CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid.
[2] Spearman rank correlation.
[3] ANCOVA, intervention group included into the model.
[4] Calculated from food records.
[5] Peak area in LC-MS analysis.

Discussion

Nontargeted LC-MS metabolite profiling showed the concomitant changes in fasting plasma metabolite profile after 2 partially overlapping dietary modifications: a WGED and a diet rich in bilberries and fatty fish in addition to whole grains (HD). After strict statistical filtering, 90 molecular features (of 3130 detected) were found to have significant changes during either one or both of the interventions when compared with the control diet. In the HD intervention, the most important findings included the impact of fatty fish, in particular on plasma concentrations of furan FAs (CMPF) and on various PUFA-containing lipids, as well as the remarkable increase in fasting plasma HA, most likely reflecting increased consumption of bilberries. In addition, 2 novel biomarkers of whole-grain consumption, glucuronidated alk(en)ylresorcinols, were found in the interventions with whole-grain products, particularly rye. Furthermore, we identified CMPF as a potential biomarker for the intake of fatty fish. Betaine and amino acid metabolism also tended to be altered in both intervention groups.

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TABLE 4 Correlation between changes in metabolites detected in LC–MS analysis and changes in food intake calculated based on a 4-d food records of participants with features of metabolic syndrome (n = 103–105)[1]

Metabolites (peak area)	MW	rt, min	Intake of whole-grain bread[2,3]		Intake of berries,[3] g/d		Intake of fish,[3] g/d	
			r	P	r	P	r	P
Hippuric acid	179.058	3.14 RP(+)	0.03	0.80	0.38	7×10^{-5}	0.12	0.23
AenR 21:1-Gln	578.382	10.90 RP(−)	0.63	1×10^{-12}	0.22	0.02	0.12	0.23
AR 19:0-Gln	552.366	10.85 RP(−)	0.47	4×10^{-7}	0.17	0.08	0.09	0.35
Pipecolic acid betaine	157.110	2.95 HILIC(+)	0.34	4×10^{-4}	0.14	0.16	−0.01	0.94
γ-Butyrobetaine	145.111	3.10 HILIC(+)	0.21	0.035	0.03	0.74	0.09	0.38
Unknown betaine	143.095	3.95 HILIC(+)	0.28	0.005	−0.06	0.55	−0.01	0.90

[1] Spearman rank correlation was used. AenR, alkenylresorcinol; AR, alkylresorcinol; Gln, glucuronide; HILIC, hydrophilic interaction; MW, molecular weight; RP, reversed phase; rt, retention time.
[2] At least 50% whole grain.
[3] Changes in dietary intake are based on 4-d food records, which were kept at study weeks 0, 3, 7, and 11.

CMPF correlated strongly with the intake of fish but not with other foods. Plasma concentrations of CMPF were strongly affected by the HD diet, and the correlation analyses with the food intake data showed an association with the intake of fish but not with other foods. The association with intake of berries was most likely due to the concomitant increase in the consumption of fish and berries, because no correlations between the respective changes during the intervention were found. CMPF belongs to the furan FAs, and fish is their richest source in the human diet (28). However, small concentrations of furan FAs were also measured in green plants, mushrooms, algae, vegetable oils, and butter (22, 28), but these foods were not associated with fasting plasma CMPF in our study. The most likely origin for plasma CMPF is fish-borne furan FAs, although the metabolic conversions are not precisely described (29).

In our study, plasma CMPF was a stronger marker for fish intake than the established biomarkers EPA and DHA. Furthermore, a model including both plasma CMPF and EPA was not able to predict the intake of fish better than plasma CMPF alone. Analytically, plasma CMPF is a good candidate for a biomarker molecule to be quantified by a targeted measurement. It elutes in the C18 RP LC column before FAs and phospholipids, exhibiting excellent retention as evidenced by the sharp peak shape. Moreover, its ionization efficiency is free from the ion suppression risk associated with LC-MS analysis of the metabolites coeluting with phospholipids. These facts support the potential application of plasma CMPF as an alternative for EPA and DHA. Indeed, several published targeted LC-MS protocols are available for measurement of plasma CMPF in renal failure (30, 31), and they could be easily adjusted for utilization in a straightforward biomarker analysis for nutritional assessment purposes.

