

# Plasma metabolomic response to postmenopausal weight loss induced by different diets

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## Abstract

**Background** Menopause is associated with increased abdominal fat and increased risk of developing diabetes and cardiovascular disease.

**Objectives** The present study evaluated the plasma metabolic response in relation to insulin sensitivity after weight loss via diet intervention.

**Methods** This work includes two studies; i) Ten women on a 5 weeks Paleolithic-type diet (PD, 30 energy percent (E%) protein, 40 E% fat, 30 E% carbohydrates), ii) 55 women on 6 months of either PD or Nordic Nutrition Recommendations diet (NNR, 15 E% protein, 30 E% fat, and 55 E% carbohydrates). Plasma metabolic profiles were acquired at baseline and post diet using gas chromatography time-of-flight/mass spectrometry and investigated in relation to insulin sensitivity using multivariate bioinformatics.

**Results** Both the PD and NNR diet resulted in significant weight loss, reduced waist circumference, improved serum lipid profiles, and improved insulin sensitivity. We

detected a baseline metabolic profile that correlated significantly with insulin sensitivity, and of which components increased significantly in the PD group compared to NNR. Specifically, a significant increase in myo-inositol (MI), a second messenger of insulin action, and  $\beta$ -hydroxybutyric acid ( $\beta$ -HB) increased while dihomo-gamma-linoleic acid (DGLA) decreased in PD compared to NNR, which correlated with improved insulin sensitivity. We also detected a significant decrease in tyrosine and tryptophan, potential markers of insulin resistance when elevated in the circulation, with the PD but not the NNR.

**Conclusions** Using metabolomics, we detected changes in the plasma metabolite profiles associated with weight loss in postmenopausal women by different diets. The metabolic profiles following 6 months of PD were linked to beneficial effects on insulin sensitivity compared to NNR.

## Graphical Abstract



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**Keywords** Metabolomics · Mass spectrometry · Multivariate analysis · Paleolithic-type diet (PD) · Nordic Nutrition Recommendation (NNR) · Type 2 diabetes mellitus (T2DM) · Insulin sensitivity · Weight loss · Myo-inositol (MI) · 1,5-anhydroglucitol (1,5-AG)

## 1 Introduction

After menopause, the incidence of cardiovascular disease (CVD) increases (Lisabeth et al. 2009). An important mediator of this increase is the redistribution of fat from peripheral to central depots due, at least in part, to a decline in estrogen. Obesity and central fat accumulation (i.e., accumulation of visceral adipose tissue) are strongly linked to an increased risk of type 2 diabetes mellitus (T2DM) and CVD (Rexrode et al. 1998, Peppas et al. 2013). Therefore, effective approaches for reducing weight and tools for identifying potential mediators of beneficial metabolic effects, notably insulin sensitivity, are important in subjects with central obesity.

A Paleolithic-type diet (PD) includes increased proportions of unsaturated fatty acids and a relatively high protein content, with demonstrated beneficial effects on metabolic imbalance, including insulin sensitivity, even when administered ad libitum compared to other types of diets, including the Nordic Nutrition Recommendations (NNR) (Jonsson et al. 2009, 2010; Lindeberg et al. 2007). We have reported that an ad libitum PD can have profound effects on weight loss and metabolic functions in obese postmenopausal women, including decreased ectopic lipid deposition and improved lipid balance and glucose tolerance. The PD was also superior to a diet based on the NNR in terms of weight loss and improvements in body composition (Ryberg et al. 2013, Mellberg et al. Mellberg et al. 2014).

Metabolomics (Fiehn et al. 2000; Kell 2004) studies have shown great promise for discovering pathways linked to disease progression (Wang et al. 2011; Newgard et al. 2009) and for dissecting putative links between food intake and metabolic functions (LeMieux et al. 2013; Brennan, 2013). Simultaneous measurement of multiple analytes (metabolites) has confirmed suggested links between key intermediates in lipid, carbohydrate, and protein metabolism and perturbations in T2DM, CVD, and cancer (Suhre 2014). The aim of the present study was to investigate the plasma metabolic profile in cohorts with diet-induced weight loss in order to find putative predictors and mediators of the outcome after these interventions, notably in relation to insulin sensitivity.

## 2 Experimental section

### 2.1 Participants and clinical protocol

The present study comprises two different dietary intervention studies: a 5-week PD pilot study (Ryberg et al. 2013) and a 6-month intervention that employed both a PD and NNR diet (Mellberg et al. Mellberg et al. 2014). The inclusion and exclusion criteria were comparable for the two studies and are presented in detailed elsewhere (Mellberg et al. 2014, Ryberg et al. 2013). Briefly, all study participants were healthy

postmenopausal women younger than 70 years of age, non-smokers, and with a body mass index (BMI) between 28 and 40 kg/m<sup>2</sup>. The studies were approved by the Regional Ethical Review Board at Umeå University, and written informed consent was obtained from all participants.

### 2.2 Five-week PD pilot study

The study participants were provided a PD ad libitum. The diet consisted of lean meat, poultry, fish, seafood, eggs, vegetables, fruit, berries, nuts, and seeds. All meals were prepared by the food service establishment at Umeå University Hospital. Participants were advised to complement the provided frozen meals with additional food items in concordance with the PD. The provided dietary recommendations aimed for an approximate intake of 30 energy percent (E%) protein, 30 E% carbohydrates, and 40 E% fat, with a high proportion of monounsaturated fatty acids (MUFA) and a low proportion of saturated fatty acids (SFA). The study participants had weekly individual meetings with the study dietician, and food intake was assessed at baseline and after 5 weeks on the PD. A detailed description of the study design, including a detailed dietary intake assessment, was presented previously (Ryberg et al. 2013).

### 2.3 Six-month intervention using a PD and NNR diet

Seventy postmenopausal women were randomized into two groups (Mellberg et al. 2014). One group was instructed to eat a PD ad libitum and the other group to eat an omnivorous diet in concordance with the NNR for 24 months (Becker, 2005). Food intake for the NNR group was ad libitum and recommendations were given for an approximate intake of 15 E% protein, 55 E% carbohydrates, and 30 E% fat (10 E% each of polyunsaturated fatty acids (PUFA), MUFA, and SFA). All participants met in groups for 12 sessions that consisted of cooking lessons, instructions regarding food records, and discussions concerning dietary changes. Plasma samples were collected at baseline and after 6 months of intervention. The largest weight reduction occurred during the first 6 months, suggesting the strongest adherence to the dietary regimens during this time period. A complete description of the study design, including a detailed dietary intake assessment for the entire intervention, was described previously by Mellberg et al. (2014). Briefly, The PD diet included a recommendation for a high intake of MUFAs and PUFAs and relative low intake of carbohydrates. The diet was based on lean meat, fish, eggs, vegetables, fruits, berries and nuts. Additional fat sources were avocado and oils (rapeseed and olive oil) used in food preparation and dressing. Dairy products, cereals, added salt and refined fats and sugar were excluded.