Overall, the impact of the HD diet on the endogenous lipid profile was very extensive, as demonstrated here with the statistical and chemometric analyses showing lipid species that were decreased in the HD group. Several findings on lipids also confirmed our earlier results of lipidomics analysis on the same sample set (6) [i.e., increases in EPA, DHA, and lysophosphatidylcholine (22:6 and 20:5)], but we discovered some additional lipid species with increases in response to the HD diet, such as lysophosphatidyl-ethanolamine (22:6 and 20:5).

Glucuronidated alk(en)ylresorcinols were the most prominent whole-grain–specific biomarkers. Glucuronidated alk(en)ylresorcinols were identified as significant biomarkers after both of the intervention diets, and alkenylresorcinol 21:1-Gln was among the most important variables contributing to the PLS-DA model clustering of the endpoint samples of the WGED intervention. In addition, we found a strong positive correlation between change in plasma concentrations of glucuronidated alk(en)ylresorcinol and change in the consumption of whole-grain bread.

The chromatographic conditions of our nontargeted analysis were most likely not suitable for analyzing the intact alkylresorcinols, but the glucuronidated forms, reported here for the first time in human plasma, are more water-soluble and thus detectable in our analysis. Alkylresorcinols are phenolic lipids with a 3,5-dihydroxy-5-alkylbenzene configuration found predominantly in the bran of rye and wheat, and they are well-established biomarkers of intake of whole-grain rye and wheat products (32). They can be detected by GC-MS from urine and plasma after whole-grain intake (33). The detection of glucuronidated alk(en)ylresorcinols in fasting plasma directly after protein precipitation in the standard RP-MS conditions could offer a straightforward alternative to the currently applied analytical methods.

Interestingly, one of the glucuronidated alk(en)ylresorcinol markers has the 21:1 alkenyl side chain, thus exhibiting 1 double bond in the carbon tail. Both alkenylresorcinols and alkylresorcinols have been reported in rye and wheat (24, 25), but thus far only alkylresorcinols have been discussed as dietary biomarkers.

The end products of endogenous metabolism of alkylresorcinols include 3,5-dihydroxybenzoic acid (3,5-DHBA), 3-(3,5-dihydroxyphenyl) propanoic acid (3,5-DHPPA) (34, 35), 5-(3,5-dihydroxyphenyl)pentanoic acid (3,5-DHPPTA), and 2-(3,5-dihydroxybenzamido)acetic acid (3,5-DHBA glycine) (36), but none of these were included as differential signals in our analysis. The estimated half-lives for alkylresorcinols in plasma range between 5 and 8 h (37). Therefore, it is possible that the glucuronidated forms of the alk(en)ylresorcinols found in fasting plasma display different bioavailability, metabolism, and kinetics in humans than the metabolites reported earlier.

Healthy diet induced an increase in fasting plasma HA. HA and pyrocatechol sulfate were among the most prominent metabolites of the diet-related polyphenols that were increased in the fasting plasma in the HD group. Because HA was not altered in the WGED group, bilberries and bilberry products were the likely sources for increased HA in the HD group, as was also suggested based on the correlation analyses with the food record data.

Several studies reported elevated HA concentrations in urine after ingestion of polyphenol-rich foods and drinks, such as red wine, grape juice, black and green tea, cranberries, and blackcurrant juice (38–41), and HA concentrations in 24-h

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urine were proposed as a potential biomarker for fruit and vegetable consumption (42). However, data on changes in plasma concentrations of HA and other phenolic acids in response to diet are limited.

Earlier reports indicate that excretion of phenolic acids and HA is increased after the intake of bilberry anthocyanins, indicating that the anthocyanins are also most likely the source of the elevated concentrations of phenolics in fasting plasma in our study (43, 44). We did not observe elevated HA after the WGED intervention, indicating different metabolism of the phenolics from this source than phenolics from berries. These results are in concordance with our earlier report, which showed no differences in urinary excretion of HA acid after ingestion of rye bread and white-wheat bread (45). Even though HA is regarded as a biomarker for dietary intake of phenolics, polyphenol metabolism by the intestinal microbiota and liver can produce numerous other metabolites into the circulation (46).

Whole-grain consumption caused alterations in amino acid and betaine metabolism. WGED intervention tended to increase fasting plasma concentrations of several amino acids such as L-lysine, L-arginine, and ornithine in accordance with an earlier report on plasma amino acids as potentially useful biomarkers for intake of grain protein (47). The diets enriched with whole grains tended to also increase betaine compounds in fasting plasma, and they associated positively with the intake of whole-grain bread in the intervention groups. Whole-grain products are known to be a major source of dietary betaine (48), and several metabolomics studies reported increased plasma betaine and altered metabolism after whole-grain intervention (49–52). In our study, the number of altered betaine-specific MS/MS signals was high, but many were not identified and need to be investigated by using modified analytical conditions. The high diversity of betaines and the large interindividual variation are most likely due to altered endogenous metabolism of betaines after the increased consumption of whole-grain products. Among the identified betaines, pipecolic acid betaine was most strongly associated with the consumption of whole-grain bread, although it has not been reported as a constituent of whole grains but only in citrus genus plants (53). One potential metabolic route for the increase in pipecolic acid betaine in the fasting plasma in the WGED group is endogenous catabolism of lysine by the intestinal bacteria, which also increased in this study group (23, 54). In addition, γ-butyrobetaine and propionylcarnitine increased in the WGED group, as was also reported in our earlier work on betaine supplementation in mice (55).

Conclusions. Novel biomarkers for intakes of selected food items included in the Nordic diet were identified in fasting plasma by using nontargeted LC-MS metabolite profiling. Correlation analyses further verified the associations of *1*) CMPF with increased consumption of fatty fish, *2*) the glucuronidated alk(en)ylresorcinols with consumption of whole grains, and *3*) HA with the intake of bilberries, thus suggesting these metabolites as biomarkers of intakes of these healthy foods. Furthermore, the observations of altered endogenous metabolism of betaine, amino acids, and lipids indicate potential beneficial effects of a healthy Nordic diet rich in whole grains, fatty fish, and berries.