## 2.4 General measurements for both interventions

Physical examinations were performed at baseline and after 5 weeks in the PD pilot study, and at baseline and after 6 months in the 6-month intervention study. Body composition, including an estimation of total body fat percentage, was measured by dual energy x-ray absorptiometry (DXA) (GE Medical Systems, Lunar Prodigy X-ray Tube Housing Assembly, Brand BX-1L, Model 8743, Madison, WI, USA). Overnight fasting blood samples were drawn from the antecubital vein after a resting period. Aliquots were frozen immediately at  $-20^{\circ}\text{C}$  until being analyzed for fasting plasma glucose (Vitros GLU Slides, Ortho-Clinical Diagnostics J&J) and fasting plasma insulin (Elecys Insulin kit, Roche Diagnostic Scandinavia AB, Sweden). Insulin sensitivity was calculated using the homeostasis model assessment (HOMA) index with the formula  $(G_0 \times I_0)/22.5$ , where  $G_0$  is fasting plasma glucose and  $I_0$  is fasting plasma insulin. High-sensitivity C-reactive protein (hsCRP) was analyzed by immunoassay (IMMULITE, Diagnostic Products Corporation, USA). A detailed description of the anthropometric measurements, including blood pressure, is found elsewhere (Mellberg et al. 2014, Ryberg et al. 2013). Aliquots for the metabolomics analysis were stored at  $-80^{\circ}\text{C}$  prior to analysis.

## 2.5 Sample preparation and metabolomics analysis

A run order design was constructed to minimize bias from sample preparation and analysis. Samples from the same participant were prepared and analyzed in close connection to each other, whereas the internal order was randomized. In addition, the sample preparation batches and analytical run order were balanced in relation to the type of diet. Thus, bias interfering with the biological variation of interest was minimized when comparing samples between diet groups or between individuals. From the 5-week PD pilot study, a total of 29 samples were analyzed in duplicate using gas chromatography time-of-flight/mass spectrometry (GC-TOF/MS). Samples from the 6-month intervention study were analyzed in two separate batches in immediate succession. Prior to GC-TOF/MS analysis, MeOH-H<sub>2</sub>O extraction followed by a two-step derivatization procedure was performed for serum metabolites as described previously (Trygg et al. 2005). The derived samples were injected in splitless mode by a CTC Combi Pal autosampler (CTC Analytics AG, Zwingen, Switzerland) into an Agilent 6890 gas chromatograph equipped with a  $10\text{ m} \times 0.18\text{ mm}$  i.d. fused silica capillary column with a chemically bonded  $0.18\text{-}\mu\text{m}$  DB 5-MS stationary phase (J&W Scientific, Folsom, CA). The column effluent was introduced into the ion source of a Pegasus III GC-TOF/MS (Leco Corp., St Joseph, MI).

## 2.6 Data treatment

Hierarchical multivariate curve resolution (HMCR) (Jonsson et al. 2005) was used to resolve overlapping metabolites/compounds that were not separated by chromatography. For the 5-week PD pilot study, all samples were processed simultaneously, except for the analytical replicates, which were predicted in the existing HMCR model (Jonsson et al. 2006). To remove instrumental biases and concentration variation, the resolved data were normalized using the means of 12 added internal standards that eluted throughout the entire chromatographic range, consisted of: [<sup>13</sup>C5]-proline, [<sup>2</sup>H<sub>4</sub>]-succinic acid, [<sup>13</sup>C<sub>5</sub>, <sup>15</sup>N]-glutamic acid, [1,2,3-<sup>13</sup>C<sub>3</sub>]-myristic acid, [<sup>2</sup>H<sub>7</sub>]-cholesterol and [<sup>13</sup>C<sub>4</sub>]-disodium -ketoglutarate were purchased from Cambridge Isotope Laboratories (Andover, MA); [<sup>13</sup>C<sub>12</sub>]-sucrose, [<sup>13</sup>C<sub>4</sub>]-palmitic acid and [<sup>2</sup>H<sub>4</sub>]-butanediamine 2HCl were obtained from Campro (Veenendaal, The Netherlands); [13C6]-glucose was purchased from Sigma Aldrich (Steinheim, Germany); [<sup>2</sup>H<sub>6</sub>]-salicylic acid was purchased from Icon (Summit, NJ); and NMethyl-N-trimethylsilyltrifluoroacetamide (MSTFA), 1 % trimethylchlorosilane (TMCS) and pyridine (silylation grade) were purchased from Pierce Chemical Co. Stock solutions of the reference compounds and IS were prepared either in Milli-Q water or methanol at the same concentration,  $0.5\ \mu\text{g}\ \mu\text{L}^{-1}$ . The area under the chromatographic peak was calculated for each internal standard using non-noisy and unique mass-channels. A principal component (PC) was then calculated based on the chromatographic peak areas for the internal standards (all variables were scaled to unit variance, non-centered). Finally, the calculated PC score value, which is comparable to the sample mean in this case, was used to normalize the resolved data by dividing each sample by the corresponding score value (Redestig et al. 2009).

## 2.7 Metabolite identification

All resolved mass spectra profiles were subjected to a spectral database search in NIST MS-Search v. 2.0.38. Match values ranking the spectra were calculated using the dot product of the two spectra (i.e., the resolved spectrum and the database spectrum); higher m/z peaks had more weight than lower m/z peaks because higher m/z values are considered more compound-specific. The match values ranged from 0 to 999, with 999 indicating an identical match. We performed positive identification by combining match values with the retention time index, which was calculated from the analytically characterized alkane series (C10–C40). Only metabolites with match value  $>700$  and retention index difference from the library match  $<20$  units were considered. However a manual inspection were performed on all components prior to assignment to compound class and or identification.

Furthermore, all metabolites were classified as amino acids and derivatives, amines, organic acids, carbohydrates, lipids, no class, or unidentified. Assignment to different compound classes was based on criteria set by the Human Metabolome Database ([www.HMDB.ca](http://www.HMDB.ca)) (Wishart et al. 2007).