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References

1. Olsen A, Egeberg R, Halkjaer J, Christensen J, Overvad K, Tjonneland A. Healthy aspects of the Nordic diet are related to lower total mortality. J Nutr 2011;141:639–44.
2. Juntunen KS, Laaksonen DE, Poutanen KS, Niskanen LK, Mykkanen HM. High-fiber rye bread and insulin secretion and sensitivity in healthy postmenopausal women. Am J Clin Nutr 2003;77:385–91.
3. Laaksonen DE, Toppinen LK, Juntunen KS, Autio K, Liukkonen KH, Poutanen KS, Niskanen L, Mykkanen HM. Dietary carbohydrate modification enhances insulin secretion in persons with the metabolic syndrome. Am J Clin Nutr 2005;82:1218–27.
4. Adamsson V, Reumark A, Fredriksson IB, Hammarstrom E, Vessby B, Johansson G, Riserus U. Effects of a healthy Nordic diet on cardiovascular risk factors in hypercholesterolaemic subjects: a randomized controlled trial (NORDIET). J Intern Med 2011;269:150–9.
5. Kolehmainen M, Mykkanen O, Kirjavainen PV, Leppanen T, Moilanen E, Adriaens M, Laaksonen DE, Hallikainen M, Puupponen-Pimia R, Pulkkinen L, et al. Bilberries reduce low-grade inflammation in individuals with features of metabolic syndrome. Mol Nutr Food Res 2012;56:1501–10.
6. Lankinen M, Schwab U, Kolehmainen M, Paananen J, Poutanen K, Mykkanen H, Seppanen-Laakso T, Gylling H, Uusitupa M, Oresic M. Whole grain products, fish and bilberries alter glucose and lipid metabolism in a randomized, controlled trial: the Sysdimet study. PLoS ONE 2011;6:e22646.
7. de Mello VD, Schwab U, Kolehmainen M, Koenig W, Siloaho M, Poutanen K, Mykkanen H, Uusitupa M. A diet high in fatty fish, bilberries and wholegrain products improves markers of endothelial function and inflammation in individuals with impaired glucose metabolism in a randomised controlled trial: the Sysdimet study. Diabetologia 2011;54:2755–67.
8. Uusitupa M, Hermansen K, Savolainen MJ, Schwab U, Kolehmainen M, Brader L, Mortensen LS, Cloetens L, Johansson-Persson A, Onning G, et al. Effects of an isocaloric healthy Nordic diet on insulin sensitivity, lipid profile and inflammation markers in metabolic syndrome—a randomized study (SYSDIET). J Intern Med 2013;274:52–66.
9. Brennan L. Metabolomics in nutrition research: current status and perspectives. Biochem Soc Trans 2013;41:670–3.
10. O'Sullivan A, Gibney MJ, Brennan L. Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. Am J Clin Nutr 2011;93:314–21.
11. Wishart DS. Metabolomics: applications to food science and nutrition research. Trends Food Sci Technol 2008;19:482–93.
12. Llorach R, Garcia-Aloy M, Tulipani S, Vazquez-Fresno R, Andres-Lacueva C. Nutrimetabolomic strategies to develop new biomarkers of intake and health effects. J Agric Food Chem 2012;60:8797–808.
13. Scalbert A, Brennan L, Manach C, Andres-Lacueva C, Dragsted LO, Draper J, Rappaport SM, van der Hooft JJ, Wishart DS. The food metabolome: a window over dietary exposure. Am J Clin Nutr 2014;99(6):1286–1308.
14. Rastas M, Seppänen R, Knuts L, Karvetti R, Varo P. Ruokien ravintoainesisältö. [Nutrient composition of foods.] 4th ed. Turku (Finland): Social Insurance Institution; 1997 (in Finnish).
15. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc B 1995;57:289–300.
16. Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J. TM4 microarray software suite. Methods Enzymol 2006;411:134–93.