## 2.8 Statistical analysis

The data sets were inspected using principal components analysis (PCA) in order to detect groupings, outliers, and trends, as well as select representative samples for training sets. Additional sample comparison modeling was performed using multivariate regression analysis with orthogonal partial least squares (OPLS). Prior to multivariate analysis, each individual's baseline sample was subtracted from its post-intervention sample to elucidate the intervention effect on the metabolic profile and minimize the influence from instrumental drift and inter-individual variation. To validate the obtained OPLS models, *P* values were calculated for the differences between the pre-defined classes. These *P* values were calculated using analysis of variance (ANOVA) based on the cross-validated OPLS scores (CV-ANOVA). Special consideration was taken to ensure proper cross-validation groups (i.e., to retain the same participant/replicate in the same group, reducing the risk of creating an over-fitted model). The importance of each variable to the multivariate model projection (variable of importance plot, VIP-value) reflects the importance of each metabolite to explaining the response (dietary difference/intervention course). A metabolite was considered to contribute significantly to the metabolite profile if it had a VIP value > 1 and/or a significant *P* value. All univariate *P* values were calculated from the Wilcoxon signed-rank test using a 95 % significance level. *P* values were obtained using univariate *t* tests, as well as CV-ANOVA and jack knifing-based confidence intervals (Efron et al. 1983) from OPLS models.

## 3 Results

### 3.1 Five-week PD pilot study

Ten overweight/obese postmenopausal women (age  $52.6 \pm 3.8$  years, BMI  $31.4 \pm 2.6$  kg/m<sup>2</sup>, total body fat  $39.1 \pm 4.5$  kg) participated in the 5-week intervention study with a PD. The effects of this intervention on anthropometric data and metabolic function were published elsewhere (Ryberg et al. 2013). Briefly, mean energy intake decreased by 25 % and was associated with a mean weight loss of 4.5 kg. BMI, waist and hip circumference, abdominal sagittal diameter, diastolic blood pressure, fasting serum glucose, and LDL/HDL cholesterol decreased, whereas hepatic insulin sensitivity increased (decreased HOMA values) (Ryberg et al. 2013).

A total of 20 plasma samples were collected and metabolically characterized in duplicate using GC-TOF/MS. One participant (Participant 3) was excluded along with her analytical replicates because of low analytical quality, incomplete chromatographic spectra due to sample contamination. The GC-TOF/MS data collected for the 18 included samples were processed by HMCR, and the analytical replicates were predictively resolved to allow the robustness of the processing to be controlled. A total of 182 putative metabolites were detected in plasma; 79 were successfully identified and/or classified using mass spectral library comparisons (Table 1). The remaining compounds not identified with enough certainty retained information regarding the fragmentation pattern and retention time index, which could be used in further identification attempts. Pearson's correlation between analytical replicates revealed a >90 % correlation for the resolved metabolites and the internal standards.

### 3.2 Plasma metabolic profile

We observed a significant difference ( $P < 0.001$ ) between metabolite profiles in plasma samples collected before and after the intervention. The existing OPLS model had 93 % model sensitivity (% correct prediction) and 100 % model specificity (% correct model classification). The OPLS model revealed a cohesive metabolic response for all participants when comparing their basal samples to those collected after 5 weeks (data not shown). Detailed information regarding identification/classification, related statistics, and responses to the intervention is provided in Table 1.

We observed an increase in circulating MUFA and PUFA [e.g., oleic acid (18:1), docosahexadecanoic acid (DHA, 22:6 n-3), palmitoleic acid (16:1), and arachidonic acid (20:4 n-6)], and a decrease in SFA, larger lipids, and sterols [e.g., cholesterol, *alpha*-tocopherol, glycerophosphocholine (GPC), lauric acid (12:0), and gamma-tocopherol]. However, we noted an increase in palmitic acid (16:0). Two of the most commonly occurring polyols in humans were significantly altered, i.e., myo-inositol (MI) increased and 1,5-anhydroglucitol (1,5-AG) decreased. The circulating concentrations of amino acids decreased in general, especially tryptophan, proline, tyrosine, threonine, and asparagine. In contrast, we detected an increase in diet-related amino acid derivatives, such as methylocysteine and pipercolic acid. The concentrations of branched chain amino acids isoleucine and valine remained unaltered.

### 3.3 Six-month intervention using a PD and NNR

Participants in both diet groups experienced a significant ( $P < 0.05$ ) decrease in BMI, waist and hip circumference,

**Table 1** Metabolites analyzed after a 5-week intervention with a Paleolithic-type diet (PD) in overweight and obese postmenopausal women

Compound class	ID	Abbreviation	Response after PD	Match value <sup>a,b</sup>	RI-RI <sub>library</sub>
Organic acid	Ketoleucine		↑	864	10
Organic acid			↑		
Organic acid			↓		
Organic acid	Glyceric acid	GA	↓	859	1
Organic acid	Fumaric acid	FA	↑	652	0
Organic acid	Malic acid	MA	↓	889	2
Organic acid	Erythronic acid	EA	↓	706	2
Organic acid			↑		
Organic acid	Threonic acid	ThrA	↓	924	1
Organic acid	Citric acid		↑*	949	1
Organic acid	Quinic acid		↓*	852	2
Amine	Putrescine	Putr	↓	658	9
Amine	Ethanolamine	EA	↑	745	33
Amine	Urea		↑	901	4
Amino acid and derivative	Valine	Val	↓	906	7
Amino acid and derivative	Isoleucine	Ile	↓	805	0
Amino acid and derivative	Proline	Pro	↓*	926	5
Amino acid and derivative	Glycine	Gly	↓	925	4
Amino acid and derivative	Threonine	Thr	↓	944	0
Amino acid and derivative	Methylcysteine	MeCys	↑*	798	5
Amino acid and derivative	Pipecolic acid	PA	↑	614	35
Amino acid and derivative	Serine	Ser	↑	800	2
Amino acid and derivative	Methionine	Met	↓	818	3
Amino acid and derivative	Pyroglutamic acid	PyrA	↓	886	2
Amino acid and derivative	Aspartic acid	AspA	↓	772	14
Amino acid and derivative		Ala	↓		
Amino acid and derivative			↓		
Amino acid and derivative	Cysteine	Cys	↓	922	1
Amino acid and derivative	Glutamine	Glu	↑	918	4
Amino acid and derivative	Ornithine	Orn	↓	924	0
Amino acid and derivative	Phenylalanine	Phe	↓	875	4
Amino acid and derivative	Asparagine	Asp	↓	699	2
Amino acid and derivative	Histidine	His	↓	909	9
Amino acid and derivative	Lysine	Lys	↑	939	6
Amino acid and derivative	Tyrosine	Tyr	↓*	929	5
Amino acid and derivative			↓		
Amino acid and derivative	Tryptophan	Trp	↓*	946	6
Amino acid and derivative	Cystine	Oxidized cys	↑	935	5
Carbohydrate			↓		
Carbohydrate	D-Galactono-1,4-lactone		↓	917	16
Carbohydrate	β-D-Methylglucopyranoside		↓	920	2
Carbohydrate	Sugar		↓		
Carbohydrate			↑*		
Carbohydrate			↓		
Carbohydrate			↓		
Carbohydrate			↓		
Carbohydrate			↓		
Carbohydrate			↓		