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17. Buré C, Ayciriex S, Testet E, Schmitter JM. A single run LC-MS/MS method for phospholipidomics. Anal Bioanal Chem 2013;405:203–13.
18. Xia YQ, Jemal M. Phospholipids in liquid chromatography/mass spectrometry bioanalysis: comparison of three tandem mass spectrometric techniques for monitoring plasma phospholipids, the effect of mobile phase composition on phospholipids elution and the association of phospholipids with matrix effects. Rapid Commun Mass Spectrom 2009;23:2125–38.
19. Murphy RC, Axelsen PH. Mass spectrometric analysis of long-chain lipids. Mass Spectrom Rev 2011;30:579–99.
20. Pinheiro J, Bates D, DebRoy S, Sarkar D. R core team. Linear and nonlinear mixed effects models. 2009; R package version 3:1–96.
21. Niwa T, Takeda N, Maeda K, Shibata M, Tatematsu A. Accumulation of furancarboxylic acids in uremic serum as inhibitors of drug binding. Clin Chim Acta 1988;173:127–38.
22. Hannemann K, Puchta V, Simon E, Ziegler H, Ziegler G, Spiteller G. The common occurrence of furan fatty acids in plants. Lipids 1989;24:296–8.
23. Fujita T, Hada T, Higashino K. Origin of D- and L-pipecolic acid in human physiological fluids: a study of the catabolic mechanism to pipecolic acid using the lysine loading test. Clin Chim Acta 1999;287: 145–56.
24. Suzuki Yesumi Y, Uramoto M, Kono Y, Sakurai A. Structural analyses of carbon chains in 5-alk(en)ylresorcinols of rye and wheat whole flour by tandem mass spectrometry. Biosci Biotechnol Biochem 1997;61: 480–6.
25. Zhu Y, Conklin DR, Chen H, Wang L, Sang S. 5-Alk(en)ylresorcinols as the major active components in wheat bran inhibit human colon cancer cell growth. Bioorg Med Chem 2011;19:3973–82.
26. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 2003;49:286–94.
27. Bruce SJ, Guy PA, Rezzi S, Ross AB. Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC-MS/MS. J Agric Food Chem 2010;58:2055–61.
28. Vetter W, Wendlinger C. Furan fatty acids? Valuable minor fatty acids in food. Lipid Technology 2013;25:7–10.
29. Toshimitsu N. 3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid. In: Desiderio DM, Nibbering NMM, editors. Uremic toxins. Hoboken (NJ): John Wiley & Sons; 2012. p. 87–98.
30. Watanabe H, Miyamoto Y, Otagiri M, Maruyama T. Update on the pharmacokinetics and redox properties of protein-bound uremic toxins. J Pharm Sci 2011;100:3682–95.
31. Boelaert J, Lynen F, Glorieux G, Eloot S, Van Landschoot M, Waterloos MA, Sandra P, Vanholder R. A novel UPLC-MS-MS method for simultaneous determination of seven uremic retention toxins with cardiovascular relevance in chronic kidney disease patients. Anal Bioanal Chem 2013;405:1937–47.
32. Ross AB. Present status and perspectives on the use of alkylresorcinols as biomarkers of wholegrain wheat and rye intake. J Nutr Metab 2012;462967.
33. Landberg R, Åman P, Kamal-Eldin A. A rapid gas chromatography–mass spectrometry method for quantification of alkylresorcinols in human plasma. Anal Biochem 2009;385:7–12.
34. Marklund M, Landberg R, Aman P, Kamal-Eldin A. Determination of alkylresorcinol metabolites in human urine by gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:888–94.
35. Landberg R, Marklund M, Kamal-Eldin A, Åman P. An update on alkylresorcinols—occurrence, bioavailability, bioactivity and utility as biomarkers. J Funct Foods 2014;7:77–89.
36. Zhu Y, Shurlknight KL, Chen X, Sang S. Identification and pharmacokinetics of novel alkylresorcinol metabolites in human urine, new candidate biomarkers for whole-grain wheat and rye intake. J Nutr 2014;144:114–22.
37. Landberg R, Linko AM, Kamal-Eldin A, Vessby B, Adlercreutz H, Aman P. Human plasma kinetics and relative bioavailability of alkylresorcinols after intake of rye bran. J Nutr 2006;136:2760–5.
38. van Dorsten FA, Grun CH, van Velzen EJ, Jacobs DM, Draijer R, van Duynhoven JP. The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. Mol Nutr Food Res 2010;54:897–908.
39. Mulder TP, Rietveld AG, van Amelsvoort JM. Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. Am J Clin Nutr 2005;81:256S–60S.