**Table 1** continued

Compound class	ID	Abbreviation	Response after PD	Match value <sup>a,b</sup>	RI-Ri <sub>library</sub>
Carbohydrate			↓		
Carbohydrate			↓		
Carbohydrate	Maltotrios		↓*	850	159
Lipid	Nonanoic acid (9:0)	NA	↑	722	1
Lipid	Lauric acid (12:0)	LauA	↓	774	2
Lipid	Palmitoleic acid (16:1)	PalA	↑	941	4
Lipid	Palmitic acid (16:0)	PA	↑	947	6
Lipid	Linoleic acid (18:2)	LA	↑	926	2
Lipid	Fatty acid		↓		
Lipid	Oleic acid (18:1)	OA	↑*	730	11
Lipid	Elaidic acid (18:1)	EA	↑	968	5
Lipid	Stearic acid (18:0)	SA	↓	876	1
Lipid	Arachidonic acid (20:4, n-6)	AA	↑	726	3
Lipid	Docosahexaenoic acid (22:6, n-3)	DHA	↑*	810	6
No class	myo-inositol		↑*	960	1
No class	Uric acid		↑	957	1
No class	Pseudouridine	Psu	↓	853	3
No class	Salicin		↓	890	3
No class	Erythritol		↓	626	2
No class	Salicylic acid		↑	807	3
No class	Phosphoric acid	PhA	↓	871	7
No class	γ-Tocopherol	GT	↓	650	5
No class	Adenosine-5-monophosphate	AMP	↓*	855	14
No class	α-Tocopherol	αT	↓*	920	9
No class	Cholesterol	Chol	↓*	829	16
No class	Ribitol		↓	613	12

Assignment to different compound classes was based on criteria set by the Human Metabolome Database ([www.HMDB.ca](http://www.HMDB.ca)) (Wishart et al. 2007). Fatty acids are also denoted by the ratio of the number of carbons to the number of double bonds. For example, if the last double bond is situated 3 and 6 carbons from the end denotation will be n-3 and n-6, respectively. Variable denoted anthropometry was not included in multivariate models

\* Non-parametric  $P < 0.05$  after 6 months of PD

<sup>a</sup> Mass spectra values according to NIST MS-Search 2.0

<sup>b</sup> Mass spectra searches were performed using in-house libraries at the Swedish Metabolomics Centre ([swedishmetabolomicscentre.se](http://swedishmetabolomicscentre.se)) and the Max Planck Institute (MPI) library in Golm (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>)

and percent body fat after 6 months. The PD group had significantly reduced waist circumference, fasting serum (fs)-insulin, and HOMA index values compared to the NNR group after 6 months of diet (Table 2). Adherence to diets was evaluated, using repeated food records and estimations of nitrogen excretion in the urine, as reported in detail in Mellberg et al. 2014. Briefly, target levels of macronutrient intake were not completely achieved. Protein intake in the PD group was lower than proposed, mirrored by a lack of difference in nitrogen excretion between groups. Furthermore, the NNR group did not reach the target amount of carbohydrate intake.

Only participants with plasma samples available at baseline and after 6 months were included in order to

elucidate the metabolic effects and their relationship to changes in insulin sensitivity and body composition. A total of 110 plasma samples (55 subjects sampled before and after dietary intervention) were characterized using GC-TOF/MS. The characteristics of the subjects included in the metabolomic analysis are provided in Table 2. A total of 117 putative metabolites were resolved from the GC-TOF/MS data; 62 of them were identified and/or assigned to a compound class (Table 3).

No significant differences, clusters, or outliers related to the different diets were detected in the metabolite profiles or clinical parameters at baseline based on either PCA or  $P$  values (data not shown). Thus, all models investigating intervention effects, not including baseline investigations,

**Table 2** Clinical and biochemical characteristics of the study population

	Participants (n)	Age (years)	BMI (kg/m <sup>2</sup> )	Waist (cm)	Hip (cm)	Percent bodyfat (%)	Glucose (mmol/L)	Insulin (mIU/L)	HOMA	hsCRP (mg/L)
PD, baseline	31	60 ± 5.4	32.4 ± 3.6	104.1 ± 9.7	112.4 ± 7	47.9 ± 3.8	5.1 ± 0.8	7.9 ± 3.8	1.8 ± 0.9	2.3 ± 2.8
NNR, baseline	24	61.1 ± 5.8	32.3 ± 3.2	105.9 ± 7.8	113.2 ± 7.3	48.9 ± 4	5.2 ± 1	9.4 ± 3.9	2.3 ± 1.1	2.8 ± 2.6
PD, 6 months	31	60 ± 5.4	29.4 ± 3.7	<b>93 ± 9.4</b>	106 ± 7.9	43.7 ± 5	5 ± 0.6	<b>7.3 ± 3.7</b>	<b>1.7 ± 1</b>	1.8 ± 2.3
NNR, 6 months	24	61.1 ± 5.8	30.8 ± 3.8	<b>98.9 ± 10.3</b>	109.6 ± 9.5	46.8 ± 5.2	5.2 ± 1	<b>9.6 ± 4.7</b>	<b>2.3 ± 1.3</b>	1.9 ± 1.6

Values are expressed as mean ± standard deviation. Bold numbers differed significantly ( $P < 0.05$ ) between dietary groups based on the Wilcoxon non-parametric significance test

were based on delta values, i.e., by subtracting baseline metabolic profiles from the post-intervention metabolic profiles to obtain delta values.

### 3.4 Baseline metabolic profiles

We identified a plasma metabolic profile at baseline that correlated significantly ( $P = 0.0045$ ) with insulin resistance (i.e., HOMA index) (Fig. 1). Specifically, ribitol, MI, beta-hydroxybutyric acid ( $\beta$ -HB), glycine, and phosphoric acid correlated negatively with the HOMA index, whereas galactono-1,4-lactone, glucose, *dihomo*-gamma-linoleic acid (DGLA, 20:3,n-6), palmitoleic acid (16:1), and amino acids valine, cysteine, and isoleucine positively correlated with the HOMA index (i.e., with decreased insulin sensitivity). We did not detect a metabolic profile that significantly correlated with the HOMA index at the remaining time-points.