40. Valentova K, Stejskal D, Bednar P, Vostalova J, Cihalik C, Vecerova R, Koukalova D, Kolar M, Reichenbach R, Sknouril L, et al. Biosafety, antioxidant status, and metabolites in urine after consumption of dried cranberry juice in healthy women: a pilot double-blind placebo-controlled trial. J Agric Food Chem 2007;55:3217–24.
41. Jin Y, Alimbetov D, George T, Gordon MH, Lovegrove JA. A randomised trial to investigate the effects of acute consumption of a blackcurrant juice drink on markers of vascular reactivity and bioavailability of anthocyanins in human subjects. Eur J Clin Nutr 2011;65:849–56.
42. Krupp D, Doberstein N, Shi L, Remer T. Hippuric acid in 24-hour urine collections is a potential biomarker for fruit and vegetable consumption in healthy children and adolescents. J Nutr 2012;142:1314–20.
43. Nurmi T, Mursu J, Heinonen M, Nurmi A, Hiltunen R, Voutilainen S. Metabolism of berry anthocyanins to phenolic acids in humans. J Agric Food Chem 2009;57:2274–81.
44. Del Bo' C, Riso P, Brambilla A, Gardana C, Rizzolo A, Simonetti P, Bertolo G, Klimis-Zacas D, Porrini M. Blanching improves anthocyanin absorption from highbush blueberry (Vaccinium corymbosum L.) puree in healthy human volunteers: a pilot study. J Agric Food Chem 2012;60: 9298–304.
45. Bondia-Pons I, Barri T, Hanhineva K, Juntunen K, Dragsted LO, Mykkanen H, Poutanen K. UPLC-QTOF/MS metabolic profiling unveils urinary changes in humans after a whole grain rye versus refined wheat bread intervention. Mol Nutr Food Res 2013;57(3):412–22.
46. Pekkinen J, Rosa NN, Savolainen OI, Keski-Rahkonen P, Mykkanen H, Poutanen K, Micard V, Hanhineva K. Disintegration of wheat aleurone structure has an impact on the bioavailability of phenolic compounds and other phytochemicals as evidenced by altered urinary metabolite profile of diet-induced obese mice. Nutr Metab (Lond) 2014;11: 7075–11-1.
47. Altorf-van der Kuil W, Brink EJ, Boetje M, Siebelink E, Bijlsma S, Engberink MF, van 't Veer P, Tome D, Bakker SJ, van Baak MA, et al. Identification of biomarkers for intake of protein from meat, dairy products and grains: a controlled dietary intervention study. Br J Nutr 2013;110:810–22.
48. Ross AB, Zangger A, Guiraud SP. Cereal foods are the major source of betaine in the Western diet—analysis of betaine and free choline in cereal foods and updated assessments of betaine intake. Food Chem 2014;145:859–65.
49. Moazzami AA, Bondia-Pons I, Hanhineva K, Juntunen K, Antl N, Poutanen K, Mykkanen H. Metabolomics reveals the metabolic shifts following an intervention with rye bread in postmenopausal women—a randomized control trial. Nutr J 2012;11:88.
50. Moazzami AA, Zhang JX, Kamal-Eldin A, Aman P, Hallmans G, Johansson JE, Andersson SO. Nuclear magnetic resonance-based metabolomics enable detection of the effects of a whole grain rye and rye bran diet on the metabolic profile of plasma in prostate cancer patients. J Nutr 2011;141:2126–32.
51. Ross AB, Bruce SJ, Blondel-Lubrano A, Oguey-Araymon S, Beaumont M, Bourgeois A, Nielsen-Moennoz C, Vigo M, Fay LB, Kochhar S, et al. A whole-grain cereal-rich diet increases plasma betaine, and tends to decrease total and LDL-cholesterol compared with a refined-grain diet in healthy subjects. Br J Nutr 2011;105:1492–502.
52. Price RK, Keaveney EM, Hamill LL, Wallace JM, Ward M, Ueland PM, McNulty H, Strain JJ, Parker MJ, Welch RW. Consumption of wheat aleurone-rich foods increases fasting plasma betaine and modestly decreases fasting homocysteine and LDL-cholesterol in adults. J Nutr 2010;140:2153–7.
53. Servillo L, Giovane A, Balestrieri ML, Ferrari G, Cautela D, Castaldo D. Occurrence of pipecolic acid and pipecolic acid betaine (homostachydrine) in citrus genus plants. J Agric Food Chem 2012;60:315–21.
54. Struys EA, Jakobs C. Metabolism of lysine in alpha-aminoadipic semialdehyde dehydrogenase-deficient fibroblasts: evidence for an alternative pathway of pipecolic acid formation. FEBS Lett 2010;584: 181–6.
55. Pekkinen J, Olli K, Huotari A, Tiihonen K, Keski-Rahkonen P, Lehtonen M, Auriola S, Kolehmainen M, Mykkanen H, Poutanen K, et al. Betaine supplementation causes increase in carnitine metabolites in the muscle and liver of mice fed a high-fat diet as studied by nontargeted LC-MS metabolomics approach. Mol Nutr Food Res 2013;57:1959–68.

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