### 3.5 Intervention-related metabolic profiles

Notably, components from the insulin resistance baseline metabolic profile were significantly altered between the diet groups after 6 months. The PD group showed improvements in insulin sensitivity as compared to the NNR group via decreased DGLA (positive correlation with the HOMA index) and an increase in MI and  $\beta$ -HB (negative correlation with the HOMA index).

Comparing the two metabolic profiles, we found a significant difference ( $P > 0.001$ ) in metabolite profiles between the PD and NNR diet. After 6 months, the PD group exhibited lower relative concentrations of 1,5-AG, DGLA (20:3, n-6), and lauric acid (12:0) and higher concentrations of MI, DHA (22:6, n-3), ascorbic acid, and  $\beta$ -HB than the NNR group (Fig. 2). The observed alterations in MI and 1,5-AG were also seen in the 5-week pilot PD study. Relevant to the aim, we also detected differences in amino acids; the concentrations of glycine, tryptophan, and tyrosine decreased, and the concentration of serine increased in the PD group versus the NNR group.

When investigating alterations in the diet-specific metabolic profiles, only the PD resulted in a significant change (OPLS CV-ANOVA,  $P > 0.001$ ) not the NNR diet (OPLS CV-ANOVA,  $P = 0.09$ ). Diet specific metabolite profiles are plotted in Fig. 3. In terms of unique metabolites, both diets resulted in a significant increase in pantothenic acid (vitamin B5) and a decrease in 1,5-AG, phosphoric acid, MI-1 phosphate, DGLA, and AA (Fig. 3b, d).

In the PD group, but not the NNR group, we observed increases in DHA, MI,  $\beta$ -HB, and oxalic acid. Concomitant decreases in SFA and n6-PUFAs [i.e.,

**Table 3** Metabolites analyzed before (0 m) and 6 months after (6 m) postmenopausal weight loss with a Paleolithic-type diet (PD) or a diet based on the Nordic Nutrition Recommendation (NNR)

Compound class	ID	Abbreviation	PD 0–6 m	NNR 0–6 m	Response after 6 m PD versus 6 m NNR	Match value <sup>a,b</sup>	RI- Ri <sub>library</sub>
Anthropometry	hsCRP			↓			
Anthropometry	Total body fat %		↓*	↓*	↓		
Anthropometry	Waist (cm)		↓*	↓*	↓*		
Anthropometry	Hip (cm)		↓*	↓*			
Anthropometry	BMI (kg/m <sup>2</sup> )		↓*	↓*	↓		
Anthropometry	SBPav (mmhg)		↓*	↓*			
Anthropometry	DPBav (mmhg)		↓*	↓*			
Anthropometry	fs-Glucose						
Anthropometry	fs-Insulin		↓		↓		
Anthropometry	HOMA				↓		
Organic acid	Pyruvic acid		↑	↓	↑		
Organic acid	Lactic acid	LacA	↓	↓	↓	913	49.5
Organic acid			↓	↓	↓		
Organic acid	Oxalic acid		↑	↑	↑	912	19.8
Organic acid	β-Hydroxybutyric acid	β-HB	↑*	↑	↑*	940	11
Organic acid	Ascorbic acid	AscA	↑	↓	↑	643	2.7
Amine	Ethanolamine	EA	↓	↑	↑	858	5.3
Amine	Urea		↓	↓	↑	881	4.8
Amine	2,5-Diaminovalerolactam		↓	↑	↓	840	4.6
Amino acid and derivative	Alanine	Ala	↓	↑	↓	818	31.3
Amino acid and derivative	Sarcosine		↓	↑	↑	881	19.9
Amino acid and derivative	2-Aminobutyric acid		↑	↓	↑	693	9.8
Amino acid and derivative	Valine	Val	↓	↓	↑	861	4.8
Amino acid and derivative	Isoleucine	Ile	↓	↓	↑	631	0
Amino acid and derivative	Glycine	Gly	↓	↑	↓	872	4.7
Amino acid and derivative	Pipecolic acid		↑	↑	↓	833	29
Amino acid and derivative	Serine	Ser	↑	↓	↓	910	2.4
Amino acid and derivative	Threonine	Thr	↓	↓	↓	893	0.6
Amino acid and derivative			↑	↓	↓	633	5.8
Amino acid and derivative	Pyroglutamic acid	PyrA	↓	↑	↓	839	5.2
Amino acid and derivative	Cysteine	Cys	↓	↑	↓	852	0.6
Amino acid and derivative	Glutamine	Glu	↓	↑	↑	869	6.55
Amino acid and derivative			↓	↓*	↑		
Amino acid and derivative	Phenylalanine	Phe	↓	↓	↓	907	0.6
Amino acid and derivative	Asparagine	Asp	↑	↑	↓	809	0.8
Amino acid and derivative	Ornithine	Orn	↑	↓	↑	883	3.05
Amino acid and derivative			↓*	↓*	↓		
Amino acid and derivative	Lysine	Lys	↑	↓	↑	875	2.4
Amino acid and derivative	Tyrosine	Tyr	↓	↑	↓	937	2.3
Amino acid and derivative	Tryptophan	Trp	↓	↓	↓	887.5	0.1
Amino acid and derivative	Cystine	oxCys	↑	↑	↓	892	0.8
Carbohydrate			↑	↑	↑		
Carbohydrate	1,5-Anhydroglucitol	1,5-AG	↓*	↓*	↓*		
Carbohydrate	Glucose	Glu	↓	↓	↓	926	7.65
Carbohydrate	D-Galactono-1,4-lactone		↓	↓	↓	914	6.7
Carboxylic acid	Citric acid	CA	↑	↓	↑	903	1



**Table 3** continued

Compound class	ID	Abbreviation	PD 0–6 m	NNR 0–6 m	Response after 6 m PD versus 6 m NNR	Match value <sup>a,b</sup>	RI- R <sub>library</sub>
Lipid	Decanoic acid (10:0)	DA	↓	↓	↓	732	1
Lipid	Lauric acid (12:0)	LauA	↓*	↓	↓	797	0.6
Lipid	Palmitoleic acid (16:1)		↑	↓	↑	813	4.6
Lipid	Palmitic acid (16:0)	PA	↓*	↓	↓	837	0.7
Lipid	Linoleic acid (18:2, n-6)	LA	↓	↓	↓	901	0.7
Lipid	Oleic acid (18:1)	OA	↓	↓	↓	927	1.7
Lipid			↓	↓*	↑		
Lipid	Stearic acid (18:0)	SA	↓	↓	↓	946	1.1
Lipid	Arachidonic acid (20:4, n-6)	AA	↓	↓	↓	879	2.6
Lipid	Dihomo-gamma-linolenic acid (20:3, n-6)	DGLA	↓*	↓*	↓*	de novo id	
Lipid			↓	↓*	↑		
Lipid	Docosahexaenoic acid (22:6, n-6)	DHA	↑	↓	↑	903	2.1
Lipid			↑	↑	↓	902	2.1
Lipid	Glycerophosphocholine	GPC	↓	↓	↓	931	4.7
Lipid	Cholesterol	Chol	↑	↑	↓	879	6.05
No class	Phosphoric acid	PhA	↓	↓*	↑	834	6.05
No class	Ribitol		↓	↓	↓	745	8.6
No class	Glycerol-3-phosphate		↑	↓	↓	903	1
No class			↓	↓	↑		
No class	Pantothenic acid		↑*	↑*	↓	850	2.7
No class	Acetyl-glucosamine		↓	↓	↑*	789	7.6
No class	Myo-inositol	MI	↑	↓	↑*	916	2.3
No class	Naproxen	NSAID	↑	↑*	↓	922	
No class	Uric acid		↑	↓	↓	885	0.95
No class	Myo-inositol-1-phosphate		↓*	↓	↓	916	
No class	Inosine		↓	↑	↓	888	8.7
No class	Unknown, replicated in Wilma		↑*	↑*	↑		

Assignment to different compound classes was based on criteria set by the Human Metabolome Database ([www.HMDB.ca](http://www.HMDB.ca)) (Wishart et al. 2007). Fatty acids are also denoted by the ratio of the number of carbons to the number of double bonds. For example, if the last double bond is situated 3 and 6 carbons from the end denotation will be n-3 and n-6, respectively. Variable denoted anthropometry was not included in multivariate models

\* Non-parametric  $P < 0.05$  after 5 weeks of PD

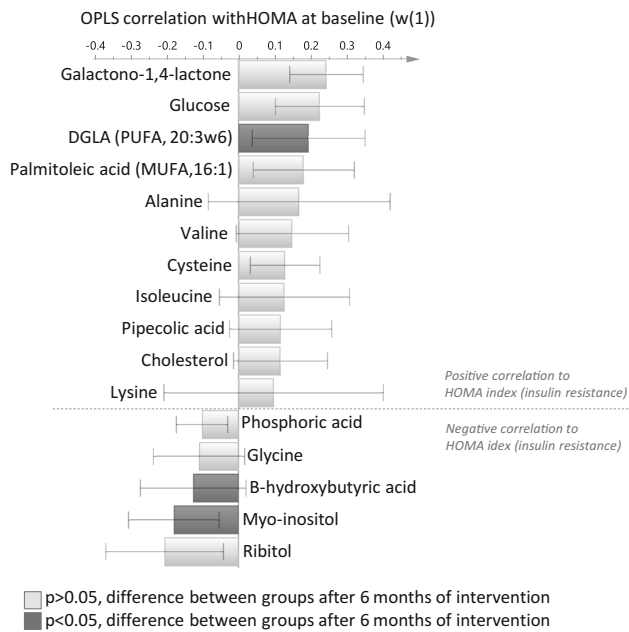
<sup>a</sup> Mass spectra values according to NIST MS-Search 2.0

<sup>b</sup> Mass spectra searches were performed using in-house libraries at the Swedish Metabolomics Centre ([swedishmetabolomicscentre.se](http://swedishmetabolomicscentre.se)) and the Max Planck Institute (MPI) library in Golm (<http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html>)

lauric acid (LauA, 12:0), palmitic acid (PA, 16:0), stearic acid (SA, 18:0), linoleic acid (18:2, n-6), and glycerophosphocholine (GPC)] were also measured. After 6 months of the PD, we also detected decreases in tyrosine and tryptophan that were not observed in the NNR group (Fig. 3b, d).

### 3.6 Individual response to the PD

The PD group revealed a robust intervention-related metabolic profile, but with large inter-individual variation after 6 months (Fig. 2a). Based on this finding, we highlighted a high responder (Participant 16) and low responder



**Fig. 1** OPLS loading describes the plasma metabolic profile in relation to the HOMA index in baseline samples from 55 obese/overweight postmenopausal women. Metabolites with positive axis values (w[1]) are associated with low insulin sensitivity (high HOMA values), whereas negative axis values (w[1]) correlate negatively with the HOMA index, i.e., high insulin sensitivity. The multivariate 95 % confidence interval is based on jack-knifing, and the bars are colored according to univariate *P* values. Dark grey bars differ significantly between diet groups after 6 months of intervention, and light grey bars do not differ significantly between groups after 6 months. Significance is based on a combination of multivariate and univariate significance testing. Table 3 provides a complete description of all metabolites included in the models, classification/identification, abbreviations, and related statistics

(Participant 41), which was verified in terms of BMI, percent body fat, and HOMA index compared to the PD group mean. Specifically, the high responder reduced her BMI by 6.5 kg/m<sup>2</sup>, body fat by 7.9 %, and HOMA index by 3.3 compared to the PD group means of 3 kg/m<sup>2</sup>, 4.2 %, and 0.4 units, respectively. In contrast, the low responder reduced her BMI by 1.3 kg/m<sup>2</sup>, percent body fat by 1.4 %, and her HOMA index increased by 5.5 units.

#### 4 Discussion

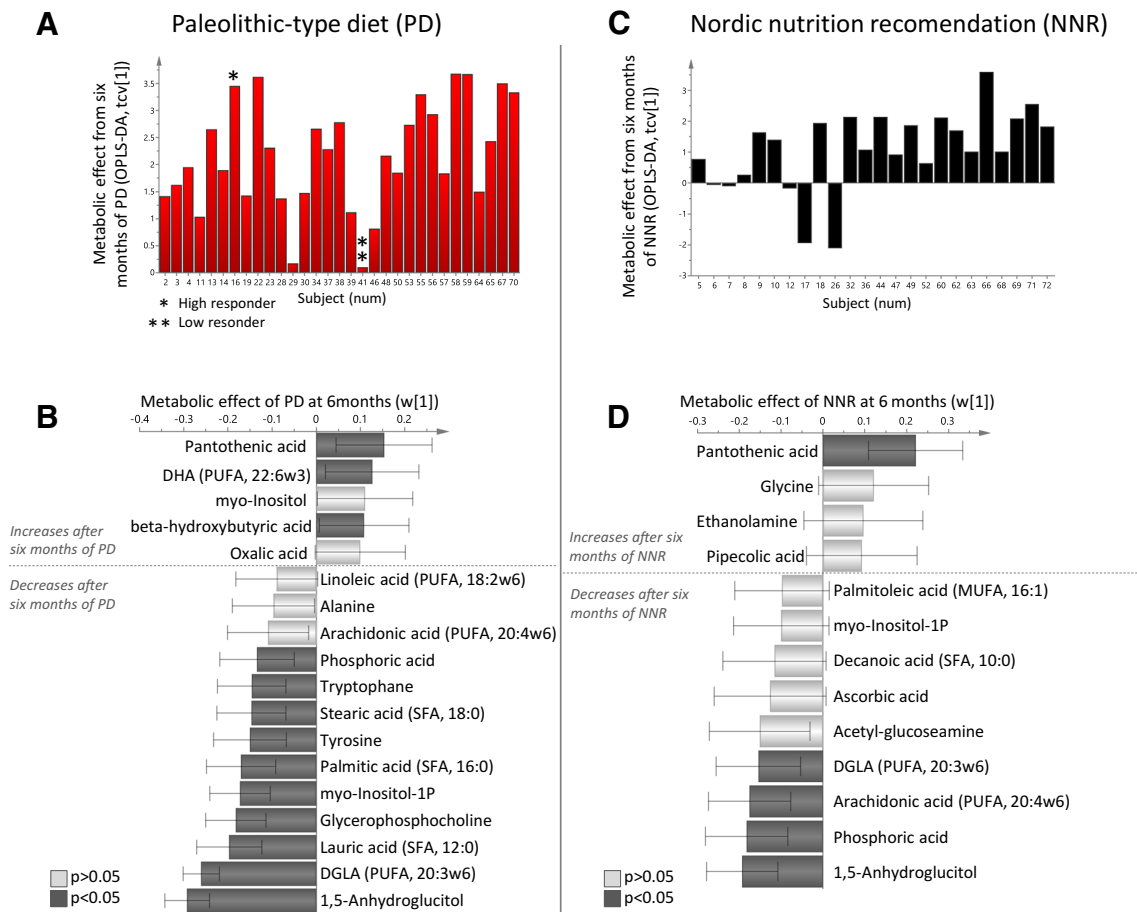
We detected an intervention-related plasma metabolic profile that differed between the PD and NNR groups. The findings in the PD group were congruent in the 5 weeks and 6 months dietary intervention studies, suggesting that they are robust. Furthermore, we observed a metabolic profile at baseline that correlated significantly with decreased insulin sensitivity (HOMA index) for both diet groups. After 6 months, the components in this metabolic

profile differed significantly between the diets and were associated with increased insulin sensitivity in the PD group compared to the NNR group. Specifically, women following the PD had a significant increase in MI and  $\beta$ -HB and reduced DGLA compared to the NNR group. This pattern correlated with improved insulin sensitivity and coincided with significant reductions in waist circumference.

Inositols, with MI as the most abundant stereoisomer, act as second messengers of insulin action and have been identified to play a vital role in activating key enzymes that control the oxidative and non-oxidative metabolism of glucose (Saltiel and Kahn, 2001). Plasma MI comes from different sources, including dietary intake (Clements and Darnell, 1980) and de novo synthesis from glucose-6-phosphate via myo-inositol-1-phosphate. Therefore, predicting where the observed increase in MI originates and whether it reflects intracellular concentrations is difficult. Interestingly, supplementation with MI (Genazzani et al. 2008) and *chiro*-inositol (CI) (Nestler et al. 1999) has been shown to reduce insulin, glucose, triglycerides, and diastolic blood pressure in young women with polycystic ovarian syndrome, a patient population at risk for developing T2DM (Dunaif, 1997). In addition, improved insulin sensitivity via MI supplementation has been confirmed in patients with gestational diabetes (Corrado et al. 2011). Notably, the menopausal state may influence the effects of MI because estrogen promotes nitric oxide, which in turn inhibits the polyol pathway (Ren and Ceylan-Isik, 2004), and a low-energy diet combined with MI supplementation has been shown to improve insulin sensitivity and blood lipids and reduce diastolic blood pressure in postmenopausal women with metabolic dysregulation (Giordano et al. 2011).

We measured significant decreases in tyrosine and tryptophan in the PD groups. Both tyrosine and tryptophan are included in the metabolic signature that is reported to be a strong predictor of insulin resistance (Newgard et al. 2009). The underlying mechanism may be the disruption of insulin signaling in skeletal muscle (Newgard et al. 2009, Wang et al. 2011a, b). Therefore, a decrease in these circulating amino acids may be beneficial for preventing T2DM. We did not find a significant correlation between tyrosine and tryptophan and the HOMA index at baseline, possibly due to the relatively well-preserved insulin sensitivity in this study group.

As no major difference was found in protein intake between the groups, based on urine nitrogen measurements, fat composition is the key difference between the PD and NNR. The PD contains greater percentages of MUFA and PUFA than the NNR. We detected significant decreases in SFA and n6-PUFA and significant increases in MUFA and n-3 PUFA after 6 months of the PD compared to the NNR.



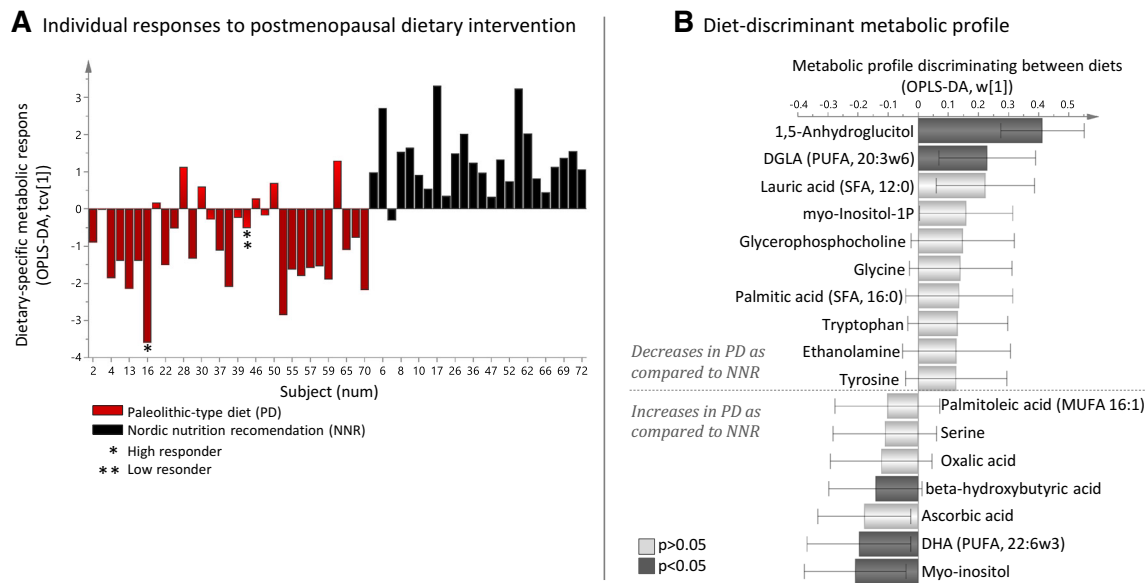
**Fig. 2** Two separate OPLS models displaying the effects of dietary intervention on plasma metabolite profiles in overweight/obese postmenopausal women after 6 months. **a** Cross-validated OPLS scores (tcv[1]) describing the metabolic effects recorded for 31 individual participants after a 6-month intervention with a Paleolithic-type diet (PD). One high\* and one low\*\* responder to PD are each highlighted in the plot. **b** OPLS model weights (w[1]) describe the metabolic profile related to the PD. Only identified metabolites with VIP > 1 are presented, and the bars are colored according to univariate P values (dark bars have a significant P value and light bars a non-significant P value). Metabolites with positive axis values increased after 6 months of PD, whereas metabolites with negative

axis values decreased. **c** Cross-validated OPLS scores (tcv[1]) describe the metabolic effects recorded for 24 participants after a 6-month intervention with a diet based on the Nordic Nutrition Recommendations (NNR). **d** OPLS model weights (w[1]) describe the metabolic profile related to the NNR intervention. Only identified metabolites with VIP > 1 are presented, and the bars are colored according to univariate P values (dark bars have a significant P value and light bars a non-significant P value). Metabolites with positive axis values increased after 6 months of the NNR, whereas those with negative axis values decreased. Table 3 provides a complete description of all metabolites included in the models, classification/identification, and their abbreviation (Color figure online)

Importantly, the n-6 PUFA DGLA, which was closely linked to insulin resistance at baseline, decreased significantly more in the PD group. DGLA has anti-proliferative and inflammatory properties through its conversion to prostaglandin (PG)H<sub>1</sub>, which is further converted to PGE<sub>1</sub>. Via this conversion, DGLA has been reported to have anti-inflammatory effects in diabetic humans (Kapoor and Huang, 2006). Because DGLA correlates with insulin resistance (i.e., via the HOMA index), diets, such as the PD, that reduce DGLA and increase MI and β-HB are expected to have beneficial effects on insulin sensitivity. As each lipid subtype has its own unique biochemical properties, including different n-6 and n-3

PUFAs (Russo, 2009, Virtanen et al. 2014), further research should focus on specific lipids and whether they are key mediators of improved insulin sensitivity and protect against CVD.

In addition to the mentioned alterations in MI, we observed an intervention-related effect of another common polyol, 1,5-AG. The intervention effects of PD on 1,5-AG (and MI) observed during the 5-week PD pilot study were verified in the long-term study; 1,5-AG decreased in both diet groups but significantly more in the PD group. 1,5-AG is generated from glycogen via 1,5-anhydrofructose by an NADPH-dependent reductase (Sakuma et al. 1998) and can act as a powerful insulin secretagogue in pancreatic β-cells



**Fig. 3** An OPLS model comparing the respective effects of dietary intervention with a Paleolithic-type diet (PD) and a diet based on the Nordic Nutrition Recommendations (NNR) on the plasma metabolic profile. **a** Cross-validated OPLS scores ( $tcv[1]$ ) describe the 55 participants' (31 PD, grey bars; 24 NNR, black bars) metabolic responses to 6 months of dietary intervention. One high responder\* and one low responder\*\* to the PD are each highlighted in the plot. **b** OPLS model weights ( $w[1]$ ) of the discriminating metabolic profile between 6 months of the PD or NNR. The multivariate 95 %

confidence interval is based on jack-knifing, and the bars are colored according to univariate  $P$  values (dark bars have a significant  $P$  value and light bars a non-significant  $P$  value). Metabolites with positive axis values decreased in the PD group relative to the NNR group, and those with negative axis values increased in the PD group relative to the NNR group. Table 3 provides complete descriptions of all metabolites included in the models, classification/identification, abbreviations, and related statistics (Color figure online)

(Yamanouchi et al. 2003). 1,5-AG has also been suggested as a marker for postprandial glycemic variability in hyperglycemic individuals (Yamanouchi et al. 1996). Therefore, the detected decrease in circulating 1,5-AG may be a consequence of a negative energy balance (which was more profound in the carbohydrate-restricted PD group), as these individuals may deplete their glycogen storage to a greater extent. Additional investigations regarding the use of 1,5-AG as a marker of postprandial glycemic variability during negative energy balance, particularly during carbohydrate restriction, are of interest.

Traditionally, nutritional recommendations to improve health have focused mainly on the whole population. However, there is major variability in individual adaptive and functional mechanisms of metabolic homeostasis. As highlighted by our results, metabolomics, if combined with suitable bioinformatics, has the potential to objectively capture the complexity of human nutritional status on both the individual and population level. This approach is a step towards assessing personalized dietary recommendations to improve health (McNiven et al. 2011, Zeisel et al. 2005). Studies of intervention effects could include normalizing the metabolic profile for each individual to baseline levels in order to more sensitively elucidate the true intervention-related metabolic effects apart from individual variability,

such as improved insulin sensitivity, in a population prior to disease onset, e.g., T2DM.

Weight loss occurred in both diet groups, despite the ad libitum conditions. Contributing factors to this may include inadequate reports of energy intake, increased physical activity energy expenditure when the volunteers were not using the accelerometers, the increased thermogenic effects of protein and other uncertainties regarding the measurement of all energy components. A major strength of this study is the validation of our findings in two independent studies. However, the study also has some limitations. First, since T2DM was an exclusion criterion, all of the included women were insulin-sensitive, which decreases the possibility of identifying effects related to improved metabolic regulation. Second, we did not include the lipidome in the analysis, which may be of future interest in relation to improvements in insulin sensitivity via lifestyle interventions.

In conclusion, we found distinct differences in the metabolite profiles of our diet study groups, which were associated with a more pronounced improvement in body composition and insulin sensitivity in the PD group. These results highlight metabolites of interest for monitoring mechanisms related to improved insulin sensitivity after weight loss in postmenopausal women. Additional studies

are warranted regarding the effects of lifestyle interventions on intermediary metabolism in participants with more pronounced metabolic dysfunction, including T2DM.

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#### Compliance with Ethical Standards

**Conflict of interest** The authors declare no conflicts of interest.

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